Comparison of the Methods of Zona Pellucida Removal and Inner Cell Mass Isolation for the Generation of Parthenogenetic Embryonic Stem Cells in HanWoo Cattle

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ABSTRACT

In general, zona pellucida (ZP) of the blastocyst has to be removed first, then either isolated the inner cell mass (ICM) or ZP-removed whole blastocyst, which is then cultured on the feeder layer to induce ICM outgrowth for the generation of embryonic stem cells (ESC). However, it is unclear whether ICM isolation before seeding on feeder layer is beneficial or not because the interaction between ICM and trophoblasts may affect cellular growth and/or pluripotency during the culture on the feeder. In the present study, two ZP removal methods (mechanically by splitting with a 28-gauge needle versus chemically by the treatment of acid-Tyrode's solution) and two ICM isolation methods (ZP-free whole blastocyst seeding versus mechanical isolation of ICM) were evaluated for the efficient isolation and culture of putative parthenogenetic bovine ESC. The number of maintained outgrown colonies was counted in each experimental group. As the result, mechanical removal of ZP with a needle and followed by whole ZP-free blastocyst seeding on feeder cells tended to attach more on the feeder layer and resulted in more outgrown colonies with its simple and less time-costing benefits. Currently we are generating ESC lines in HanWoo cattle by using this method for initial outgrowth of the parthenogenetic bovine blastocysts.

(Key words : embryonic stem cells, HanWoo cattle, parthenogenesis)

INTRODUCTION

Many researchers have attempted to generate embryonic stem cell (ESC) lines in livestock including cattle (Keefer *et al.*, 2007; Talbot *et al.*, 2008). ESC can provide valuable research tools for effecting transgenesis and developing disease models (Wilmut *et al.*, 1994; Kuroiwa *et al.*, 2004; Wall *et al.*, 2005). In our previous studies, we have shown that embryonic stem cell lines can be generated from parthenogenetic embryos in mice (Choi *et al.*, 2011). The defect in full-term development of parthenogenetic embryos enables researchers to avoid the ethical concerns related to ESC research as well. Moreover, the parthenogenetic ESC has the patient's own genetic information if the cells originate from the oocytes of the same patient. In livestock researches, parthenogenetic ESC can be a valuable research material for facilitating autologous tissue specific gene modification for bio-farming and basic developmental biology studies including imprinting and epigenetics. After the first report in mice (Kaufman *et al.*, 1983), parthenogenetic ESC was reported in non-human primate (Cibelli *et al.*, 2002), rabbits (Fang *et al.*, 2006) and human (Kim *et al.*, 2007). Currently, there is only one report of the generation of parthenogenetic bovine ESC (Pashaiasl *et al.*, 2010).

Conventionally the first step to establish ESC line is removing the zona pellucida (ZP) and then either isolating the ICM or ZP-removed whole blastocyst, which is then cultured on the feeder layer to induce ICM outgrowth for the generation of ESC (Evans *et al.*, 1981; Thomason *et al.*, 1995; Mitalipova *et al.*, 2001). Several chemicals are generally used to acquire dissociating colonies from dishes and ZP-free blastocysts, and those chemicals are important to establish and maintain the ESC line (Evans *et al.*, 1981; Thomason *et al.*, 1995). Different from other species, many reports showed bovine ESC are susceptible to chemical enzymes such as trypsin, type IV collagenase and

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protease E (Stice *et al.*, 1996; Mitalipova *et al.*, 2001). These results suggest that those chemicals may have adverse effects on the cells of the blastocyst including the ICM (Wang *et al.*, 2005; Mitalipova *et al.*, 2001). Although ICM is surrounded by trophoblastic layer in the blastocyst, it is easily exposed to external environment. So the chemicals may have detrimental effect on the ICM. In human, it is also reported that implantation rate is decreased when ZP of the blastocyst is removed chemically (Nishio *et al.*, 2006). This report implies that the chemical does not dissolve ZP but also damage to cells of the blastocyst. In ESC experiment, it is also unclear whether ICM isolation from ZP-free blastocysts before seeding on feeder layer is beneficial or not because interaction between ICM and trophoblasts may affect cellular growth and/or pluripotency during the culture on the feeder (Copp, 1978). Although several researches were reported interaction of ICM and trophectoderm such as polarity, differentiation and proliferation (Gardner *et al.*, 1973; Copp, 1978), with our knowledge, there is no direct report regarding to their interaction in ESC researches.

In the present study, two ZP removal methods (mechanically by splitting with a 28-gauge needle versus chemically by the treatment of acid-Tyrode's solution) and two embryonic cell seeding methods (whole blastocyst versus mechanical isolation of ICM) were evaluated for the efficient isolation and culture of putative bovine parthenogenetic ESC.

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 upon 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 a washing medium consisting 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. Parthenogenesis and *In Vitro* Culture (IVC)

Parthenogenetic activation was performed after IVM of the oocytes. The oocytes were incubated in CR2 medium (Rosenkrans and First, 1994) supplemented with 0.1% PVA and 10 μM Ca-ionophore for 5 min for activation and then incubated in CR2 medium with 2 mM 6-dimethylaminopurine (6-DMAP) for 3 h for post-activation treatment. After the treatments, the activated oocytes were transferred to 20 μl drop of IVC medium consisting of CR2 with 0.3% fatty acid-free BSA (ff-BSA) and 1% v/v insulin, transferrin and selenium complex (ITS) for 3 days and then transferred to CR2 medium drop with 0.15 % ff-BSA, ITS and 0.15 % FBS for 5 days at 39℃ 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. Experimental Design for ZP Removal and ICM Isolation

The ZP of parthenogenetic blastocysts on Day 8 were removed either mechanically by splitting with a 28-gauge needle or chemically by the treatment of acid-Tyrode's solution for 3~4 min (Fig. 1). The two ICM isolation methods (ZP-free whole blastocyst seeding versus mechanical isolation of ICM by splitting with two 28-gauge needles; Fig. 2) were evaluated for the efficient isolation and culture of putative bovine parthenogenetic ESC. Detailed description of experimental combination groups (from G1 to G4) is described in Table 1.

5. Culture of Putative Parthenogenetic Bovine ESC

The ZP-free whole blastocysts or isolated ICM from the blastocysts were placed and pressed onto mitomycin C-inactivated mouse embryonic fibroblast (MEF) feeder layers and cultured at 39℃ in a humidified gas atmosphere of 5% CO_2 in the bovine ESC culture medium (Pashaiasl *et al.*, 2010). The bovine stem cell culture medium is N2B27-3i medium containing N2B27 medium which is consisting of an equal volume of DMEM/F12 glutamax (Invitrogen) and Neurobasal medium (Invitrogen) with 1% (v/v) N_2 and 2% (v/v) B27 supplements

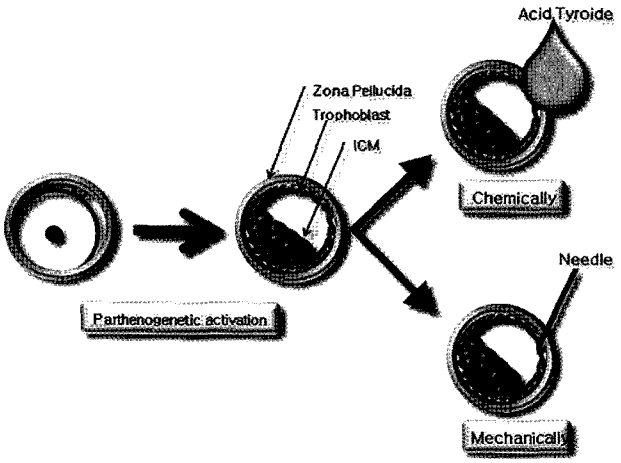

Fig. 1. Methods for zona pellucida (ZP) removal to obtain ZP-free blastocysts from the parthenogenetic bovine embryos. The ZP of the blastocysts is removed either mechanically by splitting with a 28-gauge needle or chemically by the treatment of acid-Tyrode's solution.

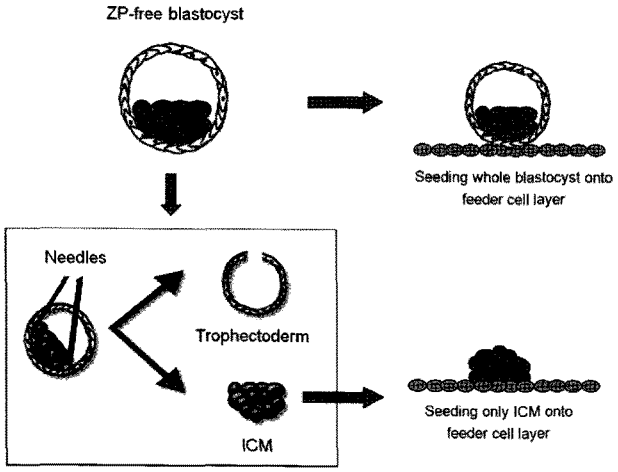

Fig. 2. Two methods of inner cell mass (ICM) isolation for the generation of parthenogenetic bovine embryonic stem cells. Either whole zona pellucida (ZP)-free blastocysts or only ICM isolated using two 28-gauge needles are seeded onto feeder cell layer (mouse embryonic fibroblast).

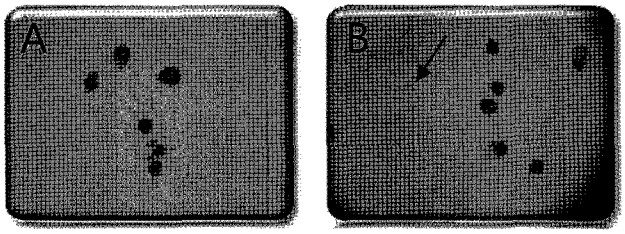

Fig. 3. The blastocysts removed their zona pellucida (ZP) chemically by the treatment of acid-Tyrode's solution (A), or mechanically by splitting with a 28-gauge needle (B). Arrow indicates empty ZP which is not shown in (A).

(Invitrogen) (Ying *et al.*, 2003) plus three inhibitors (3i), 0.8 mM PD184352 (MEK 1/2 inhibitor, Stem Cell Sciences, UK), 2 mM SU5402 (FGF receptor inhibitor, Calbiochem, USA) and 3 mM CHIR99021 (GSK3 inhibitor, Stem Cell Sciences) (Ying *et al.*, 2008). This was designated as passage zero (P0). The medium was changed every other day. After 12 days of culture, the initial outgrowths from bovine parthenotes were mechanically dissociated into clumps and 2~4 pieces from each individual colony were re-plated onto fresh MEF feeder layers and designated as P1. The colonies of the cells were passaged every 8 to 10 days.

RESULTS AND DISCUSSION

The present study was designed to find optimal condition for the isolation of pluripotent cell population from parthenogenetic bovine blastocysts. Although ZP-free blastocysts could successfully be obtained from both chemical and mechanical ZP removal methods (Fig. 3), mechanical removal of ZP with a needle and followed by whole ZP-free blastocyst seeding on MEF feeder layers (G2) tended to attach more on the feeder layer and resulted in more outgrown colonies with its simple and less time-costing benefits than the other experimental groups (Table 1). Once the colonies attached successfully, the

Table 1. Comparision of the methods of zona pellucida removal and inner cell mass isolation in cattle

Group	Zona pellucida removal	Inner cell mass isolation	Total blastocysts	Attached on feeder layer	Outgrown colonies (%)
G1	Splitting with a 28-gauge needle	Mechanically (28-gauge needles)	6	5	5 (83.3)
G2		ZP-free whole blastocyst seeding	6	6	6 (100.0)
G3	Treatment of acid-Tyrode's solution	Mechanically (28-gauge needles)	7	2	2 (28.5)
G4		ZP-free whole blastocyst seeding	6	5	5 (83.3)

propagation pattern of putative ESC following ICM outgrowth does not tend to be different among the experimental groups (data not shown).

Although ZP removal using proteolytic reagents such as protease and acid-Tyrode's solution are commonly used and also very efficient to obtain ZP-free embryos in mice (Evans *et al.*, 1981; Cosby and Dukelow, 1990), it may not be suitable for removing ZP in bovine species. In mice, less than one minute of exposure to acid-Tyrode's solution is enough for the removal of ZP whereas it took 3~4 minutes in cattle in this study. Comparably extended exposure of proteolytic factor to the embryo might affect the embryo and resulted in loss of outgrowth ability. As ZP-free whole blastocysts show more potential for the outgrowth of ICM regardless of ZP removal method, interaction between ICM and trophoblast may be one of the most important factors for the generation of putative parthenogenetic bovine ESC. Particularly, chemical ZP removal combined with mechanical ICM isolation should be avoided in bovine ESC experiment. Currently we are generating several ESC lines in HanWoo cattle by using this method for initial outgrowth of fertilized, parthenogenetic and nuclear transfer bovine blastocysts.

In conclusion, the present study shows that mechanical ZP removal and ZP-free whole blastocyst seeding on feeder layer may be the best option for the initial step of the generation of parthenogenetic ESC in HanWoo cattle.

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