In Vitro Development of Porcine Parthenogenetic Embryos under the Oil-free Culture System

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ABSTRACT

Optimization of the preimplantation mammalian embryo culture condition was widely focused on refining medium composition under the name of chemically defined media. However, recent research revealed that the alteration of physical environment can be a crucial factor to a successful embryo development. In this study, under the same embryo density, a novel culture device named oil-free micro tube culture (MTC) system was evaluated using porcine parthenogenetic embryos. The activated oocytes were placed into the 0.2 ml thin-wall flat cap PCR tube and cultured to the blastocyst stage. As a preliminary step, embryo density and culture medium volume were optimized under a standard drop culture system. The optimal embryo density range for *in vitro* culture was 0.5 embryos per μl in 20 μl drop (20.5%) and 1.0 embryos per μl in 10 μl drop (20.6%). Based on these results, we compared drop culture system and 'MTC' system in terms of the developmental rate to the blastocyst stage. In 20 μl medium volume, the 'MTC' system showed similar blastocyst formation rate when compared with drop culture system (20.2% versus 20.5%, respectively) while the 'MTC' system showed lower blastocyst formation rate than drop culture system in 10 μl one (12.7% versus 20.0%, respectively). Therefore the 20 μl MTC system may be an alternative incubation system for short-distance embryo transport without carrying the CO_2 incubator and this provides novel embryo culture device to clinical veterinary embryologists.

(Key words : oil-free, microtube culture, porcine, parthenogenesis)

INTRODUCTION

Until now, optimization of the preimplantation mammalian embryo culture condition was widely focused on refining medium composition under the name of chemically defined media (Summers and Biggers, 2003). However, recent research revealed that the alteration of physical environment can be a crucial factor to a successful embryo development. Embryo density is considered to be one of the crucial factor affecting embryo development; increasing the embryo to medium volume ratio enhanced embryo development *in vitro* (Canceco *et al.*, 1992; Lane and Gardner, 1992). Furthermore, increasing embryo density allowed growth factors to accumulate near the embryos, with effects similar to adding growth factors to the culture medium. Considering the effect of embryo density and medium volume, a few research groups reported culture devices: glass oviduct (GO; Thouas *et al.*, 2003), the well of the well (WOW; Vajta *et al.*, 2000; Taka *et al.*, 2005) and microchannel (microfluidic device; Raty *et al.*, 2004) systems that have been designed to be similar to an *in vivo* environment and showed higher developmental rates using small number of embryos.

Covering oil is another limiting factor in embryo culture *in vitro*. It is an indispensable overlay in the micro-droplet embryo culture system. Transparency of mineral oil allows monitoring the embryos and also prevents evaporation of the medium. However, an oil overlay can absorb lipophilic factors from the culture medium, and conversely, deleterious materials can diffuse into the medium (Millter *et al.*, 1994). Even a small amount of oil-covered medium can be harmful to embryonic development (Lane and Gardner, 1992). In some cases, less purified (or refined) oil arrested normal embryo development by transmitting toxic materials to embryos (Lee *et al.*, 2004). To

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eliminate or decrease its harmful effects, attempts have been made to reduce the amount of oil. For example, the GO system used only 0.2 μl of mineral oil.

Previously, we reported that oil-free microtube culture (MTC) system enhanced the developmental speed of embryos, blastocyst formation rate, and total cell number in blastocysts when compared with the conventional drop culture (Drop) system in the parthenogenetic murine embryo culture (Roh *et al.*, 2008). Because the embryos are placed together in the small area (<2 mm diameter) of the U-shaped microtube bottom, the MTC system provides embryos more opportunities to affect each other through potential paracrine actions than a conventional drop culture system. The MTC system was also effective for the generation of PESC lines in either F1-hybrid (B6D2F1) or C57BL/6 mice and also showed the promoted expression of developmentally important genes, such as IGF-1, DNMT3a and OCT-4 which are important for the generation of pluripotent stem cells (Choi *et al.*, in press).

The objective of this study is to establish a completely oil-free culture system named MTC for *in vitro* culture of porcine parthenotes. The new culture system is compared with the conventional drop culture system using the same embryo density after optimizing the number of oocytes and the volume of the culture medium.

MATERIALS AND METHODS

1. Chemicals

All inorganic and organic compounds were obtained from Sigma-Aldrich Korea (Yong-in, Korea) unless otherwise stated.

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

Slaughterhouse ovaries were collected from 5 to 6-month-old prepubertal gilts (100±10 kg of body-weight), placed in saline at 30~35℃, and transported within 2 h to the laboratory. After washing with saline three times, cumulus-oocyte complexes (COCs) were recovered by aspiration of 2- to 5-mm follicles using an 18-gauge hypodermic needle attached to a 5-ml disposable syringe. After washing three times in IVM medium, COCs that were enclosed by more than three layers of compact cumulus cells and an evenly granulated ooplasm were selected for IVM. 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 46~48 h at 38.5℃ and 5% CO_2. The IVM medium for oocytes is composed of tissue culture medium 199 with Earle's salts and L-glutamine (TCM199, Gibco Life Technologies Inc., USA) supplemented with 26.2 mM $NaHCO_3$, 3.05 mM glucose, 0.91 mM sodium pyruvate, 0.57 mM L-cysteine, 10 ng/ml epidermal growth factor, 10 IU/ml equine chorionic gonadotropin and human chorionic gonadotropin, and 0.1% (v/v) polyvinylalcohol (PVA).

3. Parthenogenesis and *In Vitro* Culture

Electrical activation was performed at room temperature using a CF-150/B electro-cell fusion system (BLS, Hungary) in a chamber that contained two stainless steel electrodes that were 1.0 mm apart and that was filled with activation buffer. Oocytes were activated with a 1.6 kV/cm DC pulse for 40 μsec in 0.26 M mannitol supplemented with 0.1 mM $MgSO_4$, 0.05 M $CaCl_2$, and 0.01% PVA. The activated oocytes were treated for 5 to 6 h in NCSU-23 supplemented with 5 μg/ml cytochalasin B, 0.04% BSA, essential amino acids and non-essential amino acids (AAs). In the first series of experiment, the oocytes were then cultured either in 10- or 20-μl micro-drops (1, 5, 10, 20, 30 or 40 embryos per drop) of NCSU-23 with 0.04% BSA and AAs for 7 days at 38.5℃ and 5% CO_2 atmosphere after rinsing with culture medium nine times. In the second series of experiment, the activated oocytes were cultured either in 10- or 20-μl micro-drops at the same number of embryos (10 embryos per tube) on a 35-mm cell culture dish (cat. no. 353002, Falcon; BD Biosciences, San Jose, CA, USA) of oil-covering or in a 250 μl microtube (the type commonly used for PCR; cat. no. PCR-02-C; Axygen, Union City, CA, USA; 10 embryos per tube), as described in the previous report (Roh *et al.*, 2008).

4. Statistic Analysis

Experiments were repeated at least five times, and the developmental rates were analyzed using Chi-square test of SPSS (Version 12.0; SPSS Inc., Chicago, IL, USA). Difference at $p<0.05$ was considered significant.

RESULTS

In the first series of experiment, embryo density and culture medium volume were optimized under the standard micro-drop culture system. After eliminating the data assessed comparatively low blastocyst formation, optimal embryo density range for *in vitro* culture was produced: 0.5 embryo per μl in 20-μl micro-drop (20.5%, Fig. 1A) and 1.0 embryo per μl in 10-μl

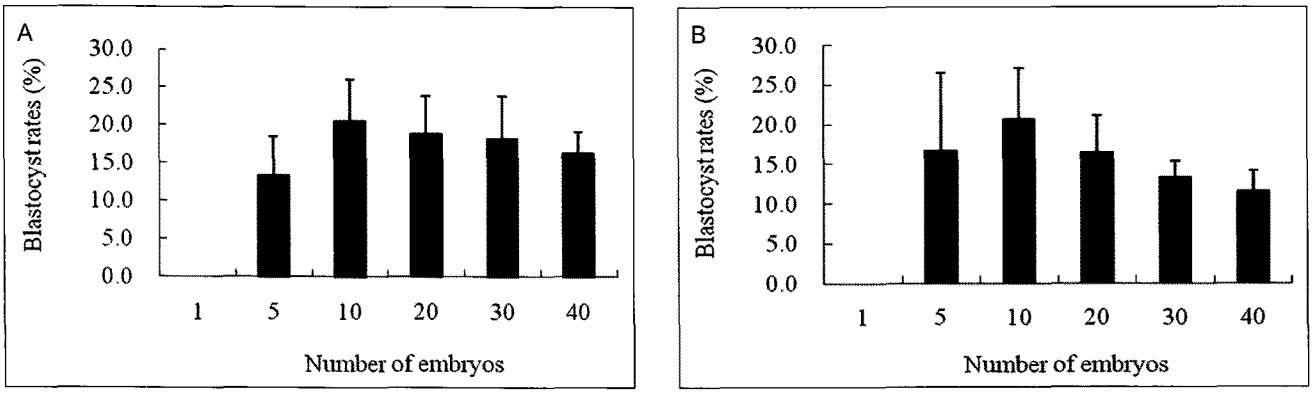

Fig. 1. The blastocyst formation rate of parthenogenetically activated porcine oocytes under different embryo density in micro-drop culture system. Parthenogenetic porcine embryos were cultured for 7 days either in 20- (A) or 10-μl (B) micro-drops: 1, 5, 10, 20 or 40 embryos per drop, respectively. Data are expressed as mean±SD.

micro-drop (20.6%, Fig. 1B) which means 10 oocytes in each drop regardless of its volume. However, there was no statistic difference among the experimental groups. Based on these results, we compared micro-drop culture system and MTC system in terms of the developmental rate to the blastocyst stage (Fig. 2). In 20-μl medium volume, the MTC system showed similar blastocyst formation rate when compared with micro-drop culture system (20.2% versus 20.5%, respectively). In 10-μl medium volume, however, the 'MTC' system showed significantly lower blastocyst formation rate than micro-drop culture system (12.7% versus 20.6%, respectively, $P<0.05$).

DISCUSSION

In the previous reports, we presented a new oil-free embryo culture system in mice, called MTC (Roh *et al.*, 2008). This novel embryo culture system enhances the development of pre-implantation stage murine embryos *in vitro* as well as providing the oil-free culturing environment. More embryos in MTC reached the blastocyst stage with a larger number of trophectodermal cells and developed faster than those in conventional micro-drop culture. In addition, we established the embryonic stem cell lines from parthenogenetic murine blastocysts (PESC) which was derived from MTC and found that the MTC system is a more effective way of generating PESC than the traditional oil-covered micro-drop culture (Choi *et al*, in press). We also generated a PESC from the parthenotes of the C57BL/6 inbred mouse strain, which is more difficult than generating a cell line from 129Sv or F1-hybrid, such as B6D2F1 or C57BL/6 × CBA (Araki *et al.*, 2009; Cheng *et al.*, 2004; Meng *et al.*, 2003).

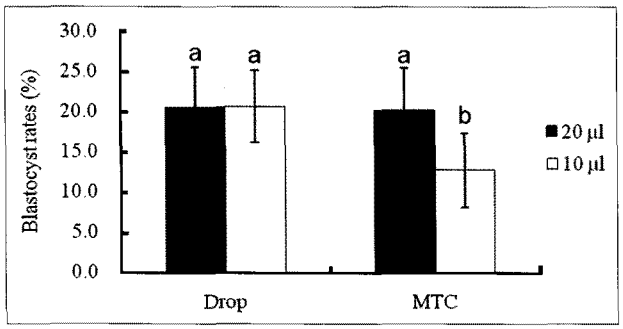

Fig. 2. Comparison of the blastocyst formation rate of parthenogenetically activated porcine oocytes between micro-drop (Drop) and microtube (MTC) systems. In each culture system, parthenogenetic porcine embryos were cultured for 7 days either in 20- or 10-μl of culture medium (10 embryos each). Data are expressed as mean±SD. $^{ab}P<0.05$.

There is a remarkable difference between conventional drop culture and MTC: the use of an oil overlay. A layer of mineral oil over the culture medium was necessary for drop cultures to prevent evaporation of the medium and maintain its three-dimensional, hemisphere structure. However, there was no significant change in the osmolarity of media in MTC (range, 281 to 286 mOsm; Roh *et al.*, 2008). Conversely, oil overlay may have affected embryo development *in vitro* because it can absorb lipophilic factors in the medium and diffuse unknown deleterious materials into the medium and in that regard, oil on the medium decreased the developmental competence of parthenogenetic embryos in the previous mouse study (Roh *et al.*, 2008). However, in the present study using porcine parthenotes, there was no positive effect on the improvement of the

in vitro embryonic development. It may be from the differences between murine and porcine embryos (or parthenotes) such as their sizes, neutralization of positive effects due to the more competition for energy substrates and the build up of metabolic by-product such as ammonium and lactate in porcine embryos. To apply MTC for porcine embryo culture, the volume of medium should be over 20 μl when 10 or more embryos are cultured in a microtube as we obtained appropriate number of blastocysts only in 20-μl MTC.

As the next step, we made a supplementary study to evaluate the effect of MTC system on CO_2 buffering in the medium. For evaluation, the culture medium which was pre-equilibrated with 5% CO_2 for over 6 h was used to make oil-overlaid micro-drop and oil-free MTC. Then these two culture devices were incubated without CO_2 supply. As a result, CO_2 buffering and retention time was longer in MTC system than in drop culture one (data not shown). This makes MTC useful for short term storage without using 5% CO_2 incubator. The MTC system is also easier to handle than embryo straws which need some device or material for their sealing to keep the embryos inside.

In conclusion, we established oil-free MTC system for porcine embryo culture and the 20-μl MTC system may be an alternative incubation system for short-distance embryo transport without carrying the CO_2 incubator and this provides a novel embryo culture device to clinical veterinary embryologists.

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