Optimization of Embryo Density and the Volume of Culture Medium for an Improvement of Mouse Parthenogenetic Embryo Development

Sangho Roh[2,3,4], Young-Joo Choi[2,4] and Byung-Moo Min[1,2,3,4,†]

[1]*Department of Oral Biochemistry*

ABSTRACT

Autocrine or paracrine mediators released by the early embryo are implicated in the support of embryonic development. Their mechanisms and optimal embryo density in the medium, however, are uncertain. This study was conducted to establish the optimal embryo density and culture medium volume in mouse parthenogenetic embryo culture. In experiment 1, culture of parthenogenetically activated oocytes at a concentration of 2~4 embryos/ μL significantly improved development to the blastocyst stage (72% ≤) compared with culture at the lower (0.2~1 embryos/ μL, 0~37.5%) and the higher (5~6 embryos/ μL, 30~53%) concentration for 120 h when the oocytes were cultured in a 5 μL drop under mineral oil. In experiment 2, the embryos cultured at a concentration of 2~4 embryos/ μL in a 10 μL drop (81.1%) showed significantly higher blastocyst rates than those in a 5 μL drop (68.5%). This study optimizes *in vitro* culture condition by modifying embryo density and the volume of culture medium. It may give appropriate level of autocrine and/or paracrine factors to enhance viability and subsequent normal development of mouse parthenogenetic embryos *in vitro*.

(Key words : Parthenogenesis, Embryo density, Culture medium volume, B6D2 F_1 mouse)

INTRODUCTION

The relative autonomy of preimplantation embryo growth, together with accumulating evidence for a role of released embryo-derived factors (Wiley *et al.*, 1986) in supporting embryonic development, has provided support for a theory of regulation of embryonic development by autocrine/paracrine growth factors. Recent studies have implicated a variety of putative growth factors including platelet-activating factor (PAF; O'Neill, 1998; O'Neill, 1997), insulin-like growth factor I (IGF-I; Schultz *et al.*, 1993; Harvey and Kaye, 1992a), IGF-II (Harvey and Kaye, 1992b), and epidermal growth factor/transforming growth factor-α (Woods and Kaye, 1989; Babalola and Schultz, 1995). Their mechanisms of action is poorly understood, while the very nature of autocrine/paracrine mediators (produced by and acting on the same cell populations) make such studies difficult. It was proposed that combined use of *in vitro* fertilization and culture at low embryo concentration provides a functional, multiple-growth factor ablation model for defining the role of autocrine/parcrine factors in early embryonic development (O'Neill, 1997). Using this model, O'Neill (1998) showed that exposure of embryos to autocrine/paracrine factors during the 2-cell stage is necessary for subsequent normal development to the blastocyst stage. Deprivation during this time led to subsequent retarded development with an increased incidence of cell death in embryos. However, the embryos cultured at higher embryo concentration may produce more metabolites or oxygen radicals than those at lower concentration, which is detrimental against further embryonic development. Mechanisms of autocrine/paracrine factors and metabolites in the medium, however, are still uncertain. Hence, optimization of embryo concentration in the medium is required to increase efficacy of viable embryo

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[2] Craniomaxillofacial Reconstructive Science Major.

[3] BK21 HLS.

[4] Dental Research Institute, Seoul National University College of Dentistry, Seoul 110-749, Korea.

† Corresponding author : Dr. B. M. Min, Department of Oral Biochemistry and Craniomaxillofacial Reconstructive Science, Seoul National University College of Dentistry, 28 Yeonkun-Dong, Jongno-gu, Seoul 110-749, Korea. TEL: +82-2-740-8661, FAX: +82-2-740-8665, E-mail: bmmin@snu.ac.kr

production *in vitro*.

This study was conducted to establish the optimal embryo density in F_1 hybrid (C57BL/6 × DBA/2; B6D2 F_1) mouse parthenogenetic embryos. In addition, we investigated whether different volume of medium with same embryo concentration affected mouse parthenogenetic embryo development.

MATERIALS AND METHODS

Reagents and Media

All inorganic and organic compounds were purchased from Sigma-Aldrich (St Louis, MO, USA) unless otherwise stated. All the media used here were based on CZB and KSOM media (Nagy *et al.*, 2003).

Recovery of Oocytes

Eight-week old B6D2 F_1 mice were superovulated by intraperitoneal injections of 7.5 IU equine chorionic gonadotropin (eCG) and 7.5 IU human chorionic gonadotropin (hCG) given 48 h apart. Superovulated females were killed by cervical dislocation at 16 h after hCG injection, and the oviducts were removed and transferred into a Petri dish containing 2 mL Hepes-buffered CZB medium (HCZB) supplemented with 300 IU/mL hyaluronidase (bovine testis). The oviduct ampullae were opened, and the cumulus-enclosed oocytes were released. After 2~3 min exposure to the medium, the cumulus-free oocytes were washed twice in HCZB before activation.

Activation and *In Vitro* Culture

Recovered oocytes were then immediately exposed to activation medium consisting of 10 mM $SrCl_2$ with 5 μg /mL cytochalasin B in calcium-free CZB for 5 h or cultured in CZB in an atmosphere of 5% CO_2 in air. Following this activation the oocytes were cultured in KSOM at 37℃ under mineral oil in an atmosphere of 5% CO_2 in air. Detailed experimental design is described belows. Activated and *in vitro* cultured oocytes were then recorded the rates of their development to the blastocyst stage after 120 h of culture.

Statistic Analysis

Differences in the mean percentages of embryonic development among the treatments were analyzed by Chi-Square test.

Experimental Designs

In experiment 1, the activated oocytes were cultured in the medium of a 5 μL drop at concentrations of 0.2, 1, 2, 3, 4, 5 and 6 embryos/μL. In experiment 2, the oocytes were cultured at a concentration of 2~4 embryos/μL either in a 5 or 10 μL drop.

Animal Ethics

All animal experiments were approved and performed under the guidelines of Institutional Animal Care and Use Committee in Seoul National University.

RESULTS

In experiment 1, culture of parthenogenetically activated oocytes at a concentration of 2~4 embryos/μL significantly improved development to the blastocyst stage (72.0% ≤) compared with culture at the lower (0.2~1 embryos/μL, 0~37.5%) and the higher (5~6 embryos/μL, 30.0~53.0%) concentration for 120 h when the oocytes were cultured in a 5 μL drop under mineral oil ($p<0.05$, Table 1). In experiment 2, the embryos cultured at a concentration of 2~4 embryos/μL in a 10 μL drop (81.1%, 73/90) showed significantly higher blastocyst rates than those in a 5 μL drop (68.5%, 202/295; $p = 0.036$, Table 2).

DISCUSSION

The present study aimed for an improvement of parthenogenetic embryo development in B6/D2 mice which is prerequisite step in SCNT programme. The strain we chose (B6/D2) is known as one of the best strain for micromanipulation since their MII chromosomes are visible without the use of fluorescence dye and they are also tolerence to nuclear injection. To optimize *in vitro*

Table 1. Effect of embryo density on *in vitro* development of mouse parthenotes

Embryo density* (embryos/μL)	Total oocytes	Development to blastocysts by 120 h (%)
0.2	6	0 (0.0)
1	40	15 (37.5)^{ac}
2	50	36 (72.0)^{b}
3	60	46 (76.7)^{b}
4	140	105 (75.0)^{b}
5	100	53 (53.0)^{a}
6	90	27 (30.0)^{c}

* Embryos were cultured in 5 μL drops under mineral oil.
$^{a\sim c}$ $p<0.05$.

Table 2. Effect of the size of medium volume on *in vitro* development of mouse parthenotes

Volume of culture medium	Total oocytes	Development to blastocysts by 120 h (%)
5 μL	295	202 (68.5)[a]
10 μL	90	73 (81.1)[b]

[ab] p=0.036.

culture condition for mouse parthenogenetic embryos, first we tested embryo density at concentrations of 0.2, 1, 2, 3, 4, 5 and 6 embryos/μL in the medium of a 5 μL drop. It has been argued that cells require paracrine or autocrine survival factors for their normal function and that deprivation of such factors causes cells to undergo cell death by default (Ishizaki *et al.*, 1995, Brison and Shultz, 1997). As described in Table 1, the embryos cultured at a concentration of 2~4 embryos/μL showed better development to the blastocyst stage than standard culture condition (a embryo/μL, O'Neill, 1998) or single embryo culture (1 embryo/5 μL = 0.2 embryo/μL). This result provides evidence that embryo-derived factors, which are limited by dilution, are required and that their autocrine/paracrine action is necessary for the subsequent normal development of the preimplantation embryos. Culturing at low embryo concentrations (0.2 to 1 embryo/μL) resulted in an obvious reduction in embryonic development evidenced by low blastocyst formation. In addition, the development of these embryos tended to be retarded (data not shown). It was previously proposed that the adverse effect of reduced embryo concentration was not affected by the absolute volume of medium, the vessel type or geometry, or the number of embryo present (O'Neill, 1997). However, in the present study, different volume of medium (5 μL versus 10 μL) caused embryos to have different developmental competence evidenced by blastocyst formation. The embryos in a 10 μL drop tended to have better developmental potential than those in a 5 μL drop. It is possible that detrimental factors released from the embryos such as oxygen radicals or metabolites diffused further in a 10 μL medium drop than in a 5 μL one. Another explanation of this result is that actual number of embryos ('20~40' embryos/10 μL vs '10~20' embryos/5 μL) or actual amount of diffusible autocrine/paracrine factors from those embryos is more important than embryo density or concentration.

In conclusion, this study optimizes *in vitro* culture condition by modifying embryo density and the volume of culture medium. It may give appropriate level of autocrine and/or paracrine factors to enhance viability and sub-sequent development of mouse parthenogenetic embryos *in vitro*. Further experiments will define the mechanisms of action of autocrine/paracrine factors required for normal development of mouse parthenogenetic embryos.

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