Existence of Amino Acids in Defined Culture Medium Influences *In Vitro* Development of Parthenogenetic and Nuclear Transfer Porcine Embryos

Cheolhee Won, Sang Kyu Park, Kiyoung Kim and Sangho Roh*

Craniomaxillofacial Reconstructive Sciences Major, Dental Research Institute and BK21 CLS, School of Dentistry, Seoul National University, Seoul 110-749, Republic of Korea

ABSTRACT

This study was designed to investigate the effect of essential amino acids (EAA) and/or non-essential amino acids (NEAA) on the development of parthenogenetic and somatic cell nuclear transfer (SCNT) porcine embryos *in vitro*. To evaluate the timing of amino acids supplementation, activated oocytes were cultured in NCSU23-PVA with EAA, NEAA or NEAA+EAA (AAs) during specific periods as below: EAA, NEAA or AAs were supplemented during Day 0 to 6 (whole culture period: ALL), Day 2 to Day 6 (post-maternal embryonic transition period: POST-MET), Day 5 to Day 6 (post-compaction period: POST-CMP), Day 0 to Day 2 (pre-maternal embryonic transition period: PRE-MET), or Day 0 to Day 4 (post-compaction period: PRE-CMP). Supplementation of NEAA decreased cleavage rates in PRE-MET and PRE-CMP and also decreased blastocyst rates in POST-CMP. On the other hand, EAA significantly enhanced blastocyst formation rate in POST-MET and no detrimental effect on embryonic development in other groups. Interestingly, NEAA and EAA had synergistic effect when they were supplemented to the medium during whole culture period. Supplementation of AAs also enhanced SCNT porcine embryo development whereas BSA-free medium without AAs could not supported blastocyst formation of SCNT embryos. In conclusion, existence of EAA and NEAA in defined culture medium variously influences the development of parthenogenetic and SCNT porcine embryos, and their positive effect are only occurred when both EAA and NEAA are supplemented to the medium during whole culture period. Additionally, AAs supplementation enhances the blastocyst formation of SCNT porcine embryos when they are cultured in the defined condition.

(Key words : amino acid, porcine embryo, *in vitro* culture, parthenogenesis, nuclear transfer)

INTRODUCTION

Chemically defined media, using purified reagents instead of biological fluids or sera, can reduce the risk of contamination by pathogens or unknown factors and can also increase our understanding of the metabolic requirements of embryos (Bavister, 1995; Keskintepe *et al.*, 1995; Liu and Foote, 1995; Holm *et al.*, 1999). Polyvinyl alcohol (PVA) has been used to establish chemically defined culture systems because PVA is an effective substitute of serum or serum albumin that efficiently supports the development of porcine embryos (Ledda *et al.*, 1992; Kim *et al.*, 1993; Van Thuan *et al.*, 2002). However, compared to BSA supplementation, PVA supplementation inhibits blastocyst formation and hatching (Biggers *et al.*, 1997), but the addition of amino acids to defined culture media could restore the problems of PVA supplementation (Biggers *et al.*, 2000). Amino acids play important roles during early embryonic development such as protein biosynthesis, stimulation of embryonic genome activation, energy production, cell signal transduction and others (Van Winkle, 2001). Although many other research groups also have reported that exogenous amino acids have beneficial effects on embryonic development in mammals (Lee *et al.*, 2004; Gardner and Lane, 1993; Miyoshi *et al.*, 1995; McKiernan *et al.*, 1991), the result from parthenogenetic or somatic cell nuclear transfer (SCNT) porcine embryo culture quite vary from other animals (Van Thuan *et al.*, 2002). Many reports related with supplementation of amino acids for porcine embryo culture focused on beneficial effect of non essential amino acids (NEAA; Gupta *et al.*, 2008; Suzuki and Yoshioka, 2006; Van Thuan *et al.*, 2002) and Van Thuan *et al.* (2002) also reported that nonpolar essential amino acids (EAA) in a protein-free medium during the first 48 h

† This study was supported by the Korea Science and Engineering Foundation (KOSEF) grant funded by the Ministry of Education Science and Technology (MEST; R01-2007-000-20326-0 and 2008-04347), Republic of Korea.

* Correspondence : E-mail : sangho@snu.ac.kr

caused the 4-cell block in parthenogenetic porcine embryos. Although the effects of amino acids on the porcine embryo development were reported by several research groups mentioned above, the information is still not enough that which stage of preimplantation porcine embryos were affected by EAA or NEAA components. First, we chose parthenogenetic embryos as the characteristics of development of diploid porcine parthenotes to the blastocyst stage resembled those of *in vitro* fertilized oocytes (Kure-bayashi *et al.*, 2000). To evaluate the timing of amino acids supplementation, porcine parthenotes were cultured in EAA, NEAA or EAA+NEAA (AAs) supplemented medium during specific culture periods (whole culture period, post-maternal embryonic transition period, post-compaction period, pre-maternal embryonic transition period, or post-compaction period). In this study, we investigated the effects of amino acid components on the development of parthenogenetic and SCNT porcine embryos to the blastocyst stage to optimize defined culture system for the production of porcine embryos *in vitro*.

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 48~50 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, Invitrogen, 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) 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 5~6 h in North Carolina State University-23 (NCSU-23) medium supplemented with 5 μg/ml cytochalasin B and 1 mg/ml PVA. The oocytes were then washed nine times with culture medium and cultured in 20-μl drops (10~15 oocytes per drop) of the appropriate culture medium for 7 days at 38.5℃ and 5% CO_2. To evaluate the timing of amino acids supplementation, activated oocytes were cultured in NCSU23-PVA with EAA, NEAA or NEAA+EAA (AAs) during specific culture periods as below: EAA, NEAA or AAs were supplemented during Day 0 to 6 (whole culture period group: ALL), Day 2 to Day 6 (post-maternal embryonic transition period group: POST-MET), Day 5 to Day 6 (post-compaction period group: POST-CMP), Day 0 to Day 2 (pre-maternal embryonic transition period group: PRE-MET), or Day 0 to Day 4 (pre-compaction period group: PRE-CMP). In each group, 150~160 oocytes were evaluated. Detailed experimental design was described in Fig. 1.

4. Preparation of Fetal Fibroblasts

Fibroblasts were obtained from porcine conceptuses that were collected between embryonic days 28~39. The head, extremities, and internal organs were removed and the remaining tissues were cut into small pieces. Cells were dispersed by ex-

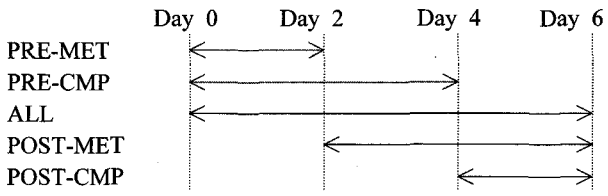

Fig. 1. Schematic explanation of amino acid supplementation to porcine embryo culture medium. Bidirectional arrows indicate the period of amino acids exposure to the embryos in culture. PRE-MET (pre-maternal embryonic transition period group), PRE-CMP (pre-compaction period group), ALL (whole culture period group), POST-MET (post-maternal embryonic transition period group), POST-CMP (post-compaction period group).

posure to 0.25% (w/v) trypsin solution (Invitrogen) and cultured in Dulbecco's minimum essential medium (Invitrogen) supplemented with 10% FBS (Invitrogen). For each passage, cells were cultured until confluent, disaggregated by incubation in a 0.25% trypsin solution for 1 min at 37℃, and fractionated into three new dishes. The cell lines were maintained in culture for at least 8 passages. For long-term storage, cells were collected after trypsinization, frozen in 10% dimethyl sulfoxide, and stored in liquid nitrogen.

5. Enucleation

To minimize the loss of mRNA and ooplasmic factors as a result of enucleation, we used our demecolcine-derived enucleation technique (Won *et al.*, 2008) modified from Yin *et al.* (2002). Briefly, matured oocytes containing the first polar body were cultured for 2 h in NCSU-23 medium supplemented with 0.4 μg/ml demecolcine and 0.05 M sucrose. Oocytes with a protruding membrane were then moved to medium containing 5 μg/ml cytochalasin B, 0.4 μg/ml demecolcine, and 0.05 M sucrose. The protrusion was aspirated with a 12-μm outer diameter beveled glass pipette.

6. Whole Cell Injection and *In Vitro* Culture

The fetal fibroblasts used as donor cells were transferred to a 10% (w/v) polyvinylpyrrolidone-supplemented Hepes-buffered TCM199 microdrop (PVP drop, 6 μl) and gently mixed at room temperature. A microdrop of Hepes-buffered TCM199 (injection drop) was placed adjacent to the PVP drop, and the drops were covered with light mineral oil to prevent evaporation. Individual fibroblasts in PVP drops were aspirated into the injection pipette (inner diameter 10~12 μm; HUMAGEN, Netherlands). The cytoplasmic membrane of the fibroblasts was ruptured by repetitive pipetting. An injection pipette, containing ruptured fetal fibroblast, was inserted into the injection drop, and the ruptured fibroblasts were injected into the enucleated oocytes through the cytoplasmic membrane. After injection, the reconstructed embryos remained in medium for 90 min before activation. The activation process for reconstructed embryos was the same as that used for parthenogenetic embryos. The reconstructed oocytes were washed with culture medium nine times and cultured in 20 μl drops (10~15 oocytes per drop) of NCSU-23 with either 1 mg/ml PVA (with or without AAs) or 4 mg/ml BSA for 7 days at 38.5℃ in 5% CO_2 in air and the number of total and hatching blastocysts were counted (Fig. 2). In total, 804 SCNT embryos were evaluated (250~300 embryos per group).

7. Statistic Analysis

Experiments were repeated 3~5 times, and mean values were analyzed by ANOVA analysis of variance. Difference at $p<0.05$ was considered significant.

RESULTS

Supplementation of NEAA decreased cleavage rates in PRE-MET (Fig. 3A) and PRE-CMP (Fig. 3B) and also decreased blastocyst rates in POST-CMP (Fig 3C). On the other hand, EAA significantly enhanced blastocyst formation and expansion rates in POST-MET while NEAA had no effect on blastocyst formation in the same group (Fig. 3D). No detrimental effect of EAA on embryonic development was found in all groups (Fig. 3A-E). Interestingly, NEAA and EAA had synergistic effect on the blastocyst formation and expansion when they were supplemented to the culture medium during whole culture period (Fig. 3E) The synergistic effect also tended to occur in PRE-MET and PRE-CMP but not significant. Supplementation of AAs to the medium without BSA but containing PVA also enhanced *in vitro* development of SCNT porcine embryos whereas BSA-free medium without AAs (group PVA) could not support blastocyst formation of SCNT embryos (Fig. 4).

DISCUSSION

The results above indicate that positive effect of AAs was occurred when both EAA and NEAA were supplemented to the medium during whole culture period. In addition, A As supplementation was essential for the development of SCNT porcine embryos when they were developed in completely de-

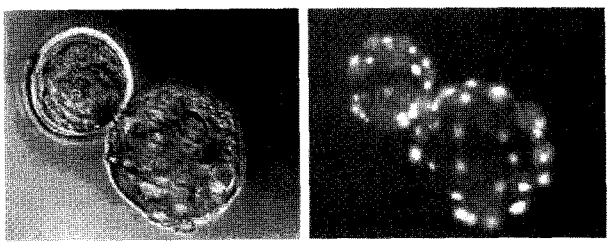

Fig. 2. A hatching stage somatic cell nuclear transfer porcine blastocyst derived from *in vitro* culture using essential and non-essential amino acids supplemented chemically defined culture medium.

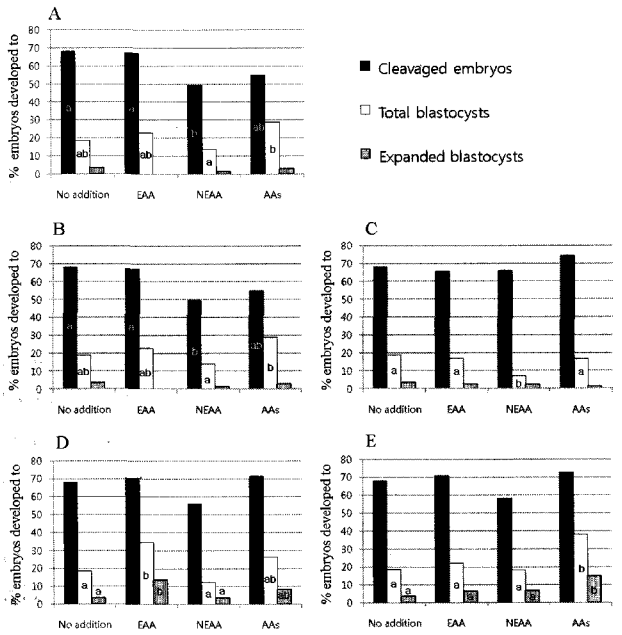

Fig. 3. Effects of the timing of essential (EAA) and non-essential (NEAA) amino acids addition on *in vitro* development of parthenogenetic porcine embryos. After electric activation, the embryos were cultured in the medium supplemented with EAA, NEAA or EAA+NEAA (AAs) or without amino acids according to the experimental groups (see Fig. 1 for experimental design and abbreviations of the groups). Values among different letters within each developmental stage are significantly different ($p<0.05$). A: PRE-MET; B: PRE-CMP; C: POST-CMP; D: POST- MET; E: ALL.

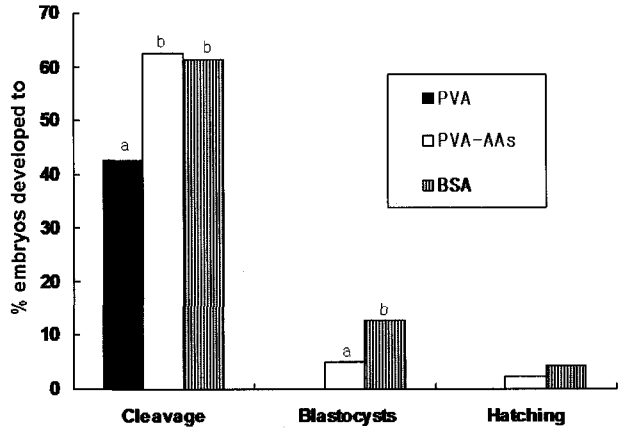

Fig. 4. Effect of amino acids supplementation on *in vitro* development of somatic cell nuclear transfer porcine embryos. Values among different letters within each developmental stage are significantly different ($p<0.05$). AAs: essential and non-essential amino acids.

fined culture condition.

As the information is still not enough that which stage of preimplantation porcine embryos were affected by EAA or NEAA components, we selected maternal embryonic transition and compaction as critical points for the supplementation of AAs. Maternal embryonic transition means the switch from maternal to embryonic genome control including 1) loss or decay of mRNA molecules of maternal origin, 2) activation of transcription of early embryonic genome, 3) developmental arrest in the presence of transcriptional inhibitors, and 4) marked qualitative changes on protein synthetic patterns (Telford *et al.*, 1990). The other point we chose was compaction of morula stage embryos. Compaction is one of the most crucial phenomena in early mammalian embryonic development (Gilbert, 2003). After compaction, morula stage embryos were polarized then formed blastocysts which initiate differentiation. In the present study, EAA, NEAA or AAs were supplemented before or after maternal embryonic transition and compaction. Embryonic day 2 and 4 after *in vitro* culture (timing of electric activation as Day 0) were considered as maternal embryonic transition and compaction respectively although the parthenotes were not at the same stage exactly.

In the present study, EAA during first 48 h after electric activation (PRE-MET group) did not inhibit development beyond the 4-cell stage and this is quite different from the previous report by Van Thuan *et al.* (2002). On the other hand, when NEAA were exposed to the embryos before maternal embryonic transition or compaction, initiation of embryonic development (cleavage) was disturbed. Lane and Gardner (1997) reported that EAA had no benefit for the development before the 8-cell stage in the mouse, whereas NEAA and glutamine significantly increased cleavage rates during the first four cell cycles. This may be caused by species specificity or different experimental conditions. We also found that NEAA and EAA should be supplemented together because they had synergistic effect on the blastocyst formation and expansion when they were supplemented to the culture medium during whole culture period whereas the other research group reported that only NEAA could enhance blastocyst formation rate (Gupta *et al.*, 2008). In our study, synergistic effect of EAA and NEAA supplementation was remarkable to the expansion of blastocysts in the parthenogenetic embryos. Van Thuan *et al.* (2002) also reported that NEAA after 4-cell stage produced favorable condition for the expansion of blastocysts. However, we could obtain similar results when we supplemented EAA to the me-

dium, not NEAA. Those differences may be from the different conditions of *in vitro* maturation, activation, and/or culture. As second step, we cultured SCNT porcine embryos using AAs supplemented protein-free, chemically defined culture medium. When AAs were supplemented to the defined culture medium, the cleavage rate was comparable to that of BSA supplemented counterpart. As no SCNT blastocyst was obtained in PVA group, it was clear that addition of AAs to defined medium supported blastocyst formation and hatching in SCNT porcine embryos although the rates were still lower than those in BSA group. As BSA uptaken by the embryos can be utilized as amino acids, supplementary beneficial effect of BSA other than protein biosynthesis may be the reason for difference of developmental competence between BSA and PVA groups.

In conclusion, existence of EAA and NEAA in defined culture medium variously influenced *in vitro* development of parthenogenetic and SCNT porcine embryos, and their positive effect were only occurred when both EAA and NEAA were supplemented to the medium during whole culture period. In addition, AAs supplementation was essential for the development of SCNT porcine embryos when they were developed in defined culture condition.

REFERENCES

Bavister BD. 1995. Culture of preimplantation embryos: Facts and artifacts. Hum. Reprod. Update 1:91-148.

Biggers JD, McGinnis LK and Raffin M. 2000. Amino acids and preimplantation development of the mouse in protein-free potassium simplex optimized medium. Biol. Reprod. 63: 281-293.

Biggers JD, Summers MC and McGinnis LK. 1997. Polyvinyl alcohol and amino acids as substitutes for bovine serum albumin in culture media for mouse preimplantation embryos. Hum. Reprod. Update 3:125-135.

Gardner DK and Lane M. 1993. Amino acids and ammonium regulate mouse embryo development in culture. Biol. Reprod. 48:377-385.

Gupta MK, Uhm SJ, Lee SH and Lee HT. 2008. Role of non-essential amino acids on porcine embryos produced by parthenogenesis or somatic cell nuclear transfer. Mol. Reprod. Dev. 75:588-597.

Holm P, Booth PJ, Schmidt MH, Greve T and Callesen H. 1999. High bovine blastocyst development in a static *in vitro* production system using SOFaa medium supplemented with sodium citrate and myo-inositol with or without serum-proteins. Theriogenology 52:683-700.

Keskintepe L, Burnley CA and Brackett BG. 1995. Production of viable bovine blastocysts in defined *in vitro* conditions. Biol. Reprod. 52:1410-1417.

Kim JH, Niwa K, Lim JM and Okuda K. 1993. Effects of phosphate, energy substrates, and amino acids on development of *in vitro*-matured, *in vitro*-fertilized bovine oocytes in a chemically defined, protein-free culture medium. Biol. Reprod. 48:1320-1325.

Kure-bayashi S, Miyake M, Okada K and Kato S. 2000. Successful implantation of *in vitro*-matured electroactivated oocytes in the pig. Theriogenology 53:1105-1119.

Lane M and Gardner DK. 1997. Differential regulation of mouse embryo development and viability by amino acids. J. Reprod. Fertil. 109:153-164.

Ledda S, Loi P, Branca A, Cappai P, Dattena M and Naitana S. 1992. Use of a protein-free medium for the *in vitro* development of sheep embryos. Boll. Soc. Ital. Biol. Sper. 68: 305-309.

Lee ES, Fukui Y, Lee BC, Lim JM and Hwang WS. 2004. Promoting effect of amino acids added to a chemically defined medium on blastocyst formation and blastomere proliferation of bovine embryos cultured *in vitro*. Anim. Reprod. Sci. 84:257-267.

Liu Z and Foote RH. 1995. Effects of amino acids on the development of *in-vitro* matured/*in-vitro* fertilization bovine embryos in a simple protein-free medium. Hum. Reprod. 10:2985-2991.

McKiernan SH, Bavister BD and Tascar RJ. 1991. Energy substrate requirements for *in vitro* development of hamster 1- and 2-cell embryos to the blastocyst stage. Hum. Reprod. 6:64-75.

Miyoshi K, Abeydeera LR, Okuda K and Niwa K. 1995. Effects of osmolarity and amino acids in a chemically defined medium on development of rat one-cell embryos. J. Reprod. Fertil. 103:27-32.

Scott FG. 2003. Developmental Biology. 7th ed, Sinauer Associates, Inc., Sunderland, MA., pp. 366-367.

Suzuki C and Yoshioka K. 2006. Effects of amino acid supplements and replacement of polyvinyl alcohol with bovine serum albumin in porcine zygote medium. Reprod. Fertil. Dev. 18:789-795.

Telford NA, Watson AJ and Schultz GA. 1990. Transition from maternal to embryonic control in early mammalian

development: a comparison of several species. Mol. Reprod. Dev. 26:90-100.

Van Thuan N, Harayama H and Miyake M. 2002. Characteristics of preimplantational development of porcine parthenogenetic diploids relative to the existence of amino acids *in vitro*. Biol. Reprod. 67:1688-1698.

Van Winkle LJ. 2001. Amino acid transport regulation and early embryo development. Biol. Reprod. 64:1-12.

Won C, Park SK, Cho SG, Min BM and Roh S. 2008. Kinetin enhances *in vitro* development of parthenogenetic and nuclear transfer porcine embryos. Mol. Reprod. Dev. 75: 1701-1709.

Yin XJ, Kato Y and Tsunoda Y. 2002. Effect of enucleation procedures and maturation conditions on the development of nuclear-transferred rabbit oocytes receiving male fibroblast cells. Reproduction 124:41-47.

(접수일: 2008. 10. 24 / 채택일: 2008. 11. 11)