Effect of Concentration and Exposure Duration of FBS on Parthenogenetic Development of Porcine Follicular Oocytes

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ABSTRACT

The aim of present experiment was to examine hatching rate as *in vitro* indicator of viability of porcine embryos before early stage embryo transfer such as zygotes or 2-cell stage embryos. Cumulus-oocyte complexes (COCs) collected from ovaries were matured in North Carolina State University 23 (NCSU-23) containing 10% porcine follicular fluid (pFF), 10 ng/ml epidermal growth factor (EGF), 10 μg/ml follicle stimulating hormone (FSH), 35 μg/ml luteinizing hormone (LH), and 1mg/ml cysteine. After 24 hours, the COCs were transferred to the same medium without hormones. After 65h of maturation, oocytes were exposed to phosphate buffered saline (PBS) with 7% ethanol (v/v) for 7 minutes, and then the oocytes were washed and cultured in tissue culture medium (TCM) 199 containing 5 ug/ml cytochalasin B for 5h at 38.5℃ in an atmosphere of 5% CO_2 and 95% air with high humidity. After cytochalasin B treatment, the presumptive parthenotes were cultured in porcine zygote medium (PZM)-5 and cleavage of the parthenotes was assessed at 72h of activation, Normally cleaved parthenotes were cultured for an additional 8 days to evaluate their ability to develop to blastocyst and hatching stages. The fetal bovine serum (FBS) were added at Day 4 or 5 with concentrations of 2.5, 5 or 10%. The blastocyst rates were ranged within 39.1~70% in each treatment. However hatching rate was dramatically decreased in non-addition group. In this experiment, embryo viability in female reproductive tract may be estimated before embryo transfer with *in vitro* culture adding FBS by hatching ability.

(Key words : porcine, parthenotes, fetal bovine serum, *in vitro* culture, hatching rate)

INTRODUCTION

The establishment of a reliable culture system for pig embryos derived from *in vitro* matured and oocytes, including animal cloning and production of transgenic pigs for human organ transplantation using somatic cell nuclear transfer, has vast postretinal for research and commercial use (Kim *et al.*, 2006). *In vitro* production of porcine embryos has become routine in most laboratories but the yield and quality of the resultant blastocysts remain suboptimal (Gupta *et al.*, 2007). Improvement of *in vitro* culture systems for the production of high-quality blastocysts is a very imperative step (Okada *et al.*, 2003).

Hatching is a good diagnostic indicator of embryo culture system because of a strong relationship between blastocyst quality and implantation as implantation-competence (Balaban *et al.*, 2000; Nomura *et al.*, 2007; Urman *et al.*, 2007). However, pig blastocyst is hard to hatching in culture system when we do not use the serum (Robl and Davis, 1981). Biphasic effect of serum on embryo development was demonstrated. Exposure of early bovine or porcine embryos to serum was detrimental to blastocyst development *in vitro* and significantly reduced the formation of hatched blastocysts (Robl and Davis, 1981; Pinyopummintr and Bavister, 1991; Bavister, 1995; Dobrinsky *et al.*, 1996; Wang and Day, 2002). In contrast, adding serum to culture medium from Day 5 accelerated the development of bovine blastocysts (Thompson *et al.*, 1998; Choi *et al.*, 2002). In addition, 80% of porcine *in vivo* zygotes developed into hatched blastocysts when serum was added on Day 5 (Dobrinsky *et al.*, 1996).

The aim of present experiment was to examine blastocyst formation and hatching rate as testing tool of porcine embryos viability before early stage embryo transfer such as zygotes or

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2-cell stage embryos. We evaluated the optimal concentrations and exposure durations of FBS on porcine parthenotes.

MATERIAL AND METHODS

1. Transportation of Ovaries

Ovaries were obtained from prepubertal gilts at a local abattoir and brought to the laboratory in physiological saline (0.85% [w/v] NaCl) with 100 μg/ml streptomycin (Sigma, USA) and 100IU/ml penicillin (Sigma, USA) at 30~33℃.

2. *In Vitro* Maturation of Porcine Follicular Oocytes

Ovaries were obtained from prepubertal gilts at a local abattoir and brought to the laboratory in physiological saline with antibiotics at 30~33℃. The ovaries were washed and wiped, and then cumulus-oocytes complexes in the follicular fluid were aspirated form surface-visible follicles (2~6 mm in diameter) with 10ml syringe fitted with an 18-gauge needle. After being washed three times with modified phosphate-buffered saline (DPBS; Gibco, USA) containing 0.3% bovine serum albumin (BSA), the COCs were suspended in maturation medium, NCSU-23 containing 10% (v/v) porcine follicular fluid, 10 ng/ml epidermal growth factor (EFG; Sigma, USA), 10 μg/ml follicular stimulating hormone (FSH; Sigma, USA) and 35 μg/ml luteinizing hormone (LH; Sigma, USA), cysteine 1 mg/ml (Sigma, USA), 100IU/ml penicillin G, and 100 μg/ml streptomycin sulfate (Gibco, USA). After 24 hours, the COCs were transferred to the same medium without hormones. The COCs were incubated for 65h in four-well dishes (Nunc, Denmark) at 38.5℃ in an atmosphere of 5% CO_2 and 95% air with high humidity. We chose duration of maturation culture of 65h based on our previous report on parthenogenetic activation of porcine IVM oocytes (Kim *et al.*, 2005).

3. Oocyte Activation and Cytochalasin B Treatment

After 48h maturation culture, the COCs in DPBS containing 300 IU/ml (w/v) hyaluronidase (Sigma, USA) were vortexed for 3 minutes to eliminate cumulus cells. After treatment with hyaluronidase, the denuded oocytes were selected with polar body and then the polar body extruded oocytes returned to the maturation medium without hormones. After 65 h of maturation, oocytes were exposed to PBS with 7% ethanol (v/v) for 7 minutes, and then the oocytes were washed and cultured in TCM 199 containing 5 μg/ml cytochalasin B (Sigma, USA) for 5 h at 38.5℃ in an atmosphere of 5% CO_2 and 95% air with high humidity.

4. *In Vitro* Culture of Parthenotes

After cytochalasin B treatment, the presumptive parthenotes were cultured in PZM-5 medium (IFP, Japan) and cleavage of the parthenotes was assessed at 72h of activation. Normally cleaved parthenotes were cultured for an additional 8 days to evaluate their ability to develop to blastocyst and hatching stages. The FBS were added at Day 4 or 5 with concentrations of 2.5, 5 or 10%.

5. Statistical Analysis

A randomized block design was utilized, and data were collected from the replicates. The data were analyzed by Duncan's multiple range tests using the SAS program. Differences at a probability value (P) of 0.05 or less were considered significant.

RESULTS AND DISCUSSION

The effect of serum on developmental ability in parthenotes was shown in Table 1. Results were not different in blastocyst

Table 1. Effect of concentration and exposure duration of FBS on parthenogenetic development of porcine follicular oocytes

Concentration (%)	Day	No. oocytes	No. blastocyst (%)	No. hatched Bl/total (%)	No. hatched Bl/blastocysts (%)
0	–	124	58 (46.8)	2/124 (1.6)[a]	2/58 (3.4)[a]
2.5	4	67	34 (50.7)	17/ 67 (25.4)[b]	17/34 (50.0)[b]
2.5	5	60	42 (70.0)	20/ 60 (33.3)[b]	20/42 (47.6)[b]
5	4	66	38 (57.6)	21/ 66 (31.8)[b]	21/38 (55.3)[b]
5	5	60	27 (45.0)	13/ 60 (21.7)[b]	13/27 (48.1)[b]
10	4	64	25 (39.1)	14/ 64 (21.9)[b]	14/25 (56.0)[b]
10	5	65	35 (53.8)	19/ 65 (29.2)[b]	19/35 (54.3)[b]

[a,b] Values with different superscripts are significantly different in each column (P<0.05).

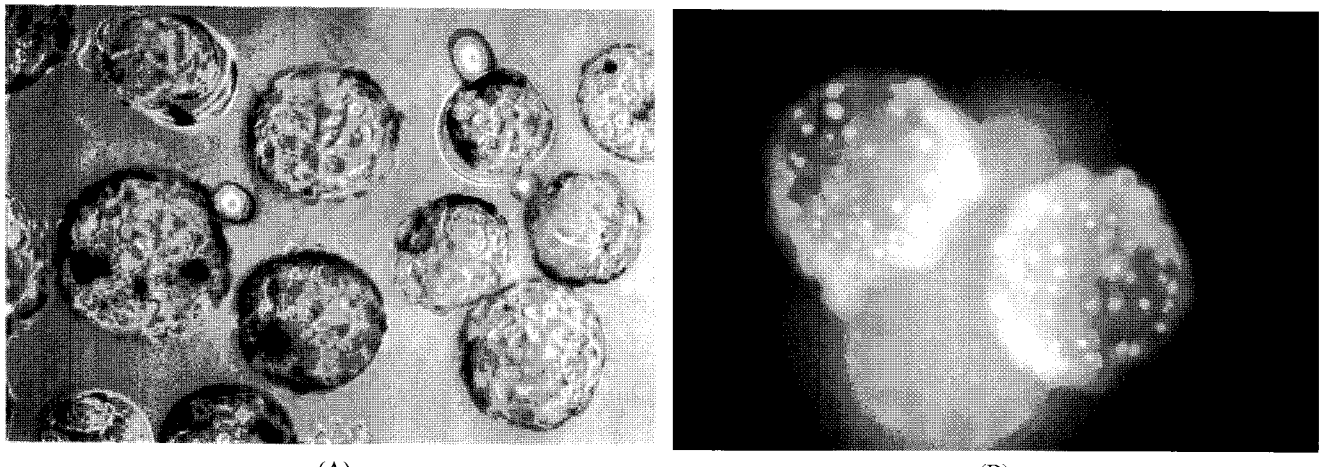
rates as serum addition of 0%, 2.5% from D4 to D8, 2.5% from D5 to D8, 5% from D4 to D8, 5% from D5 to D8, 10% from D4 to D8 and 10% from D5 to D8 as 46.8, 50.7, 70.0, 57.6, 45.0, 39.1 and 53.8% respectively. However hatching rate was different in non-addition group as 3.4% from serum-addition group as 50.0, 47.6, 55.3, 48.1, 56.0 and 54.3%.

Concentration effect was combined with blastocyst rates of Day 4 and Day 5 and duration effect was combined with blastocyst rates of serum concentration of 2.5, 5, and 10%. The percentages of blastocysts were also not statistically different among the concentration of 0, 2.5, 5 and 10% as 46.8, 60.4, 51.3 and 46.5% and duration of Day 4 to Day 8 and Day 5 to Day 8 as 49.1 and 56.3%, respectively. Hatching rates from blastocyst rates were also not different in serum-addition group of the concentration of 2.5, 5 and 10% as 48.8, 51.7, and 55.2% and duration of Day 4 to Day 8 and Day 5 to Day 8 as 53.8 and 50.1%. However hatching rate was dramatically decreased in non-addition group as 3.4%.

Pig embryos would be an excellent tool for human biomedical approaches, i.e. xenotransplantation, stem cell technologies and so on (Prather *et al.*, 2003). Although there were intensive efforts, the culture protocols for *in vitro* production of porcine embryos are not satisfactory. The current serum-free systems for porcine embryo production are not optimal. The average number of embryonic cells in Day 6 blastocysts produced in these systems was less than one-third of those produced *in vivo* (Wang *et al.*, 1999). The diploid parthenotes have advantage of homogeneous quality for precise culture experiment from pig embryos that have relatively high incidence of polyspermy that occurs during *in vitro* fertilization (Cui *et al.*, 2004). The objectives of the present study were to evaluate the effect of concentration and exposure duration of FBS on parthenogenetic development of the oocytes.

Embryo culture can serve as a strong diagnostic tool, yielding useful information regarding the implantation potential of the embryo. The information thus gained is useful for quality control of the embryology laboratory and success of the IVF/intracytoplasmic sperm injection (ICSI) programme. Zygotes can be scored and zygote quality has been associated with further embryonic development and cleavage stage embryo quality. Early cleavage, cleavage rate, cleavage stage embryo grade and subsequent progression of these embryos to the blastocyst stage have all been shown to be individual and collective markers for the implantation-competent embryo (Balaban and Urman, 2003). The developmental ability to blastocyst formation and hatching will be good indicator of implantation-competence. However, *in vitro* produced porcine embryos were hardly hatching. So, we examined effect of serum addition on developmental potential up to blastocyst formation and hatching as indicator.

Basic culture media for embryos of domestic animals are usually supplemented with various types of serum or serum derivatives because these components are known to be important for embryo development by serving as nutrients, pH buffers, anti-oxidants and other functions (Barvister, 1995; Gardner and Lane, 1997). Serum and serum derivatives (e.g. BSA) in culture media are reported to have the most influence on embryo development, morphology and metabolism among the



(A) (B)

Fig. 1. Hatching or hatched blastocysts at day 8 supplemented with 10% serum at day 4 after activation treatment (A) and their Hoechst 33342 staining for counting the cell number.

components added to culture media to improve embryo development (Carolan *et al*, 1995; Thompson, 1997). However, The effect of FBS supplementation on embryo development is shown to be biphasic. Supplementation of medium with FBS throughout the culture period inhibited cleavage and subsequent development of porcine embryos. while supplementing with FBS in the late stage of development increased the rates of hatched blastocyst formation (Kim *et al.*, 2004). The increased rates of hatching in the presence of serum could be due to the presence of plasminogen, which is converted to plasmin that can proteolytically degrade the zona pellucida and facilitate hatching (Menino Jr and Williams, 1987; Kaaekuahiwi and Menino Jr., 1990). In our experiment, 2.5%, 5% and 10% of serum addition form D3 to D8 were harmful to blastocyst formation as 93.3 (28/30), 26.7 (8/30) and 23.3% (7/30) and hatching as 46.4 (13/28), 62.5 (5/8) and 28.6% (2/7) in dose dependent manner (data not shown). In contrast, 10% FBS addition at Day 4 of *in vitro* embryo culture was beneficial to generate a higher proportion of high-quality blastocysts (Okada *et al.*, 2006) and to survive cryopreservation by stimulatory effect on the mitosis of porcine embryos cultured *in vitro* (Men *et al.*, 2005). Using FBS from Day 4 to 7, the rate of development to the blastocyst stage and the cell numbers also increased (Dobrinsky *et al.*, 1996; Kim *et al.*, 2004; Okada *et al.*, 2006). These results suggest that certain further modifications and optimization of culture conditions will be improve the embryo quality. In this experiment, improved hatching ability by addition of FBS may be estimated as indicator of embryo viability in female reproductive tract.

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