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Effect of Porcine Serum as Macromolecule on the Meiotic Maturation and Embryonic Development of Porcine Oocytes

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ABSTRACT

This study was conducted to establish an *in vitro* maturation (IVM) system by selection of efficient macromolecule in the porcine *in vitro* production (IVP) technology. To choose the efficient macromolecules in the development of porcine embryos, the effects of 3 kinds of macromolecules (porcine serum; PS, porcine follicular fluid; pFF, and polyvinyl alcohol; PVA) supplemented in IVM media on the maturation, cleavage, and development rates to blastocyst of parthenogenetic activation (PA) and *in vitro* fertilization (IVF) embryos were examined. The maturation rates of porcine oocytes in media supplemented with PS were significantly higher than those with pFF and PVA (92.4% vs. 85.4%, 77.1%; $p<0.05$). In the cleavage and development to blastocyst rates, supplement with PS or pFF in the IVM media was more effective than PVA. However, there were no significant differences in cleavage and development to blastocyst between PS and pFF group. From the results of this study, it was demonstrated that PS was optimal macromolecule in the porcine IVM media.

(Key words : porcine serum, follicular fluid, IVM, IVF, parthenogenetic activation)

INTRODUCTION

It has been a longstanding objective to establish optimal conditions for IVM of follicular oocyte from domestic animals to improve basic agricultural research and biotechnology. Even if porcine oocytes can develop to the blastocyst stage following maturation and fertilization *in vitro*, their developmental potential is more variable and lower than that of oocytes that mature *in vivo* (Beckmann and Day, 1993). These results suggest that failure in previous attempts of fertilization and further development of oocytes matured *in vitro* were due to insufficient oocyte culture systems. Several attempts have been made to modify the culture conditions, including the addition of hormones and macromolecules to the maturation medium for IVM. It has been common practice to include macromolecules from various sources in bovine IVM culture media; bovine serum, bovine follicular fluid (BFF) or BSA has usually been used for that purpose (Gordon 2003) and used likewise for porcine IVP (Abeydeera and Day, 1997a; Abeydeera and Day, 1997b; Ding and Foxcroft, 1992; Dode and Graves, 2001; Yoshida *et al.*, 1992; Zheng and Sirard, 1992).

Follicular fluid is primarily the transudate of plasma that contains specific components such as steroids, glycosaminoglycans and many other metabolites synthesize the cells of the follicle wall (Wise, 1987). Several recent studies have claimed the beneficial effects of pFF on the IVM and early embryonic development of porcine oocytes (Dode and Graves, 2001; Naito *et al.*, 1988; Naito *et al.*, 1989). During follicular growth, equilibrium is established between serum and follicular fluid and the metabolite concentrations in the two compartments are similar and are, in turn, similar to those in blood serum (Gordon, 2003). Follicular fluid is known to contain the putative oocyte maturation inhibitor (OMI) which is believed to be responsible for the maintenance of meiotic arrest in bovine oocytes (Sirard and First, 1988) and the presence of meiotic inhibitors in follicular fluid from pigs and mice has been well established (Downs *et al.*, 1985; Eppig and Downs, 1987). On the other hand the ammonia concentration in BFF decreased as follicle size increased, there was no evidence of an adverse effect on the subsequent developmental competence of oocytes (Hammon *et al.*, 2000a; 2000b).

Bovine serum, in the form of FBS or estrus cow serum

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(ECS) has been used as the main protein source in domestic IVM studies for many years (Gordon, 2003; Younis *et al.*, 1989; Zheng and Sirard, 1992). Moreover, ECS had a significant effect, compared with FBS, on the subsequent developmental competence of oocytes (Gordon, 2003).

BSA supplementation supported the IVM of LH-enhanced oocytes reflected by increased oocyte fertilizability and embryonic viability following IVF (Zuelke and Brackett, 1990). However BSA can be regarded as a variable component of a maturation medium, since it has been recognized for some time that the commercial preparation is a chemically impure product (Gordon, 2003) and any IVM medium containing commercial BSA cannot be regarded as being chemically defined (Kane, 1987).

As mentioned above, it is a well-known fact that growth factors and hormones, in addition to many other factors, are abundant in serum and follicular fluid (Gordon, 2003; Younis *et al.*, 1989). However, pFF is not using commercially now and there is variability in the properties of the pFF which is depending on the batch. Moreover, pFF may be a source of infection but FBS maturation system was advantageous because it can produce porcine IVP embryos uncontaminated by potential pathogens in pFF (Noakes *et al.*, 2001; Sur *et al.*, 2001; Suzuki *et al.*, 2006). On the other hand, some study used PS instead of pFF for IVM supplementation, however there was no report whether predominant evidence between porcine serum and pFF (Wu *et al.*, 2001).

As mentioned above, supplementation of IVM media with pFF undoubtedly discorded with proper quality control and repeatability among laboratories. In order to eliminate such variability it is imperative to develop a more defined culture system for mammalian IVM and as an alternative, many studies have been reported about new maturation media supplemented with polyvinyl alcohol (PVA), as a substitute for pFF. In order to be effective, media using PVA should be supplemented with growth factors such as EGF and or essential and nonessential amino acids. However, the developmental competence of embryos derived from the porcine IVP system which supplemented with PVA was still low and fluctuated (Abeydeera *et al.*, 1998; Abeydeera *et al.*, 2000; Jeong *et al.*, 2008; Kishida *et al.*, 2004; Marques *et al.*, 2007; Saeki *et al.*, 1991).

To establish an optimal IVM culture system for porcine oocytes, this study was conducted to select an efficient macromolecule by comparison of the effects of 3 macromolecules (PS, pFF and PVA) supplemented with IVM media on nuclear maturation and development to the blastocyst stage after PA and IVF.

MATERIALS AND METHODS

1. Culture Media

Unless otherwise state, all chemicals used in this study were purchased from Sigma Chemical Company (St. Louis, MO). The defined basic medium used for oocyte maturation were Tissue culture medium (TCM)-199 with Earle's salts, L-glutamine, and 2.2 g/L sodium bicarbonate, 0.8 mM L-cysteine, 0.4 mM Na-pyruvate, 1.13 mM kanamycin, 10 ng/ml Epidermal growth factor and 1 μg/ml Insulin. The changes of components in the defined basic medium were made according to each aspect to be examined in oocyte maturation. The basic medium used for IVF was modified Tris-buffered medium (mTBM) (Abeydeera and Day, 1997a) and the embryo culture medium for embryo development was North Carolina State University (NCSU)-23(Petters and Wells, 1993).

2. Preparation of Follicular Fluid and Porcine Serum

pFF was withdrawn with a syringe from superficial follicles 3～8 mm in diameter in prepubertal gilt or sow ovaries, centrifuged at 3,000×g for 10 min, filtered sequentially through a 1.2, 0.45, and 0.2 μm syringe filter (Gelman Sciences, Ann Arbor, MI, USA), and stored in aliquots at −20℃ until use. PS was prepared by centrifugation of venous blood from prepubertal pigs (3 months from birth) at 4,000×g for 20 min, and filtered sequentially through a 1.2, 0.45, and 0.2 μm syringe filter (Gelman Sciences) and stored in aliquots at −20℃ until use.

3. Oocyte Collection

Ovaries were obtained from prepubertal gilts and sows at a local slaughterhouse and transported to the laboratory. The ovaries were collected from gilts within 10 min after death and placed in 0.9% saline at 30～37℃ for not more than 3 h, subsequently washed twice in sterile saline. Granulose-cumulus-oocyte (GCOCs) and cumulus-oocytes complex (COCs) were aspirated from follicles (3～8 mm diameter) using an 18-gauge needle attached to a 10 ml disposable syringe. After pooling follicular contents in a 15 ml conical tube for 5 min, supernatant was discarded. COCs in the sediment were washed three times in Hepes buffered Tyrode's lactate medium within 0.05% (w/v) polyvinyl alcohol (P- 8136; PVA) (TLH-PVA).

Only COCs with more than two layers of intact cumulus cells and uniform cytoplasm were collected to use in the IVM.

4. *In vitro* Maturation (IVM)

Approximately 50 COCs were transferred to each well of a 4 - well multidish (Nunc, Nalge Nunc International, Roskilde, Denmark) containing 500 μl of IVM medium with gonadotropic hormones (GTH) including 200 IU/ml pregnant mare serum gonadotropin (PMSG; Intervet, Boxmeer, The Netherlands) and 200 IU/ml human chorionic gonadotrophin (hCG; Intervet) at 39℃ in a humidified atmosphere of 5% CO_2. After culturing for 22 h, COCs were transferred to PMSG- and hCG-free IVM medium and cultured for another 22 h. After IVM, COCs were treated with 0.1% (w/v) hyaluronidase (H- 3506) and oocytes were freed from cumulus cells by repeated pipetting.

5. Frozen Semen Processing

The sperm-rich fractions of Landrace were collected from pig AI (Artificial insemination) center (DARBY AI center, Anseong, Korea). Semen was slowly chilled to 15℃ by 2 h after collection. One volume of sperm was diluted with two volumes of Hulsenberg Ⅷ extender (Richter *et al.*, 1975), centrifuged at 15℃ for 10 min at 400×g, and the supernatant solution was poured off. The pellet was resuspended with the first diluted solution (BF_5; 52.3 mM TES, 16.5 mM Trizma base, 177.8 mM D (+)-glucose, 5 ml/L OEP (orvus es paste), 0.02 g/L gentamycin sulfate and 200 ml/L egg yolk) and diluted to provide 1.0×10^9 sperm/ml. Semen was cooled to 5℃ over a 1 h period and one volume of a BF_5 + 2% (v/v) glycerol (G-9012) (second diluted solution) was mixed to one volume of cooled semen. Straws (IMV Technologies, L' Aigle, France) were immediately filled with 2.5 ml of semen and needle holder was used to seal the ends of the straws. The straws were horizontally placed on an aluminum rack and set into a liquid nitrogen tank containing liquid nitrogen (LN_2). The straws were situated 4 cm above the LN_2, and kept at that level for 20 min before the straws were thrown into LN_2 storage.

6. Parthenogenetic Activation of Oocytes

For electrical activation, matured oocytes with 1st polar body were equilibrated for 5 min in activation solution (280 mM mannitol, 0.1mM $CaCl_2 \cdot 2H_2O$ and 0.05mM $MgCl_2 \cdot 6H_2O$) and transferred to a chamber consisting of two electrodes 1 mm apart which was overlaid with the same activation solution. Oocytes were exposed to a single pulse of 1.2 KV/cm for 60 μs, on a BTX Electro cell manipulator 2001 (BTX, San Diego, CA). After activation by DC pulse, oocytes were washed twice with TLH-PVA and then transferred into NCSU-23 medium supplemented with 5 μg/ml cytochalasin B (C-6762) for 5~6 h at 39℃ in a humidified air of 5% CO_2.

7. *In vitro* Fertilization

Denuded oocytes were randomly selected from each treatment and washed three times in IVF medium, and 15 oocytes were placed into each of 45 μl drops of the same medium, which had been covered with warm mineral oil in a 60×15 mm^2 polystyrene petri dish (Falcon 1007, Becton Dickinson Labware, USA). The dishes were kept in the incubator until spermatozoa were added for fertilization. A semen pellet was thawed and washed three times by centrifugation at 1,900×g for 4 min in sperm washing solution (Dulbecco's PBS(Gibco-BRL), 0.1% (w/v) BSA and 0.1% (w/w) penicillin-streptomycin solution). At the end of the washing procedure, the sperm pellet was resuspended in IVF medium. After appropriate dilution, 5 μl of this sperm suspension was added to 45 μl of the medium that contained oocytes to give a final sperm concentration of 2.0×10^6/ml. Oocytes were coincubated with spermatozoa for 6 h at 39℃ in an atmosphere of 5% CO_2 in air.

8. Embryo Culture

After fertilization or PA, the embryos were washed three times with TLH-PVA. Each 10 oocytes was then cultured in the 30 μl NCSU-23 droplets containing 4 mg/ml BSA under mineral oil at 39℃ in a humidified air of 5% CO_2. The rates of cleavage and development to blastocyst were checked on day 2 and day 7, respectively. Blastocysts were stained with Hoechst 33342 to examine the cell number to investigate of developmental competence of blastocyst.

9. Experimental Design

In this study, the effect of porcine serum and follicular fluid was evaluated on nuclear maturation and development to the blastocyst stage after parthenogenetic activation and IVF. As a control, 10% (v/v) pFF was added to the defined basic medium. GCOCs and COCs were cultured in defined basic medium supplemented with 10% (v/v) of PS (PGS used) or 0.1% (w/v) PVA.

10. Statistical Analysis

Data were analyzed by a general linear model procedure

using the Statistical Analysis System (version 9.1.3; SAS Institute, USA), followed by the least significant difference mean separation procedure when treatments differed at $p<0.05$. Cell number was expressed as mean±SEM.

RESULTS

1. Effects of Different Macromolecules in IVM Media on the Maturation Rates of Porcine Oocytes

Maturation rates of porcine oocytes matured in IVM media supplemented with 3 macromolecules were shown in Table 1. The maturation rates of PS group were significantly higher than that of pFF and PVA group ($p<0.05$). Although maturation rates of PVA group was significantly lower than other two groups, relatively high maturation rates were shown ($p<0.05$).

2. Effects of Different Macromolecules in IVM Media on Development of Porcine PA Embryos

Developmental potential of porcine PA oocytes matured in media supplemented with three kinds of macromolecule was shown in Table 2. The cleavage and development to blastocyst rate of PS and pFF group were significantly ($p<0.05$) higher than that of PVA group, however there was not significantly difference between PS group and pFF group. There were no significant differences among the three groups in the cell number of blastocyst.

3. Effects of Different Macromolecules in IVM Media on Development of Porcine IVF Embryos

Developmental potential of porcine IVF oocytes matured in media supplemented with three kinds of macromolecule was shown in Table 3. The cleavage and development to blastocyst rate of PS group were significantly ($p<0.05$) higher than that of PVA group, however there was not significantly difference between PS and pFF group and between pFF and PVA group. There were no significant differences among the three groups in the cell number of blastocyst.

Table 1. Effects of different macromolecules in IVM media on the maturation rates of porcine oocytes

Macromolecule	No. of oocytes	
	Examined	Matured (%)
PS	542	501 (92.4)[a]
pFF	527	450 (85.4)[b]
PVA	550	424 (77.1)[c]

[a~c] Values in the same column with different superscripts are different ($p<0.05$).

Table 2. Effects of different macromolecules in IVM media on development of porcine parthenogenetic activation embryos

Group	No. of oocytes			No. of cells
	Cultured	Cleaved	Develop to bl.	
PS	262	238 (90.84)[a]	64 (24.4)[a]	23.1±6.47
pFF	253	220 (86.95)[a]	60 (23.7)[a]	23.2±5.48
PVA	265	180 (67.92)[b]	39 (14.7)[b]	22.9±3.62

[a,b] Values in the same column with different superscripts are different ($p<0.05$).

Table 3. Effects of different macromolecules in IVM media on development of porcine IVF embryos

Group	No. of oocytes			No. of cells
	Cultured	2-cell	Blastocyst	
PS	280	229 (76.6)[a]	30 (10.7)[a]	22.5±7.31
pFF	274	206 (75.1)[ab]	25 (9.1)[ab]	22.6±6.51
PVA	285	205 (71.9)[b]	15 (5.3)[b]	22.3±6.72

[a,b] Values in the same column with different superscripts are different ($p<0.05$).

DISCUSSION

In this study, the addition of PS to IVM medium enhanced the maturation rate and developmental capacity of porcine oocytes after PA and IVF which comparable to that of follicular fluid and PVA. The results of this study demonstrated that the IVM of porcine oocytes was significantly higher in the PS group than pFF and PVA group. However, there were no significant differences in the cleavage and development to blastocyst rate between PS and pFF group.

The most common serum preparations used for bovine oocyte maturation are FBS or ECS and some laboratories used PS for porcine IVM media supplementation (Dode and Graves, 2001; Funahashi and Day, 1993; Nagyova *et al.*, 1999; Na-

gyova *et al.*, 2004; Wu *et al.*, 2001; Zheng and Sirard, 1992). There were shown that FBS as a supplement provided a superior environment for rat, bovine and hamster oocyte maturation when compared with BSA (Leibfried-Rutledge *et al.*, 1986; Vanderhyden and Armstrong, 1989; Younis *et al.*, 1989). Moreover, the IVM media supplemented with BSA was considered as semidefined media although it is commercially available. However serum contains substances such as cell proliferation inhibitor, antibodies cross-reacting with the culture, and complement, which may decrease cumulus cells expansion and oocytes maturation abilities (Freshney, 1987).

The use of follicular fluid to replace serum in order to increase the maturation rate has been proposed, which were similar with *in vivo* maturation system (Dode and Graves, 2001; Tatemoto *et al.*, 2004). Follicular fluid which contains various types of hormones, such as proteins, amino acids, enzymes, carbohydrates, glycoproteins, gonadotropins, steroids, prostaglandins, peptides, and various growth factors gives assistant to the nutritional and developmental support of the oocyte and follicular maturation and the maturation of its oocyte are parallel events and also functionally related (Hafez and Hafez, 2000). Nuclear and cytoplasmic maturation is an important process for fertilization and subsequent embryo development and nuclear maturation is regulated by the activity of a cytoplasmic maturation-promoting factor (MPF) which is activated at the onset of germinal vesicle breakdown (GVBD) and chromosome condensation, with peak activity at metaphase Ⅰ& Ⅱ (Norbury and Nurse, 1992). Follicular fluid might alter the expression of MPF in oocytes through cumulus cell communication after luteinizing hormone (LH) stimulation and has a positive effect on *in vitro* fertilization and embryonic development (Kim *et al.*, 1996; Naito *et al.*, 1988, 1989; Romero-Arredondo and Seidel, 1996; Yoshida *et al.*, 1992). Moreover, pFF added to IVM medium had beneficial effects on the resumption of meiosis and on MPN formation in combination with follicle stimulating hormone (FSH) (Naito *et al.*, 1988; Rath *et al.*, 1995) and significantly increased the rate of nuclear maturation, normal fertilization, and normal cleavage (Yoshida *et al.*, 1992). However, high concentrations of BFF in bovine IVF suppress fertilizability and developmental capacity, although BFF may also function to reduce polyspermy (Choi *et al.*, 1998; Kim *et al.*, 1996), even though a low concentration (10%) of follicular fluid obtained from large follicles in the maturation medium increased the subsequent cleavage rate and the ability to develop into blastocysts and hatched blastocyst (Dode and Graves, 2001; Kim *et al.*, 1996). Besides follicular fluid obtained 20 h after LH surge gave better results in the *in vitro* maturation than the 0 h follicular fluid (Romero-Arredondo and Seidel, 1996).

The higher maturation rates when using the pFF as macromolecules obtained from the large - size follicle (6 mm to 8 mm in diameter) than FBS suggest that there may be an effective substances in large-size follicle which was lack or is present in lower concentration in FBS and medium-size follicle (2 mm to 5 mm in diameter) (Dode and Graves, 2001). Moreover, porcine IVM in NCSU-37 supplemented with FBS reduced cumulus expansion and maturational ability, however the embryos produced from those oocytes had the same developmental ability as those produced from oocytes matured with pFF (Suzuki *et al.*, 2006). In the present study, likewise pFF group, porcine IVM in mTCM- 199 supplemented with PS did not reduce cumulus expansion and the complex of GCOCs and COCs was more floating in the maturation medium than PVA group. The oocytes were surrounded by a light colored fully expanded cumulus mass. This result was similar with studies reported by Nagyova *et al.* (1999; 2004). In addition, the maturation rate was significantly higher in the PS group than pFF and PVA group. This result was out of accordance with previous studies that the maturation rates were similar (both were > 70%) or reduced to about 30 to 40% after IVM in medium supplemented with FBS (Suzuki *et al.*, 2006; Tatemoto *et al.*, 2004). They found that FBS has a decreased level of activity of superoxide dismutase (SOD), which protect from oxidative stress caused by the hypoxanthine-xantine-oxidase system (Tatemoto *et al.*, 2004). Although we did not inspect the difference in activity of SOD between pFF and PS, this may be one elucidation for the difference of maturation rates. Growth factors such as EGF, which was low in FBS - supplemented media, also stimulate cumulus expansion and oocyte maturation in pFF - supplemented media (Prochazka *et al.*, 2000). However, in the present study, the addition of EGF to maturation medium supplemented with both FBS and pFF enhanced rates of maturation and cleavage.

In the present study, the cleavage and developmental rates to blastocyst stage of PS group were not significantly different to that pFF group after PA and IVF. Moreover, there was no obvious difference in morphology or in total cell numbers in blastocysts derived from PA and IVF. These results were similar with the study reported by Suzuki *et al.* (2006), even though embryo production system was different and PS was

used instead of FBS for IVM supplementation. However, the effects of FBS and PS for porcine IVM supplementation were not significantly different in maturation and fertilization rates (Zheng and Sirard, 1992).

In order to understand the factors that play roles in maturation rates and developmental capacity of porcine oocytes after IVF, PA, and somatic cell nuclear transfer (SCNT), a defined maturation system with PVA has been introduced in several studies. However, the developmental ability of oocytes matured in defined media which supplemented with PVA still tends to be lower than that of oocytes matured in media supplemented with pFF (Hong *et al.*, 2004; Jeong *et al.*, 2008; Kishida *et al.*, 2004; Song and Lee, 2007). In this study, although maturation rates were shown in the PVA group was significantly lower than PS and pFF groups, relatively high maturation rates were shown ($p<0.05$). Moreover, the cleavage and development to blastocyst rate of PVA group from IVF were significantly ($p<0.05$) lower than that of PS group, however there was no significant difference between pFF and PVA group. This result was in of accordance with previous studies and further research is needed to improve the embryo production in defined conditions.

The method of producing high-quality porcine IVP embryos for medical biotechnologies such as xenotransplantation must take due account of the risks of disease transmission. pFF cannot be used commercially but it could be only obtained from abbatoir-derived ovaries. Therefore, the properties of pFF were variable with laboratory staff and the batch and may be a source of infection. In order to eliminate such variability and pathogenicity, it is imperative to develop a more defined culture system for IVM. However, at present, the chemical defined medium significantly reduced the maturation rates and developmental capacity of porcine oocytes fertilized *in vitro* or parthenogenetic activated when compared to the IVM medium supplemented with pFF or serum. This result is consistent with the previous findings that the developmental ability of oocytes matured in defined media still tends to be lower than that of oocytes matured media supplemented with pFF (Hong *et al.*, 2004; Kishida *et al.*, 2004; Song and Lee, 2007) and the successful use of defined media for porcine IVP has not yet been reported. If PS could be obtained from the germfree or gnotobiotic pigs, it will be more useful for porcine IVP than FBS and pFF. Concerns regarding the transmission of potential xenozoonotic agents, such as porcine endogenous retroviruses, still exist, but recent studies revolve around the control and elimination of porcine endogenous retroviruses and other potential xeonzoonotic agents to prevent the infection of pigs whose organs may be used for xenotrasnplantaion. Furthermore, ECS had a significant and marked effect compared with FBS in the bovine IVP system, on the subsequent developmental competence of oocytes and some studies suggesting that pro-estrus cow serum (PECS) witch was collected on the day prior to estrus might be more effective in maturation media than ECS (Gordon, 2003). However, the ESS and PGS for porcine IVP maturation medium supplementations was not compared up to now and further studies are need to improve this IVM system.

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