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In Vitro Fertilization and Embryonic Development of Porcine Oocytes Mtured in mSOF

J. M. Koo, S. H. Hyun, B. C. Lee, S. K. Kang and W. S. Hwang†

Department of Theriogenology and Biotechnology, College of Veterinary Medicine, Seoul 151-742, Korea

SUMMARY

Embryos derived from pig oocytes matured in mSOF are able to develop to blastocysts after IVF. Experiment 1 evaluated the effects of two maturation media (TCM-199 vs mSOF) on maturation rate, fertilization parameters, including penetration, polyspermy, male pronuclear formation, and the mean number of sperm penetrated per oocyte. Experiment 2 and Experiments 3 examined the effects of two maturation media on zona pellucida solubility and cortical granule distribution by transmissible electron microscopy, respectively. Experiment 4 assessed the effects of two maturation media on the *in vitro* embryo cleavage rate and development to blastocyst. Lastly, experiment 5 examined the cell number of blastocyst. An effect of media ($P<0.05$) was detected for mSOF on the mean number of sperm per oocyte. In TCM group, zona digestion time (196.5±15.5 vs 131.6±20.1 before IVF, 397.5±30.3s vs 185.3±16.4s after IVF, $P<0.05$) was higher in TCM-199 group. No significant effects of media was observed on cortical granule distribution between two groups by TEM. An effect ($P<0.05$) was observed on embryo development to blastocyst (16% vs 8%) but not on cleavage rates. No significant effects of media was observed on total cell number of blastocyst. We found that the high mean number of sperm penetrated per oocyte and the weaker zona pellucida on the basis of the digestion time was shown in pig oocytes matured in mSOF, however, porcine oocyte maturation with supplemented synthetic oviduct fluid medium (mSOF) resulted in blastocyst cell numbers comparable to those observed with Tissue Culture Medium 199.

Key words: porcine, IVF, mSOF

INTRODUCTION

Porcine oocytes have been generally cultured *in vitro* in various media supplemented with 10% serum, hormones, growth factors such as pig follicular fluid, estrogen, and epidermal growth factor (EGF) (Fukui and Ono, 1989; Naito et al., 1988; Tervit et al., 1972). These culture media were aimed to establish the optimal condition for meiotic and cytoplasmic maturation of oocytes *in vitro* and affect maturation rate of oocyte, developmental competence of embryo and fetal growth (Ho et al., 1995; Tomson et al., 1995; Funahashi et al., 1994; Xu et al., 1992; Zheng and sirard, 1992; Mcgaughey, 1977). In oocyte maturation, both

† Correspondence : E-mail: hwangws@snu.ac.kr
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meiotic and cytoplasmic maturation are considered importantly (Wang et al., 1998; Wang et al., 1997; Mattioli et al., 1988). In fact, cytoplasmic maturation of porcine oocytes is significantly affected by *in vitro* maturation (IVM) system (Wang et al., 1998; Mattiol; et al., 1988; Sato et al., 1982). The effects of maturation media on the fertilization parameters and development have been studied (Funahashi et al., 1996). Inappropriate cytoplasmic maturation of porcine oocytes matured *in vitro* results in low developmental competence. One of the main reasons of low development in porcine in vitro fertilization (IVF) is polyspermy. The polyspermic rate is higher in porcine oocytes *in vitro* matured than *in vivo* derived oocytes (Niwa, 1993). The reason for high polyspermy in IVF system is still unclear. Wang et al. (1988) found that *in vitro* matured oocytes have weaker zona and is weakly labeled by a lectin that is specific for glycoprotein compared to *in vivo* oocyte.

In order to seek alternative *in vitro* maturation medium for porcine oocytes, in this study, mSOF (modified synthetic oviductal fluid) with amino acid (mSOFaa) and Tissue Culture Medium (TCM) were employed. The mSOF medium was designed from salts and energy metabolites found in sheep oviductal fluid as simple defined medium and widely used for bovine embryo culture and *in vitro* maturation media (Watson et al., 2000). Watson et al. found that bovine oocyte maturation in mSOF resulted in blastocyst cell numbers comparable to those observed with TCM199. The aims of this study were 1) to examine the effects of mSOF on fertilization parameters, 2) to investigate the effect of mSOF on zona solubility and cortical granule distribution with particular correlation to the polyspermy ; 3) to determine if mSOF can be employed for porcine oocyte maturation after IVF.

MATERIALS AND METHODS

Culture Media

Two maturation media (mSOF and TCM-199) were used in this study; protein-free TCM199 (Gibco BRL, Grand Island, NY) (Abeydeera, 1998b) supplemented with 10 ng/ml EGF, 0.57 mM cysteine, 0.1% polyvinyl alcohol (PVA), 0.5 μg/ml LH, 0.5 μg/ ml FSH, 75 μg/ml potassium penicillin G, and 50 μg/ml streptomycin sulfate and slightly modified by the addition of 3.05 mM D-glucose and 0.91 mM sodium pyruvate. The mSOF was supplemented with ITS 10 (g/ml and 0.57mM cysteine/L. All two maturation media were supplemented with 10% (v/v) porcine follicular fluid (pFF). The pFF was collected from follicles (3~6mm diameter) of pig ovaries. After centrifugation at 300g for 30 min at 4℃, the supernatant was filtered through 1.2μm syringe filters and stored at −20℃ until use. The same pool of pFF was used for two maturation media. The IVF medium was described by Wang et al. (1993), which was designated as modified Tris- buffered medium (mTBM: pH, 7.2~7.4; 39℃; 5% CO_2 [v/v] in air). The mTBM consisted of 113.1 mM NaCl, 3 mM KCl, 7.5 mM $CaCl_2 \cdot 2H_2O$, 20 mM Tris (crystallized free base; Fisher), 11 mM glucose, 5 mM sodium pyruvate, and no antibiotics. Embryos were cultured in North Carolina State University (NCSU)-23 medium (20), which was designated as *in vitro* culture (IVC) medium supplemented with 4 mg/ml BSA. Media for IVF and IVC were covered with paraffin oil and equilibrated at 39℃, 5% CO_2 (v/v) in air, at least 12 h before use.

Oocyte Collection and IVM

Ovaries collected from prepubertal gilts at a local abattoir were transferred in 0.9% (w/v) NaCl containing 100 mg/L penicillin at 37℃ within 2h. The ovaries were washed three times in saline. Cumulus oocytes complexes (COCs) were collected from follicles 3~6 mm in diameter and washed

three times with DPBS (Dulbecco's phosphate buffered solution, Gibco) supplemented with 4mg/mL BSA (Sigma; A-8802). After washing, COCs were washed twice more in two maturation medium (TCM or mSOF) equilibrated for a minimum 3 h at 39℃ under 5% CO_2 in air. A group of 50 COCs was cultured in 500-μL of each IVM medium supplemented with 10 IU eCG and hCG, 10 μg/ml EGF at 39℃ in a humidified atmosphere of 5% CO_2 and 95% air. After culturing for 22 h, oocytes were washed once and then cultured in maturation medium without hormone for another 22 h at 39℃, 5% CO_2 in air.

In Vitro Fertilization and Embryos Culture

After 46 h IVM, all oocytes were freed from cumulus and corona cells by treatment with maturation media containing 0.1% (w/v) hyaluronidase (Sigma H-3506), and then washed three times. A group of 15 oocytes were transferred into a 40 μl droplet of mTBM covered with mineral oil (Sigma ; M- 8806). The 0.25 mL frozen straw were thawed at 39℃ and washed three times by centrifugation at 300g for 3 min in DPBS supplemented with 0.1% BSA . After washing, the sperm pellet was resuspended in mTBM. The 5 μl of sperm suspension was added to 40 μl droplet of mTBM to give a final sperm concentration of 1 to 1.5×10^6 cells /ml. Oocytes and sperm were coincubated for 5～6 h at 39℃ in an atmosphere of 5% CO_2 until examination. At 6 h after IVF, putative embryos were washed three times in embryo culture medium and 10 embryos were transferred to 25 μl drops of NCSU-23 medium and incubated at 39 in 5% CO_2 and 5% O_2. At 48 h and 168 h after insemination, the rates of cleavage and blastocyst formation were monitored.

Experiment 1: Assessment of Meiotic Maturation and Fertilization Parameters

After IVM or 12h after IVF, oocytes were mounted and fixed for 24 h in 25% acetic acid in ethanol at room temperature. The fixed oocytes were then stained with 1% (w:v) orcein in 45% (v:v) acetic acid and examined at (Leitz) with phase-contrast optics under ×200 or ×400 magnification. Oocytes were considered penetrated when they had one or more swollen sperm heads and male pronuclei and corresponding sperm tails.

Experiment 2: Zona Pellucida Solubility after IVM and IVF

To test zona pellucida solubility, *in vitro* matured or *in vitro* fertilized oocytes were washed three times in TL-Hepes-PVA at 39℃ and placed (n = 10/group) into 100 μl of a 0.1% (w/v) pronase solution in DPBS. Zona digestion was observed continuously at room temperature with an inverted microscope. When the zona pellucida was no longer visible at ×200 magnification, the zona pellucida dissolution time was recorded (Kim et al., 1996). The Experiment 2 was repeated four times.

Experiment 3: Examination of Transmissible Electron Microscopy

Two different groups of oocytes were prepared for transmissible electron microscopy (TEM). Oocytes with a polar body were examined by Hoechst staining and fixed after IVM 48h. (TCM199 vs mSOF). The 40 oocytes per group were prepared. All oocytes were washed twice immediately after IVM 48 h in 1% phosphate buffer and fixed in 2% combined osmium glutaldehyde, 2% formaldehyde, 90 mM potassium oxalate and 1.4% sucrose for 24 h at 4℃. Subsequently, oocytes were embedded in 1% agar in distilled water. Agar cubes containing the oocytes were washed several times in phosphate buffer and then transferred to 1% OsO_4 for 3 h at 4℃. Following washing in distilled water, oocytes were dehydrated in 50, 70, 90 and 100% ethanol and embedded in agar. After polymerization for 48

h, the blocks were cut into semithin and ultra thin sections. Ultra thin sections were collected on copper rids, contrasted with uranyl acetate and lead citrate and examined on a JEOL JEM 100 CX2 electron microscope.

Experiment 4: Assessment of Embryonic Development

Oocytes matured *in vitro* and devoid of cumulus cells were washed and randomly assigned to each of two groups: control (TCM199) or mSOF. Pre-equilibration, preincubation, and fertilization were performed as described earlier. Six hours after fertilization, control putative zygotes were washed three times in 20 μl drops of IVC medium, transferred to a fresh 20 μl drop (n = 15 oocytes/drop), and incubated at 39℃, 5% (v/v) CO_2 in air. The zygotes were washed three times in 20 μl of IVC medium, and randomly assigned to 20 μl IVC drops. At 48 and 144 h after insemination, respectively, the cleavage rate and blastocyst formation were evaluated under a stereomicroscope. The Experiment 4 was repeated seven times, with multiple observations within each replicate and the percentage blastocyst formation determined from the number of oocytes inseminated.

Experiment 5: Differential Staining

Numbers of ICM and TE nuclei of pig blastocysts were investigated by a modified immunosurgery method (Machaty et al., 1998). Briefly, blastocysts were treated with 0.5% protease to remove zona pellucida. Zona-free blastocysts were incubated in 50 μl of TL-Hepes-PVA medium supplemented with 10% (v:v) rabbit anti-pig whole serum at 39℃ for 40 min. The embryos were incubated in 50 μl of TL-Hepes-PVA medium supplemented with 10% (v:v) guinea pig serum as complement, 4 μg/ml bisbenzimide (Hoechst 33342), and 100 μg/ml propidium iodide. After 1 h, the embryos were washed with TL-Hepes-PVA medium and individually mounted on a glass slide. The number of ICM and TE nuclei in a single embryo was counted by using a fluorescence microscope (Nikon Corp., Tokyo, Japan).

Statistical Analysis

The volume fraction and the number of organelles per unit volume for cellular organelles were analyzed by General Linear Models procedures of SAS 6.12 program (SAS Institute Inc., Cary, NC). All percentage data were subjected to arc-sine transformation before statistical analysis. When detected the significance of main effects in each experimental parameter, When a significant model effect was found in each experimental parameter, data were compared by the least squares method. Statistical significance was determined where P value was less than 0.05. The data represented as means±SD.

RESULTS

Experiment 1: Effect of mSOF Media on Nuclear Maturation and IVF

To examine the effects of mSOF on IVM, nuclear maturation of pig oocytes matured in two

Table 1. Effect of maturation media on nuclear status of oocyte

Maturation medium	Oocytes examined N	% nuclear status of oocytes (mean±S.D.)		
		GV	M- I	M-II
mSOF	89	5.3 ± 2.9	10.1 ± 2.9	68.2 ± 3.3
TCM199	91	9.7 ± 2.3	11.6 ± 3.9	65.1 ± 3.4

No significant difference (P>0.05).

Table 2. Sperm penetration and male pronuclear formation of porcine oocytes matured in mSOF *vs* TCM-199 at 12h after insemination

Maturation medium	No. of Oocytes examined	No. of oocytes		No.of polyspermic oocytes (%)[b]	No.of oocytes formed MPN (%)[b]	Mean no. spermatozoa in penetrated oocyte [c]
		Matured (%)	Penetrated (%)[a]			
mSOF	72	63(88)	52(72)	20(38)	40(76)	2.9 ± 4.8 [d]
TCM	68	56(82)	41(60)	12(29)	26(63)	1.9 ± 2.9 [e]

a; percentage of metaphase II oocytes.
b; percentage of oocytes penetrated.
c; mean±SD
d,e; different superscripts within columns denote significant ($P<0.05$) differences.

media were stained with aceto-orcein. As shown in Table 1, no significant differences were observed in the percentages of maturation between oocytes matured in mSOF and TCM199. Effects of mSOF on fertilization parameters are shown in Table 2. No significant differences were shown in penetration, formation of the male pronucleus and polyspermy between oocytes matured in mSOF and TCM199. A significant effect of mSOF maturation medium ($P<0.05$) was found in the mean number of spermatozoa (MNS) in penetrated oocyte. Significantly increased MNS in penetrated oocyte sperm penetration compared with the TCM group (2.9±4.8 vs. 1.9±2.9; Table 2).

Experiment 2: Effects of mSOF Media on Zona Pellucida Solubility

Effects of mSOF on zona pellucida solubility were examined to determine the association of high MNS per oocyte after IVF. The ZPs of all oocytes matured in mSOF were dissolved within 2.5 min before IVF and 3 min after IVF after exposure to a 0.1% pronase solution. However, In TCM groups, zona digestion time was higher (196.5±15.5 vs 131.6±20.1 before IVF, 397.5± 30.3s vs 185.3± 16.4s after IVF, $P<0.05$) in TCM-199 group. While zona hardening detected in both groups, the zona hardening in mSOF group was weaker than that of TCM group.

Experiment 3: Effects of mSOF Media on the Cortical Granule Distribution

In both groups, cortical granules in oocytes derived from both maturation media were arranged at the peripheral region along the oolemma. The number, size and distribution of cortical granules were not different in two groups (Fig. 1).

Experiment 4: Effects of mSOF on Embryonic Development

To test whether mSOF as IVM medium had an effect on embryonic development *in vitro*, both cleavage rate and development to blastocyst were examined. Although mSOF showed slightly higher (77.1±3.5 vs 60.1±3.9, $P=0.07$) rate of cleavage rate, no statistically significant differences were observed on cleavage rates (Table 4). However, an effect ($P<0.05$) of mSOF was found in development of embryos to blastocysts. A significant difference ($P<0.05$) in number of blastocysts was found between two groups (Table 4).

Experiment 5: Effects of mSOF Media on the Inner Cell Mass and Trophectoderm

Total cell number was slightly higher (41.8±2.7 vs 34.9±2.1, $P=0.07$) in blastocyst from TCM199

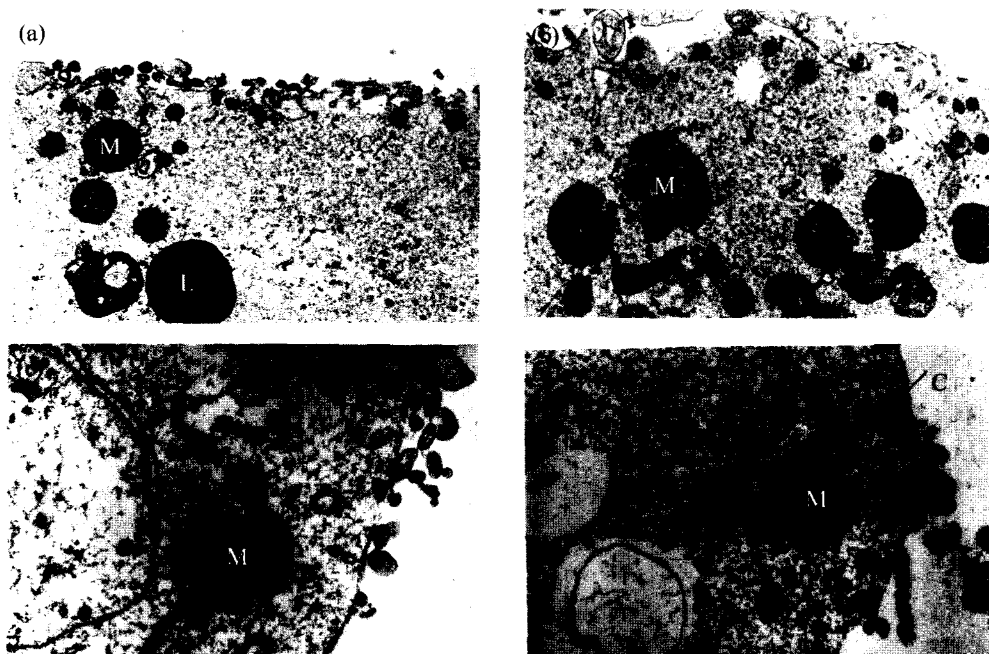

Fig. 1. Electron micrograph of peripheral region of fully grown porcine oocyte matured in TCM-199 and mSOFaa (×18000) a) showing pleomorphic mitochondria(pM) b) hooded mitochondria (hM). Cortical granules were in the solitary positions along the oolemma. No difference in number and shape of cortical granules c), d) the density of peripheral cytoplasm is higher in TCM-199. (×30000)
CG : *cortical granule*, M : *mitochondria*, L : *Lipid droplet*, ZP : *zona pellucida*.

Table 4. Comparison of developmental ability of porcine oocytes matured in mSOF *vs* TCM-199[a].

Maturation medium	No. of oocytes examined	No. and (%)* of IVF embryos developed to			
		2-cell [48][b]	8-cell [96][b]	Morula [144][b]	Blastocyst [168][b]
mSOF	315	242(77)	91(29)	69(22)[c]	50(16)[b]
TCM	320	192(60)	64(20)	42(13)[c]	26(8)[c]

[a] Oocytes were cultured in NCSU-23 with 1% (w/v) BSA for 5 days after IVF.
[b] Numberd in brackets indicate the time of examination after insemination.
[b,c] Within columns, values with different superscripts differ at $P<0.05$.

Table 5. Allocations of cells (mean±SD) in porcine blastocysts to ICM and TE

IVM medium	No. blastocysts examined	Cell no. of blastocysts			ICM/total cells
		ICM	TE	Total	
mSOF	20	13.35±1.4	18.25±1.5	34.9±2.1	0.43±2.4
TCM	20	14.55±1.3	25.35±1.9	41.8±2.7	0.36±1.2

No significant difference ($P>0.05$).

(Table 5). No significant effect of mSOF as IVM medium was detected on the number of ICM and TE of blastocysts, even thought it seem to lower ICM (13.4±1.4 vs 14.6±1.3, P=0.08) in mSOF group.

DISCUSSION

The present study demonstrated that mSOF could be employed for *in vitro* pig oocytes maturation. Penetration, polyspermy and the formation of male pronucleus were comparable to pig oocytes matured in TCM199. However, the MNS per oocyte was significantly higher in oocytes matured in mSOF and the zona of porcine oocyte matured in mSOF was weaker on the basis of dissolution time before and after IVF.

In the mouse, these modified zona receptors appear shortly after fertilization and are cleavage products of ZP2 and ZP3, resulting in the inhibition of sperm binding (Kuoba et al., 2000). Kuoba et al. reported that added pOSP (porcine oviduct specific glycoprotein) decreased the number of spermatozoa bound to the zona pellucida during IVF and pOSP may play an important role *in vivo* in the fertilization process, including a block of polyspermy (Kuoba et al., 2000). Oviductal-derived oocytes and embryos are more resistant to *in vitro* proteolytic degradation than either follicular oocytes or embryos recovered from the uterine lumen (Kuoba et al., 2000). Similarly, the zona pellucidae of oocytes matured *in vitro* are more susceptible to protease digestion by pronase than nonfertilized oocytes flushed from the oviduct on Day 2 of the estrous cycle (Wang et al., 1998). Therefore, an unknown factor of oviductal origin may be involved in providing zona stability that also contributes to the functional block of polyspermy *in vivo.* Kim et al. (1996) also reported ZP hardening after incubation of *in vitro* matured pig oocytes in oviductal fluid. Recently, it also has been reported that exposure of bovine ZPs to ovine and bovine oviducts prevented digestion by pronase, and the changes in the ZPs were correlated with the block to polyspermy (Duby et al., 1997). Coy et al. found that the zona pellucida of oocytes incubated in TCM199 was harder than that of oocytes cultured in TALP or TBM as IVF media (Coy et al., 2002). Weak zona of pig oocyte matured in mSOF could not harden the zona immediately and cause polyploidy in embryo after IVF. Our results indicate that *in vitro* maturation media affect on zona pellucida hardening. Oocytes matured in TCM199 might modify zona pellucida sperm receptors immediately after fertilization in "zona hardening." Polyspermy has remained a persistent problem of pig oocytes matured and fertilized *in vitro*, often reaching levels greater than 50% (Wang et al., 1998; Abeydeera et al., 1998b; Wang et al., 1997; Wang et al., 1993).

A recent study reported that pig oocytes flushed from the oviduct on Day 2 of the estrous cycle and subsequently fertilized *in vitro* had a much lower incidence of polyspermy (28%) than oocytes matured and fertilized *in vitro* (62%) (Wang et al., 1991). Monospermic embryos having diploid chromosome may have better developmental competence than polyspermic ones having polyploid chromosome(Han et al., 1999). Polyspermy in pig may be the main reason of low development. However, Han et al. showed that the development to blastocyst was not different between polyploid or diploid embryos *in vivo* or *in vitro* and blastocyst derived from polyploid embryos that possess significantly lower ICM. Our results agreed with Han's report that the polyploid porcine embryo can developed to blastocyst.

Cortical granule (CG) density and distribution are also important cytoplasmic markers during oocyte meiotic maturation (Hyttel et al., 1986). The CG exocytosis of pig oocytes matured *in vivo* (Yoshida et al., 1993; Cran and Cheng, 1985) and

in vitro (Kim et al., 1996; Wang et al., 1993) have been examined, and is thought to be one of the main reasons for polyspermy. In the pig, oocytes fertilized *in vitro*, when compared with those fertilized *in vivo*, showed an incomplete cortical granule exocytosis (Kim et al., 1996). In contrast, Wang et al. reported no differences in the ability to release CGs after IVF between *in vivo* and *in vitro* oocytes (Wang et al., 1998). In the present study, oocytes derived from both groups showed cortical granules are distributed along the oolemma, indicating the sign of the fully matured oocyte (Hyttel et al., 1997; Ernst and Jimmy, 1994; Hyttel et al., 1989; Hyttel et al., 1986; Cran, 1985; Fleming and Saacke, 1972). Although cortical exocytosis was not examined in this study, the pattern of CG distribution in oocytes matured in both media was very similar to *in vivo* oocyte.

Beckmann and Day (1993) demonstrated successful culture of porcine zygote to blastocyst stages in modified Whitten's medium which is low-salted medium. Funahashi et al. (1994) found that intracellular glutathione concentration was higher in oocytes matured in low-salted Whitten's medium (68mM) than those matured in Media 199 (116mM) with a correlation with male pronuclear formation. The mSOF medium is simply defined medium and contains lower (96mM) concentration of NaCl than TCM-199 (116Mm). In this study, when compared these two media, no significant difference in male pronucleus formation between two groups was detected. These results suggest that 96mM and 116mM NaCl concentration does not make difference in formation of male pronucleus. It can be argued that mSOF medium is not completely optimized for porcine maturation. Since, however, it is a completely defined simple medium (Watson et al., 2000), it can be easily modified to investigate the effects of specific components on the zona pellucida weakness and cytoplasmic maturation of porcine oocyte.

The results of this study further demonstrated that oocytes matured in mSOF provided a significant increase in post-cleavage development from embryo to blastocyst. This observed increase in blastocyst development might result from metabolic effects. In electronmicrograph it was observed many lipid droplets exist in oocyte matured in TCM 199. In embryos, this increase occurred regardless of the composition of the medium in which the embryos were cultured. It has been suggested that an increased amount of lipid in embryos cultured *in vitro* results from uptake of lipid from serum in the culture medium (Gardner et al., 1994). Lipid may have accumulated as a result of insufficient metabolism by mitochondria present in bovine oocyte matured *in vitro* (Chow et al., 1994; Dorland et al., 1994). Adrienne E. et al.(Crosier et al., 2000) found that increases in lipid density occurred equally in compact morulae cultured in a completely serum-free medium and in serum-supplemented media. However, on the basis of electron micrograph presented in this study, the increased volume density of lipid in oocytes cultured *in vitro* may result from the culture medium (both groups were pig follicular fluid as serum supplemented).

In summary, the results of this study indicates that pig oocyte matured in mSOF produced that higher MNS per oocyte compared to those in TCM 199 after IVF. This higher MNS may result from weaker zona pellucida on the basis of proteolysis of oocyte matured in mSOF. A post-cleavage increase in the number of embryos developing to blastocysts was observed in mSOF group. These data indicate that mSOF medium can be employed for pig oocyte maturation not for IVF but for somatic cell nulear transfer and lipid droplet research for pig oocyte.

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