ISSN 2508-755X (Print) / ISSN 2288-0178 (Online)
J. Emb. Trans. (2017) Vol. 32, No. 4, pp. 275~285
https://doi.org/10.12750/JET.2017.32.4.275

Effects of High Dose Lysophosphatidic Acid Supplement during IVC on Preimplantation Development of Porcine Embryos

Minghui Jin[1], Il-Jeoung Yu[2] and Yubyeol Jeon[2,†]

[1]*Laboratory of Veterinary Embryology and Biotechnology (VETEMBIO), College of Veterinary Medicine, Chungbuk National University, Cheongju 361-763, Chungbuk, Republic of Korea.*
[2]*Department of Theriogenology and Reproductive Biotechnology, College of Veterinary Medicine and Bio-safety Research Institute, Chonbuk National University, Iksan 54596, Republic of Korea.*

ABSTRACT

Lysophosphatidic acid (LPA) is an important signaling molecule. Here, the effect and mechanism of LPA on the preimplantation development of porcine embryos during *in vitro* culture (IVC) was examined. Porcine embryos were cultured in porcine zygote medium (PZM-3) supplemented with 30 μM LPA during different days. There was a significantly higher cleavage rate in Day 1-7 and significantly higher total cell number of blastocysts in Day 1-3 and Day 4-7. It was also found that messenger RNA (mRNA) expression level of *PCNA*, *BCL2* and *BAX* in blastocysts obtained from D1-7 group were significantly higher and *BCL2/BAX* mRNA ratio in D1-3 group was significantly lower than control group but Day 4-7 and Day 1-7 groups were comparable with control group. Treatment with 20 μM PLC inhibitor significantly decreased the embryo cleavage rate and blastocyst formation rate. Moreover, LPA as an activator of PLCs, enhanced the 30 μM LPA + 20 μM U73122 group embryo cleavage rate which similar with control group. In conclusion, the results suggest that treatment with LPA during IVC improves the porcine early embryo cleavage by activation of PLC signaling pathway and regulate the mRNA expression that contribute to total cell number of blastocysts during blastocyst formation.

(Key Words : Lysophosphatidic acid (LPA), Porcine, Embryo cleavage, Phospholipase C (PLC), U73122)

INTRODUCTION

The phospholipid derivative lysophosphatidic acid (LPA) is an important signaling molecule that shows growth factor-like and hormone-like functions via the activation of a series of six G protein-coupled receptors (LPA1-6) (Choi *et al.*, 2010; Moolenaar *et al.*, 1992; Mutoh *et al.*, 2012). It is detectable in various body fluids, and LPA (at the 60nM ~ 70μM level) has been detected extracellularly in biological fluids, such as serum (Tigyi and Miledi, 1992), follicular fluid (Tokumura *et al.*, 1999), and uterine luminal fluids (Seo *et al.*, 2008). LPA can evokes various cellular responses, including cell proliferation, survival, motility, cytoskeletal rearrangement, and differentiation (Moolenaar, 2000).

The effect of LPA on the preimplantation development of embryos has been investigated in mice (Jo *et al.*, 2014; Kobayashi *et al.*, 1994; Liu and Armant, 2004), pigs (Zhang *et al.*, 2015), and cows (Boruszewska *et al.*, 2016; Torres *et al.*, 2014). It was reported that in mice, supplementation of *in vitro* maturation (IVM) medium with 30 μM LPA enhanced the developmental competence of *in vitro* matured oocytes, making them more similar to *in vivo* matured control oocytes, and LPA increased the cleavage rate and blastocyst formation rate significantly in pigs (Jo *et al.*, 2014; Kim *et al.*, 2016). The addition of LPA to the culture medium at the pronuclear stage during *in vitro* culture (IVC) increased the blastocyst rate, and supplementation at the morula stage accelerated blastocyst differentiation, based on blastocyst outgrowth rates in mice (Kobayashi *et al.*, 1994; Liu and Armant, 2004).

The LPA receptor couples to multiple independent effector pathways in a G protein-dependent manner, including G alpha subunit families ($G_{12/13}$, $G_{q/11}$, $G_{i/o}$, and Gs) (Choi *et al.*, 2010; Riaz *et al.*, 2016; Yung *et al.*, 2014). Supplementation of IVM medium with LPA altered the maturation rate in mice that was

† Correspondence: Yubyeol Jeon (ORCID: 0000-0003-0328-2974)
Phone: +82-63-850-0934, Fax: +82-63-850-0910
E-mail: ybjeon@jbnu.ac.kr

repressed by a phospholipase C (PLC) inhibitor and a protein kinase C (PKC) inhibitor, suggesting that PLC and PKC are involved in LPA-induced signal transduction, leading to nuclear maturation (Komatsu *et al.*, 2006). The G_i protein is involved in LPA induced meiotic maturation, as suggested by the inhibition of LPA's action on the maturation rates by the pertussis toxin (Hinokio *et al.*, 2002). In pigs, LPA can activate the mitogen-activated protein (MAP) kinase pathway and decrease in cAMP levels in oocytes, which influences both nuclear and cytoplasmic maturation of porcine oocytes positively (Zhang *et al.*, 2015). Supplementation with LPA in IVC medium had demonstrated that LPA activates the G_i protein, because an inhibitor of the G_i protein reversed LPA's action on the blastocyst rate during IVC (Kobayashi *et al.*, 1994). The addition of LPA also can activate ROCKs (Rho-kinases), because LPA weakens the inhibition of a specific inhibitor of ROCKs, leading to the embryos developing into blastocysts as in normal conditions (Zhang *et al.*, 2014). In addition, a chelator of free cytosolic Ca^{2+} attenuated the effect of LPA on murine blastocyst outgrowth, which indicated that LPA's effect on trophoblast differentiation depends on its ability to induce intracellular Ca^{2+} mobilization (Moolenaar, 1995).

Although these studies have reported the effect of LPA on the preimplantation development of embryos in many animals, the concentration of LPA used in the most reports was limited to physiological concentrations, ranging from 0.01 to 10 μM and few reports used concentrations higher than physiological concentrations. A previous study reported that treatment with 30 μM LPA in IVM medium was comparable to the *in vivo* matured control (Jo *et al.*, 2014), therefore, it is necessary to test and verify the effect of 30 μM LPA on the preimplantation development of embryos. In the present study, the effects of 30 μM LPA on the development of porcine preimplantation embryos during IVC were determined and the involvement of the LPA signaling pathway was investigated in the early stage of porcine embryonic development.

MATERIALS AND METHODS

1. Chemicals

All chemicals and reagents used in this study were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA), unless otherwise stated.

2. Oocyte collection and *in vitro* maturation (IVM)

Ovaries of prepubertal gilts were collected at a local abattoir and transported to the laboratory within 2 h in a 0.9% (wt/vol) NaCl solution supplemented with 100 IU/L of penicillin G and 100 mg/mL of streptomycin sulfate at 32 to 35 ℃. The cumulus–oocyte complexes (COCs) in the ovaries were aspirated from 3 to 6-mm diameter superficial follicles using an 18-gauge needle attached to a 10 mL disposable syringe and allowed to settle in 15 mL conical tubes at 37 ℃ for 5 min. The supernatant was discarded, and the precipitate was resuspended in HEPES-buffered Tyrode's medium (TLH) containing 0.1% (wt/vol) polyvinyl alcohol (TLH-PVA). Next, only those COCs with three or more uniform layers of compact cumulus cells and homogenous cytoplasm were selected and washed three times in TLH-PVA. Approximately 60 COCs were placed into each well of a four-well Nunc dish (Nunc, Roskilde, Denmark) containing 500 μL of culture medium (TCM-199; Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 0.6 mM cysteine, 0.91 mM sodium pyruvate, 10 ng/mL of epidermal growth factor (EGF), 75 μg/mL of kanamycin, 1 μg/mL of insulin, 0.1% (vol/vol) PVA, 10 IU/ mL equine chorionic gonadotropin (eCG), and 10 IU/mL of human chorionic gonadotropin (hCG) (Intervet, Boxmeer, Netherlands), which were incubated at 39 ℃ with 5% CO_2 in a 95% humidified chamber. Oocyte maturation was performed with (21-22 h) or without (18-20 h) hormones in IVM medium.

3. *In vitro* fertilization (IVF) and *in vitro* culture (IVC)

For IVF, matured oocytes were denuded with 0.1% hyaluronidase and washed in TLH-PVA. Groups of 15 oocytes at the MII stage were placed randomly in 40 μL droplets of modified Tris-buffered medium (mTBM) (Abeydeera *et al.*, 1998) in a 35 × 10 mm Petri dish (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ, USA) and covered with prewarmed mineral oil. Next, liquid semen supplied weekly from the Veterinary Service Laboratory (Department of Livestock Research, Gyeonggi-do, Republic of Korea) was stored at 17 ℃ for 5 days before use. The semen sample was washed twice with Dulbecco's phosphate buffered saline supplemented with 0.1% BSA via centrifugation at 2000 × *g* for 2 min. After washing, the sperm pellet was resuspended in mTBM, which had been preequilibrated for 18 h at 39 ℃ in 5% CO_2. After appropriate dilution, 5 μL of the sperm suspension was added to a 40 μL drop of fertilization medium (mTBM) to set the final sperm

concentration at 1×10^6 sperm/mL. Just before fertilization, sperm motility was assessed, and more than 80% motile sperm were used in each experiment. To use stored liquid semen, a modified two-step culture system (Gil *et al.*, 2004) was applied. The oocytes were coincubated with the sperm for 20 min at 39 ℃ in a humidified atmosphere of 5% CO_2 and 95% air. After 20 min of coincubation with the sperm, the loosely attached sperm cells were removed from the zona pellucida via gentle pipetting. Next, the oocytes were washed three times in mTBM and incubated in mTBM without sperm for 5 to 6 h at 39 ℃ in a humidified atmosphere of 5% CO_2 and 95% air. Thereafter, the gametes were washed three times with IVC medium (porcine zygote medium 3, PZM-3) and cultured in 25 μL microdrops (10 gametes/drop) of PZM-3 (Yoshioka *et al.*, 2002) with prewarmed mineral oil. The embryos with cultured drops were incubated at 39 ℃ for 168 h under a humidified atmosphere of 5% O2, 5% CO_2, and 90% N_2. Cleavage and blastocyst formation were evaluated under a stereomicroscope (Olympus, Tokyo, Japan) at 48 and 168 h after insemination, respectively.

4. Embryo evaluation and total cell count of blastocysts

The day of IVF was considered Day 1. The embryos were evaluated for cleavage using a stereomicroscope on Day 3 (48 h). Evenly cleaved embryos were classified into three groups (two to three, five to eight, and six to eight cells). Blastocyst formation was assessed on Day 7 (168 h) after IVF, and blastocysts were classified according to degree of expansion and hatching status (Cheng *et al.*, 2004) as follows: an early blastocyst (a small blastocyst with a blastocele equal to or less than half of the embryo volume), an expanded blastocyst (a large blastocyst with a blastocele greater than half of the embryo volume or a blastocyst with a blastocele completely filling the embryo), and a hatched blastocyst (a hatching or already hatched blastocyst). To quantify the total cell number of the blastocysts, at Day 7, the blastocysts were collected and washed in 1% (wt/vol) PBS-BSA and stained with 5 μg/mL of Hoechst 33342 (bisbenzimide) for 5 min. After final wash in PBS-BSA, the embryos were fixed briefly in 4% paraformaldehyde in PBS. Next, the blastocysts were mounted on glass slides in a drop of 100% glycerol, covered gently with a coverslip, and observed using a fluorescence microscope (Nikon Corp.) at 400 × magnification. The experiment was repeated at least three times.

5. Quantitative real-time polymerase chain reaction (qRT-PCR)

For the gene expression study, blastocysts from each group (without LPA: control, LPA treated Day 1-3, Day 4-7, and Day 1-7) were sampled separately using a stereomicroscope. All samples were stored at -80 ℃ until analyzed. The expression levels of *POU5F1*, *CDX2*, *PCNA*, *BCL2*, *BAX*, and *CASP3* mRNA in the blastocysts were analyzed via quantitative RT-PCR. Total RNA was extracted using the NucleoZOL reagent (Macherey-Nagel GmbH & Co. KG, Germany), according to the manufacturer's protocol. Complementary DNA (cDNA) was prepared by subjecting total RNA to reverse transcription using Moloney murine leukemia virus reverse transcriptase (Invitrogen Corporation) and random primers (9-mers; Takara Bio, Inc., Otsu, Shiga, Japan). To determine the conditions for the logarithmic-phase PCR amplification of the target mRNA, 1-μg aliquots were amplified using differing numbers of cycles. The housekeeping gene *RN18S* was PCR amplified to rule out the possibility of RNA degradation and to control for the variation in mRNA concentrations in the reverse transcription reaction. A linear relationship between PCR product band visibility and the number of amplification cycles was observed for the target mRNAs. *RN18S* and the target genes were quantified using 40 cycles. The cDNA was amplified in a 20 μL PCR reaction containing 1 U Taq polymerase (Intron BioTechnologies, Co., Ltd., Seongnam, Korea), 2-mM deoxyribonucleoside triphosphates mix, and 10 pM of each gene-specific primer. The quantitative RT-PCR was performed with 1 μL of cDNA template added to 10 μL of 2 × SYBR Premix Ex Taq (Takara Bio, Inc.) containing specific primers at a concentration of 10 pM each. The reactions were carried out for 40 cycles, with the following cycling parameters: denaturation at 95 ℃ for 5 s, annealing at 57 ℃ for 15 s, and extension at 72 ℃ for 15 s. All oligonucleotide primer sequences are presented in Table 1. The fluorescence intensity was measured at the end of the extension phase of each cycle. The threshold value for the fluorescence intensity of all samples was set manually. The reaction cycle at which the PCR products exceeded this fluorescence intensity threshold was deemed the threshold cycle (Ct) in the exponential phase of the PCR amplification. The expression of each target gene was quantified relative to that of the internal control gene (*RN18S*). The relative quantification was based on a comparison of Cts at constant fluorescence intensity. The amount of transcript present was

Table 1. Sequences of the oligonucleotide primers and probes used in real-time PCR.

Messenger RNA	Primer sequences	Product size (bp)	GenBank accession number
RN18S	F: 5'-CGCGGTTCTATTTTGTTGGT-3' R: 5'-AGTCGGCATCGTTTATGGTC-3'	207	NR_046261
POU5F1	F: 5'-GCGGACAAGTATCGAGAACC-3' R: 5'-CCTCAAAATCCTCTCGTTGC-3'	200	NM_001113060
CDX2	F: 5'-GGAACCTGTGCGAGTGGATG-3' R: 5'-GCTCGGCCTTTCTCCGAATG-3'	168	NM_001278769
PCNA	F: 5'-CCTGTGCAAAAGATGGAGTG-3' R: 5'-GGAGAGAGTGGAGTGGCTTTT-3'	187	XM_003359883
BCL2	F: 5'-AGGGCATTCAGTGACCTGAC-3' R: 5'-CGATCCGACTCACCAATACC-3'	193	NM_214285
BAX	F: 5'-TGCCTCAGGATGCATCTACC-3' R: 5'-AAGTAGAAAAGCGCGACCAC-3'	199	XM_003127290
CASP3	F: 5'-CGTGCTTCTAAGCCATGGTG-3' R: 5'-GTCCCACTGTCCGTCTCAAT-3'	186	NM_214131

inversely related to the observed Ct and, for every twofold dilution in the amount of transcript, the Ct was expected to increase by one. The relative expression (R) was calculated using the following equation: $R=2^{-[\triangle Ct\ sample\ -\ \triangle Ct\ control]}$. To determine a normalized arbitrary value for each gene, every obtained value was normalized to that of *RN18S*. The experiments were repeated at least three times.

6. Experimental design

In experiment 1, IVF embryos at Day 1 to Day 7 (D1-7; full term), Day 1 to Day 3 (D1-3; early stage), Day 4 to Day 7 (D4-7; late stage), or without LPA (control group) were cultured in PZM-3 medium supplemented with 30 μM LPA. The blastocysts derived from IVF after treatment with LPA for different periods were collected on Day 7, and the mRNA expression of *POU5F1*, *CDX2*, *PCNA*, *BCL2*, *BAX*, and *CASP3* were investigated. The experiments were repeated three times. In experiment 2, IVF embryos at the zygote stage were cultured in PZM-3 medium supplemented with 0, 5, 10, and 20 μM U73122, a specific PLC inhibitor, to identify the optimal inhibitor concentration. According to the inhibitor concentration, the IVF embryos at the zygote stage were divided evenly into four groups as follows: a control group, a 30 μM LPA group, a 20 μM U73122 group, and a 30 μM LPA + 20 μM U73122 group. Each treatment group was treated for 2 days and then cultured in non-treatment PZM-3 for 5 days. The rate of cleavage was evaluated on Day 2, and the blastocyst formation and total cell count of blastocysts were evaluated on Day 7.

7. Statistical analysis

The statistical analyses were performed using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). Percentage data (e.g., rates of cleavage, blastocyst formation, and number of nuclei) were compared using one-way ANOVA, followed by Duncan's multiple range tests. All results are expressed as the mean ± standard error of the mean. Probability values less than 0.05 were considered statistically significant, unless otherwise stated.

RESULTS

1. Effects of LPA-supplemented IVC media on embryonic development after IVF

There was a significantly higher cleavage rate in the full-term group with that in the control (71.25% and 57.46%, respectively; Table 2 and Fig. 1A) and significantly higher total cell number in the blastocysts of the early-stage and late-stage groups compared with that in the control (56.07, 56.53, and 45.19, respectively; Table 2). There were no significant differences in the blastocyst formation rate in the treatment groups (early-stage, 32.81%; late-stage, 33.54%; full-term, 28.83%) compared with that in the control (32.51%; Fig. 1B).

Table 2. Preimplantation development of IVF porcine embryos cultured with 30 μM LPA over time.

LPA treatment	No. of embryos cultured, N*	No. (%) of embryos developed to		Total cell number (*n*)
		≥ 2-cell	blastocysts	
Control	196	113(57.46±2.55)[a]	63(32.51±2.36)	45.19±2.08(21)[a]
Day 1 - 3	193	120(61.99±2.95)[a,b]	63(32.81±3.89)	56.07±3.73(15)[b]
Day 4 - 7	196	129(65.91±1.98)[a,b]	66(33.54±3.07)	56.53±2.79(19)[b]
Day 1 - 7	199	142(71.25±4.95)[b]	57(28.83±4.44)	52.11±3.53(19)[a,b]

Values with different superscript letters within same column are significantly different (P<0.05).
*5 times replicated.

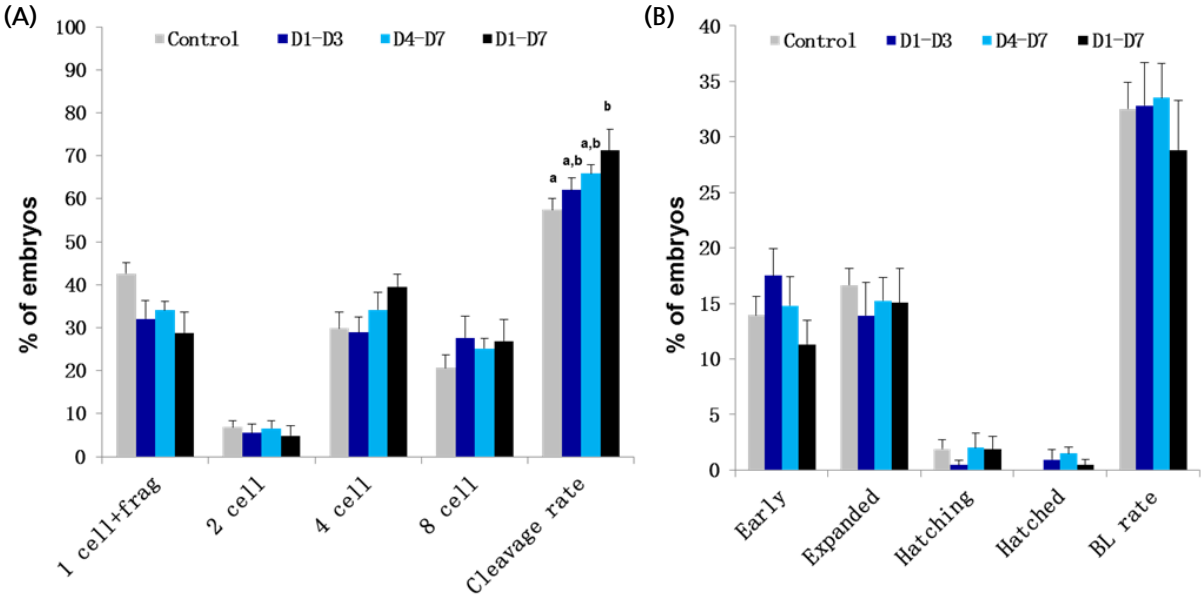

Fig 1. Effect of LPA treatment during IVC on the cleavage pattern of IVF at day 2 (A) and blastocysts formation pattern of IVF at day 7 (B). Values with different superscript letters within same column are significantly different (P<0.05). *5 times replicated.

2. Effects of LPA treatment during IVC on gene expression in blastocysts

To examine the effect of LPA on the expression of pluripotency-related, differentiation-related, DNA repair-related, and apoptosis-related genes, the mRNA expression levels of *POU5F1*, *CDX2*, *PCNA*, *BAX*, *BCL2*, and *CASP3* were evaluated in the blastocysts from each group. As shown in Fig. 2A, the expressions of *PCNA*, *BCL2*, and *BAX* in blastocysts obtained from the D1-7 group were significantly higher and the expression level of *BAX* in the Day 1-3 group was also significantly higher than that in the control (P< 0.05). The *BCL2/BAX* mRNA ratio in the D1-3 group was significantly lower than that in the control; however, the ratios in the D4-7 and D1-7 groups were comparable with that in the control group (P< 0.05). No significant differences in *POU5F1*, *CDX2*, and *CASP3* transcript levels were observed in the LPA-treated blastocyst group compared with that in the control group (Fig. 2B).

3. Effects of phospholipase C inhibitor, U-73122, treatment on IVF-derived embryo development

The cleavage rate and blastocyst formation rate were significantly lower in the 20 μM U73122-treated group (53.91% and 27.67%, respectively) than those in the control group (71.46% and 40.54%, respectively) after IVF (Table 3). The proportion of one-cell embryos and the fragmentation rate increased significantly and the four-cell-stage embryos were reduced significantly in the 20-μM U73122-treated group compared with that in the control group (Fig. 3A). The proportion of the expanded blastocysts was reduced significantly compared with that in the control group (Fig. 3B).

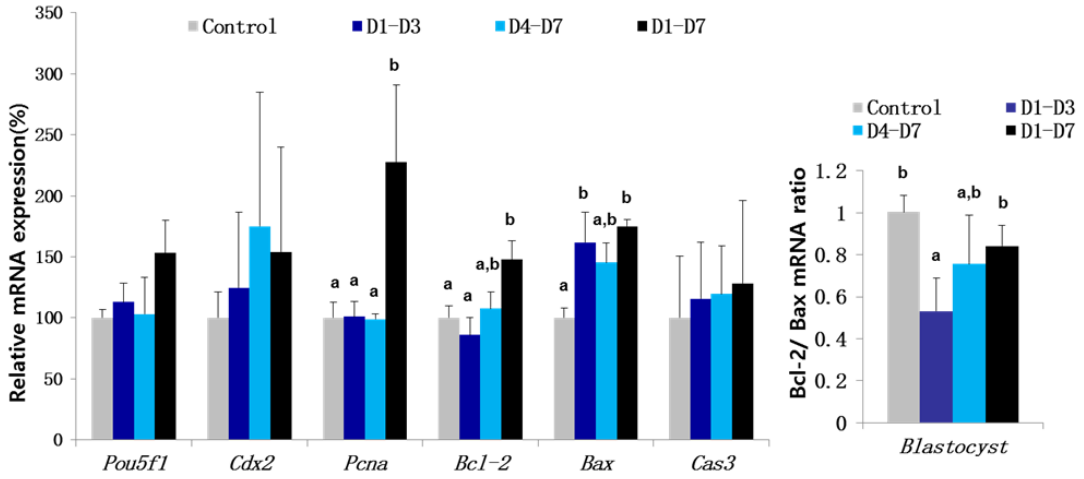

Fig 2. mRNA expression levels (Mean ± SEM) of POU5F1, PCNA, BCL2, BAX and CASP3 in blastocysts treated with LPA during in vitro culture (IVC) (A) Bcl-2/Bax mRNA ratio. (B) Within the same target mRNA, values with different superscript letters (a and b) are significantly (P<0.05) different. The experiment was replicated at least three times.

Table 3. Embryonic development of porcine embryos derived from IVF treated with PLC inhibitor during Days 1 to 3.

U73122 Treatment (μM)	No. of embryos cultured, N*	No. (%) of embryos developed to		Total cell number (n)
		≥ 2-cell	blastocysts	
0	109	78(71.46±1.49)^{b}	44(40.54±3.33)^{b}	43.85±2.06(27)
5	110	68(61.88±2.41)^{a,b}	40(36.46±1.64)^{a,b}	45.32±2.63(25)
10	109	68(62.22±3.25)^{a,b}	36(32.83±3.18)^{a,b}	42.91±2.36(22)
20	111	60(53.91±4.42)^{a}	31(27.67±3.61)^{a}	45.16±3.14(19)

Values with different superscript letters within same column are significantly different (P<0.05).
* 3 times replicated.

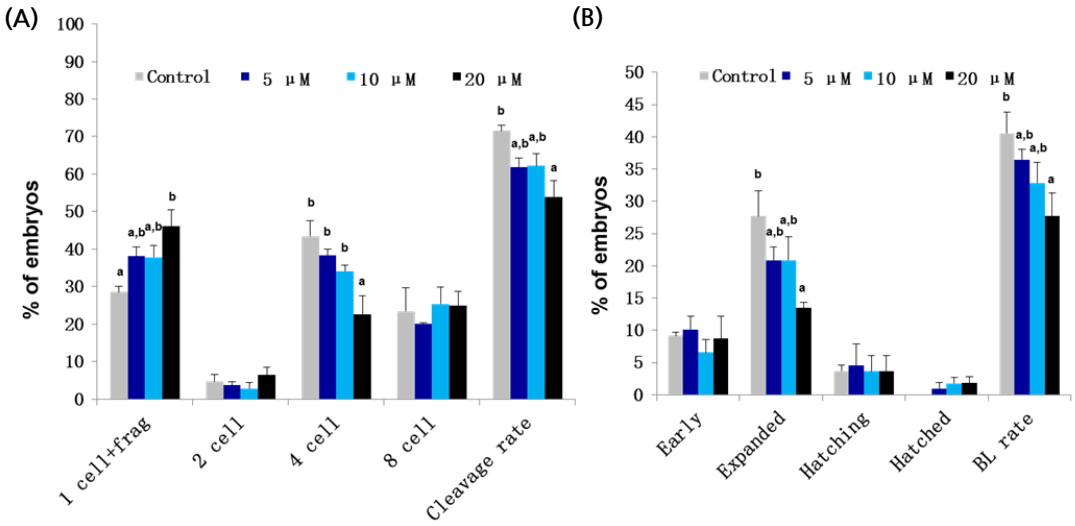

Fig 3. Effect of PLC inhibitor during IVC on the cleaved pattern of IVF at day 2 (A) and blastocysts formation pattern of IVF at day 7 (B). Values with different superscript letters within same column are significantly different (P<0.05). *3 times replicated.

4. Effects of U73122 and LPA co-treatments on porcine pre-implantation embryonic development

As shown in Table 4, the cleavage rate of the 30 μM LPA-treated group (71.92%) was significantly higher and that of the 20 μM U73122-treated group (57.22%) was significantly lower, compared with those in the other groups; the cleavage rate of the 30 μM LPA + 20 μM U73122-treated group showed no significant difference compared with that of the control group (65.47% and 65.59%, respectively). The proportion of one-cell embryos and the fragmentation rate were reduced significantly in the 30 μM LPA-treated group and were increased significantly in the 20 μM U73122-treated group, but showed no significant difference in the 30 μM LPA + 20 μM U73122-treated group (Fig. 4A). The hatched blastocyst rate increased significantly in 30-μM LPA-treated group compared with that in the control (Fig. 4B).

DISCUSSION

LPA has growth factor and hormone-like activities in various animal cells and influences the survival, proliferation, differentiation, migration, adhesion, and morphology of a range of cell types during development (Choi *et al.*, 2010; Mutoh *et al.*, 2012). In the reproductive system, LPA produced in the uterine endometrium might play a critical role in uterine endometrial function and conceptus development (Seo *et al.*, 2008). To date, supplementation of the culture medium with

Table 4. Effect of LPA on PLC inhibited porcine embryos derived from IVF during Days 1 to 3.

LPA and U73122 treatment (μM)	No. of embryos cultured, N*	No. (%) of embryos developed to		Total cell number (*n*)
		≥ 2-cell	blastocysts	
Control	247	162(65.59±1.15)^{b}	97(39.37±3.67)^{a,b}	47.90±1.69(49)
LPA	244	173(71.92±1.95)^{c}	115(46.59±2.33)^{b}	46.62±1.69(53)
U73122	244	138(57.22±2.08)^{a}	82(33.74±2.55)^{a}	46.29±2.05(41)
LPA+U73122	243	159(65.47±2.07)^{b}	84(38.59±1.96)^{a}	48.93±2.22(40)

Values with different superscript letters within same column are significantly different (P<0.05).
* 6 times replicated.

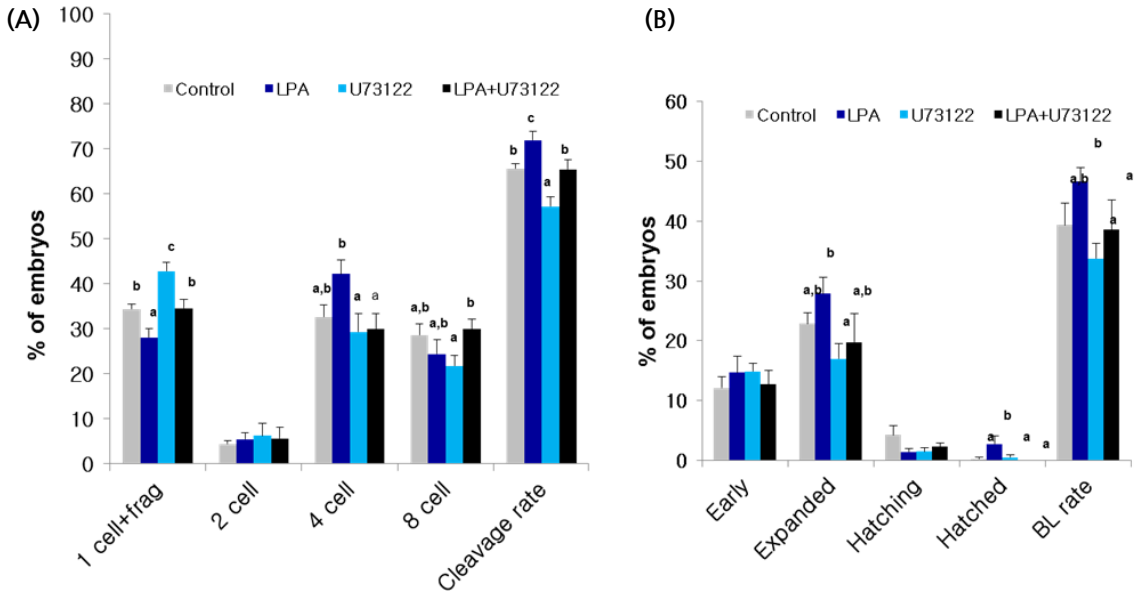

Fig 4. Effect of LPA and PLC inhibitor co-treatment during IVC on the cleaved pattern of IVF at day 2(A) and blastocysts formation pattern of IVF at day 7(B). Values with different superscript letters within same column are significantly different (P<0.05). *6 times replicated.

LPA during embryo development has been reported in several studies. However, the timing and concentration of the LPA treatment were different in these studies, which led to different results. The addition of 10 μM LPA to the culture medium at the pronuclear stage increased the blastocyst rate, and treatment for 1 h with 10 μM LPA at the morula stage accelerated trophoblast differentiation in mouse embryos (Kobayashi *et al.*, 1994; Liu and Armant, 2004). In addition, exposure of one-cell stage porcine embryos in the culture medium to 10μM LPA improved the blastocyst rates, inducing an increase in the inner cell mass, trophectoderm, and total cells in porcine blastocysts (Zhang *et al.*, 2015). In contrast, supplementation of the culture medium with 10 μM LPA during the late cleavage stage had no effect on embryo yield or quality (Torres *et al.*, 2014). However, no reports have studied the contribution of LPA to preimplantation porcine embryo development comprehensively. In the present study, the contribution of LPA to porcine IVC was investigated via supplementation of LPA at the early stage, late stage, and full term embryonic development during IVC, and it showed that LPA supplementation during IVC has beneficial effects on the early embryo cleavage and cell number of blastocysts.

The effects of LPA supplementation during IVC on mRNA expression in blastocysts have been reported in cattle and pigs (Torres *et al.*, 2014; Zhang *et al.*, 2015). In cattle, the results showed that exposure to 10 μM LPA reduced the transcript level of *BAX* significantly and increased transcription levels of *BCL2* and *IGF2R* significantly compared with control blastocysts (Torres *et al.*, 2014). Similarly, in pigs, exposure to 10 μM LPA reduced the expression levels of *CASP3* and *BAX* significantly, but increased that of *BCL2L1* (Zhang *et al.*, 2015). However, in the present study, both *BCL2* and *BAX* mRNA levels increased significantly in the full-term 30 μM LPA-treated group. *BCL2* family members are noted for their regulation of apoptosis, playing a pivotal role in deciding whether a cell will live or die (Gross *et al.*, 1999). Therefore, we analyzed the *BCL2/BAX* ratio as an indication of cell survival or death (a lower ratio indicates less susceptibility to apoptosis). The results showed that the *BCL/BAX* ratio was significantly lower in D1-3 group, but was rescued in the D1-7 group, which was similar to that in the control group. Otherwise, this study also noted significantly increased *PCNA* mRNA expression in the D1-7 group. The PCNA protein is a central molecule in the life and death cycle of a cell. PCNA is involved in DNA repair, including nucleotide excision repair, base excision repair, and mismatch repair (Paunesku *et al.*, 2001). Thus, the results suggested that the balance between cell proliferation and apoptosis disturbed in the blastocysts, and that the functions of *PCNA* might be switched on significantly within the blastocysts of D1-7 group, thereby increasing the *BCL2/BAX* ratio significantly.

Numerous studies have demonstrated that activators of G protein-dependent PLC or tyrosine kinase-dependent PLC, including LPA, induce upregulation of PLC and the release of intracellular Ca^{2+} (Lapetina, 1982; Margolis *et al.*, 1989; Moolenaar, 1995). Those studies showed that potentially, LPA could influence embryonic development by modulating intracellular Ca^{2+} signaling through the PLC pathway. In fertilized eggs, the release of intracellular Ca^{2+}, acting through calmodulin, is critical for numerous developmental events, including egg activation, fertilization, and cell division, representing a key regulator of mitosis and cytokinesis (Hepler, 1989; Keating *et al.*, 1994; Whitaker and Patel, 1990). Intracellular Ca^{2+} transients occur concomitantly with cell division during early embryogenesis, providing an additional link between Ca^{2+} and early embryonic development (Grandin and Charbonneau, 1991; Keating *et al.*, 1994; Stricker, 1995). Moreover, intracellular Ca^{2+} is also critical for growth and differentiation in mouse embryos (Stachecki and Armant, 1996). Based on the observation that the differentiation of the trophoblast begins with the process of compaction, previous reports showed that maintaining the compaction of the morula is regulated by $[Ca^{2+}]_i$, presumably acting on the cytoskeleton, and that LPA accelerates blastocyst differentiation through its ability to induce Cai^{2+} transients and heparin-binding EGF-like growth factor (HB-EGF) autocrine signaling (Liu and Armant, 2004; Pey *et al.*, 1998). However, the PLC signaling pathway induced by LPA in the early stage of porcine embryo development has yet to be determined. Although the intracellular level of Ca^{2+} or PKC were not measured directly after LPA exposure in this study and it cannot be certain that their intracellular levels were increased, the inhibition of PLC by U73122 resulted in a cleavage rate similar to the control after LPA exposure during IVC, suggesting that the PLC signaling pathway is activated by LPA leading to an increased cleavage rate.

Finally, although this study revealed a beneficial effect of LPA on the early embryo cleavage rate and the total cell number

of blastocysts, some limitations should be taken into account when interpreting the results. LPA stimulation had no effect on the blastocyst formation rate, which agreed with previous results in cattle, but contrasted with those in mice (Kobayashi *et al.*, 1994; Torres *et al.*, 2014). Therefore, the effect of 30 μM LPA supplementation in IVC medium on the blastocyst rate during porcine embryo development is probably species-specific or is related to difference in LPA concentrations used among the studies. Moreover, it can be noted that a discrepancy in the results for the blastocysts total cell number between Table 2 and Table 4. The concentration of DMSO, as the solute of U73122 used in this study was 0.20% and was adjusted to be the same in the other groups in Table 4. DMSO concentrations of 0.10% and 0.20% used during *in vitro* culture influence the quality of embryos at the morphological level and supplementation of 0.10% DMSO was suitable for working with bovine embryos *in vitro*. Thus, it is possible that the toxic effects of the higher DMSO concentration might be the key factor in the discrepancy between two outcomes.

In summary, it focused on the effect of LPA supplementation at different stages of porcine embryonic development during IVC. Furthermore, this study indicated that the PLC signaling pathway was activated by LPA in the early embryo cleavage stage and contributed to an increased cleavage rate. The schematic drawing of the proposed hypothetical mechanism of LPA was shown in Fig. 5. However, the mechanisms of PLC downstream signaling in early embryo cleavage stage and embryo genomic activation period are not well understood. In a future study, the effect of LPA supplementation on the mechanism of PLC downstream signaling should be determined.

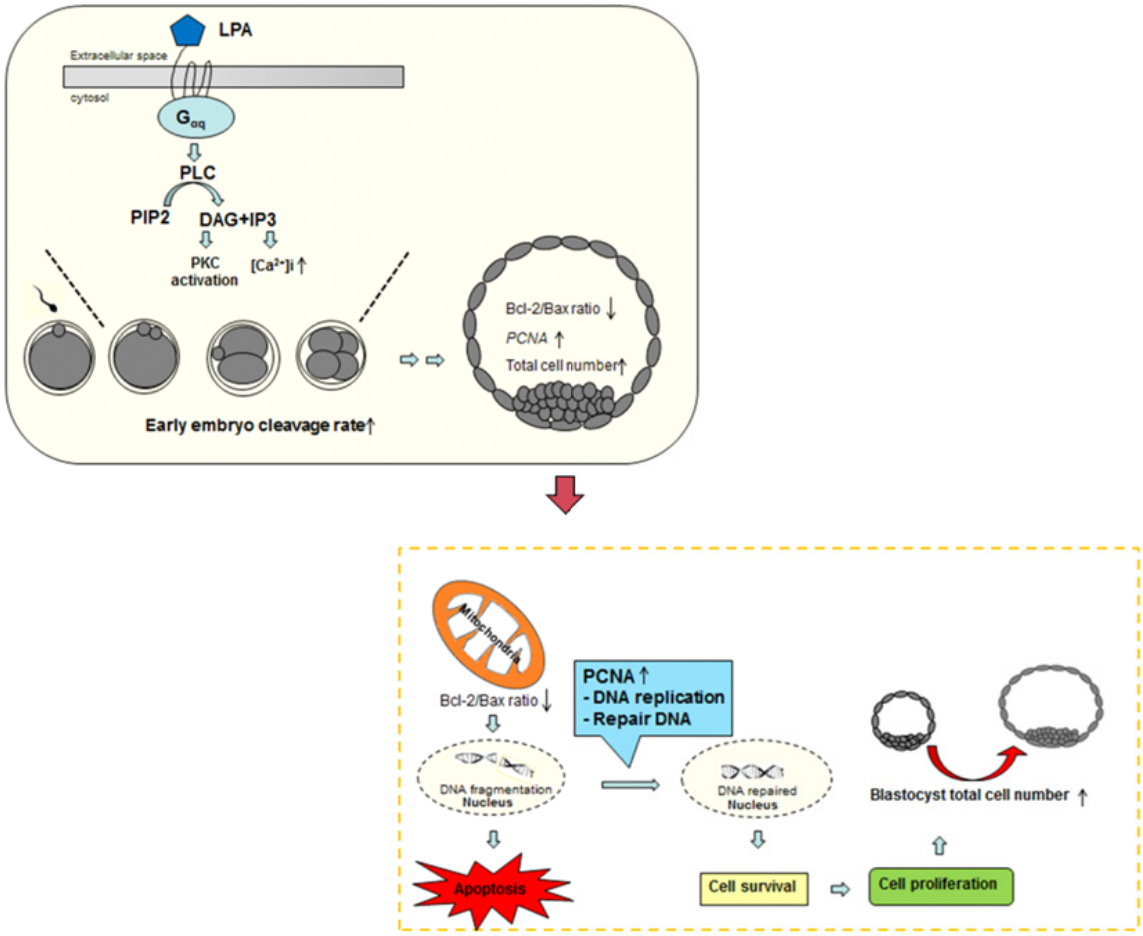

Fig 5. Schematic drawing of the proposed hypothetical mechanism of LPA in porcine embryonic development during in vitro culture. Treatment with LPA during IVC improved the porcine early embryo cleavage after IVF by activating the PLC signaling pathway and regulate the mRNA expression to increase the total cell number of blastocysts during blastocyst formation.

REFERENCES

Abeydeera LR, Wang WH, Cantley TC, Rieke A, Prather RS, and Day BN. 1998. Presence of epidermal growth factor during in vitro maturation of pig oocytes and embryo culture can modulate blastocyst development after in vitro fertilization. Mol. Reprod. Dev. 51:395-401.

Boruszewska D, Sinderewicz E, Kowalczyk-Zieba I, Grycmacher K, and Woclawek-Potocka I. 2016. Studies on lysophosphatidic acid action during in vitro preimplantation embryo development. Domest. Anim. Endocrinol. 54:15-29.

Cheng T, Huang C, Huang L, Chen C, Lee M, and Liu J. 2004. Evaluation of mouse blastocyst implantation rate by morphology grading. Chin. J. Physiol. 47:43.

Choi JW, Herr DR, Noguchi K, Yung YC, Lee C-W, Mutoh T, Lin M-E, Teo ST, Park KE, and Mosley AN. 2010. LPA receptors: subtypes and biological actions. Annu. Rev. Pharmacol. Toxicol. 50:157-186.

Gil MA, Ruiz M, Vazquez JM, Roca J, Day BN, and Martinez EA. 2004. Effect of short periods of sperm-oocyte coincubation during in vitro fertilization on embryo development in pigs. Theriogenology 62:544-552.

Grandin N, and Charbonneau M. 1991. Cycling of intracellular free calcium and intracellular pH in Xenopus embryos: a possible role in the control of the cell cycle. J. Cell Sci. 99:5-11.

Gross A, McDonnell JM, and Korsmeyer SJ. 1999. BCL-2 family members and the mitochondria in apoptosis. Genes. Dev. 13:1899-1911.

Hepler PK. 1989. Calcium transients during mitosis: observations in flux. J. Cell Biol. 109:2567-2573.

Hinokio K, Yamano S, Nakagawa K, Irahara M, Kamada M, Tokumura A, and Aono T. 2002. Lysophosphatidic acid stimulates nuclear and cytoplasmic maturation of golden hamster immature oocytes in vitro via cumulus cells. Life. Sci. 70:759-767.

Jo JW, Jee BC, Suh CS, and Kim SH. 2014. Addition of lysophosphatidic acid to mouse oocyte maturation media can enhance fertilization and developmental competence. Hum. Reprod. 29:234-241.

Keating TJ, Cork RJ, and Robinson KR. 1994. Intracellular free calcium oscillations in normal and cleavage-blocked embryos and artificially activated eggs of Xenopus laevis. J. Cell Sci. 107:2229-2237.

Kim K-J, Lee E, and Hyun S-H. 2016. Effect of lysophosphatidic acid on porcine oocyte in vitro maturation and subsequent embryonic development after parthenogenetic activation and ivf. Reprod. Fertil. Dev. 28:159.

Kobayashi T, Yamano S, Murayama S, Ishikawa H, Tokumura A, and Aono T. 1994. Effect of lysophosphatidic acid on the preimplantation development of mouse embryos. FEBS Lett. 351:38-40.

Komatsu J, Yamano S, Kuwahara A, Tokumura A, and Irahara M. 2006. The signaling pathways linking to lysophosphatidic acid-promoted meiotic maturation in mice. Life Sci. 79: 506-511.

Lapetina E. 1982. Platelet-activating factor stimulates the phosphatidylinositol cycle. Appearance of phosphatidic acid is associated with the release of serotonin in horse platelets. J. Biol. Chem. 257:7314-7317.

Liu Z, and Armant DR. 2004. Lysophosphatidic acid regulates murine blastocyst development by transactivation of receptors for heparin-binding EGF-like growth factor. Exp. Cell Res. 296:317-326.

Margolis B, Rhee S, Felder S, Mervic M, Lyall R, Levitzki A, Ullrich A, Zilberstein A, and Schlessinger J. 1989. EGF induces tyrosine phosphorylation of phospholipase C-II: a potential mechanism for EGF receptor signaling. Cell 57:1101-1107.

Moolenaar W, Van der Bend R, Van Corven E, Jalink K, Eichholtz T, and Van Blitterswijk W (1992). Lysophosphatidic acid: a novel phospholipid with hormone-and growth factor-like activities. Paper presented at: Cold Spring Harb Symp Quant Biol. (Cold Spring Harbor Laboratory Press).

Moolenaar WH. 1995. Lysophosphatidic acid, a multifunctional phospholipid messenger. J. Biol. Chem. 270:12949-12952.

Moolenaar WH. 2000. Development of our current understanding of bioactive lysophospholipids. Ann N Y Acad. Sci. 905:1-10.

Mutoh T, Rivera R, and Chun J. 2012. Insights into the pharmacological relevance of lysophospholipid receptors. Br J. Pharmacol 165:829-844.

Paunesku T, Mittal S, Protić M, Oryhon J, Korolev S, Joachimiak A, and Woloschak G. 2001. Proliferating cell nuclear antigen (PCNA): ringmaster of the genome. Int. J. Radiat. Biol. 77:1007-1021.

Pey R, Vial C, Schatten G, and Hafner M. 1998. Increase of intracellular Ca2+ and relocation of E-cadherin during experimental decompaction of mouse embryos. Proceedings

of the National Academy of Sciences 95:12977-12982.

Riaz A, Huang Y, and Johansson S. 2016. G-protein-coupled lysophosphatidic acid receptors and their regulation of AKT signaling. International journal of molecular sciences Front Biosci. (Landmark Ed) 17:215.

Seo H, Kim M, Choi Y, Lee C-K, and Ka H. 2008. Analysis of lysophosphatidic acid (LPA) receptor and LPA-induced endometrial prostaglandin-endoperoxide synthase 2 expression in the porcine uterus. Endocrinology 149:6166-6175.

Stachecki JJ, and Armant DR. 1996. Transient release of calcium from inositol 1, 4, 5-trisphosphate-specific stores regulates mouse preimplantation development. DEVELOPMENT-CAMBRIDGE-122:2485-2496.

Stricker SA. 1995. Time-lapse confocal imaging of calcium dynamics in starfish embryos. Dev. Biol. 170:496-518.

Tigyi G, and Miledi R. 1992. Lysophosphatidates bound to serum albumin activate membrane currents in Xenopus oocytes and neurite retraction in PC12 pheochromocytoma cells. J. Biol. Chem. 267:21360-21367.

Tokumura A, Miyake M, Nishioka Y, Yamano S, Aono T, and Fukuzawa K. 1999. Production of lysophosphatidic acids by lysophospholipase D in human follicular fluids of in vitro fertilization patients. Biol. Reprod. 61:195-199.

Torres AC, Boruszewska D, Batista M, Kowalczyk-Zieba I, Diniz P, Sinderewicz E, Saulnier-Blache JS, Woclawek-Potocka I, and Lopes-da-Costa L. 2014. Lysophosphatidic acid signaling in late cleavage and blastocyst stage bovine embryos. Mediators Inflamm. 2014.

Whitaker M, and Patel R. 1990. Calcium and cell cycle control. Development 108:525-542.

Yoshioka K, Suzuki C, Tanaka A, Anas IM, and Iwamura S. 2002. Birth of Piglets Derived from Porcine Zygotes Cultured in a Chemically Defined Medium 1. Biol. Reprod. 66:112-119.

Yung YC, Stoddard NC, and Chun J. 2014. LPA receptor signaling: pharmacology, physiology, and pathophysiology. J. Lipid. Res. 55:1192-1214.

Zhang JY, Dong HS, Oqani RK, Lin T, Kang JW, and Jin DI. 2014. Distinct roles of ROCK1 and ROCK2 during development of porcine preimplantation embryos. Reproduction 148: 99-107.

Zhang JY, Jiang Y, Lin T, Kang JW, Lee JE, and Jin DI. 2015. Lysophosphatidic acid improves porcine oocyte maturation and embryo development in vitro. Mol. Reprod. Dev. 82:66-77.

Received November 29 2017, Revised December 15, 2017, Accepted December 20, 2017