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Effect of lysophosphatidic acid on the follicular development and the expression of lysophosphatidic acid receptor genes during *in vitro* culture of mouse ovary

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Abstract

Lysophosphatidic acid (LPA) known as a serum-derived growth factor, is involved in several cell physiological functions in the female reproductive system including: oocyte maturation, *in vitro* fertilization and embryo implantation by its transmembrane G protein-coupled receptors. The aim of the present study was to examine the effect of LPA on *in vitro* follicular development of mouse ovarian tissue. Neonatal mouse ovarian tissues were cultured in five different concentrations of LPA (0, 5, 10, 20 and 40 μM). The developmental competence and the function of cultured ovarian tissue were assessed by morphological study using hematoxylin and eosin staining and hormonal analysis. The expression of LPA receptor (LPAR 1-4) genes were analyzed by real-time RT-PCR. The proportion of preantral follicles and the level of E_2 hormone were significantly higher in the 20 μM LPA-treated group than those in the other treatment groups. There was a significant difference in the expression of LPAR 1-4 genes in 20 μM LPA treated group in comparison with 0 μM LPA (control group) treated and non-cultured groups. In addition, the expression of LPAR1 gene was higher than other receptor genes in all studied groups. In conclusion supplementation of the media with 20 μM LPA, could improve the survival and developmental potential of follicles and it had positive effects on cell function and stimulation of E_2 synthesis in mouse whole ovarian tissues.

اثر لیزوفسفاتیدیک اسید بر روی تکوین فولیکولی و بیان ژن‌های گیرنده لیزوفسفاتیدیک در طول کشت آزمایشگاهی بافت تخمدان موش

چکیده

لیزوفسفاتیدیک اسید که به‌عنوان فاکتور رشد برگرفته از سرم شناخته می‌شود در بسیاری از عملکردهای فیزیولوژیک سیستم تولیدمثل جنس ماده از جمله بلوغ سیتوپلاسمی و هسته‌ای تخمک، لقاح آزمایشگاهی و لانه گزینی جنین بوسیله گیرنده‌های غشایی همراه شده با پروتئین G نقش دارد. هدف از مطالعه حاضر بررسی تأثیر لیزوفسفاتیدیک اسید بر روی تکوین فولیکولی بافت تخمدان موش در آزمایشگاه می‌باشد. بافت تخمدان‌های موش هفت روزه در حضور پنج غلظت مختلف از لیزوفسفاتیدیک اسید (صفر، پنج، ۱۰، ۲۰ و ۴۰ میکرومول) کشت داده شدند. پتانسیل تکوین و عملکرد بافت تخمدانی توسط مطالعه مورفولوژیک (رنگ آمیزی هماتوکسیلین و ائوزین) و آنالیز هورمونی مورد ارزیابی قرار گرفت. میزان بیان ژن رسپتورهای لیزوفسفاتیدیک اسید (LPAR1-4) با استفاده از روش RT–PCR کمی آنالیز شد. تعداد فولیکول های پره آنترال و سطح هورمون ۱۷–بتا استرادیول در گروهی که تحت تأثیر دوز ۲۰ میکرومول لیزوفسفاتیدیک اسید کشت داده شده بود در مقایسه با دوزهای دیگر افزایش معنی‌داری داشت. بیان ژن‌های LPAR1-4 در گروهی که با دوز ۲۰ میکرومول لیزوفسفاتیدیک اسید کشت داده شده بود در مقایسه با گروه کشت نشده و گروه کشت شده با دوز صفر میکرومول (گروه کنترل) به طور معنی داری متفاوت بود .علاوه بر این ژن LPAR1 بالاترین میزان بیان ژن را نسبت به سایر ژن‌های رسپتورها در همه گروه های مورد مطالعه داشت. غنی‌سازی محیط کشت با لیزوفسفاتیدیک اسید، به ویژه در دوز ۲۰ میکرومول ، میزان زنده ماندن و پتانسیل تکوین فولیکولی را بهبود بخشید و تأثیرات مثبتی روی عملکرد سلولی و تحریک سنتز E_2 در بافت‌های کامل تخمدان موش گذاشت.

واژه های کلیدی: تخمدان، کشت آزمایشگاهی، گیرنده لیزوفسفاتیدیک اسید، لیزوفسفاتیدیک اسید، موش

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Introduction

In vitro culture (IVC) of ovarian tissue has been introduced as an alternative approach for follicular development in the field of reproductive biology.[1-4] However, the low survival rate of follicles, maturation rate of oocytes and embryo development was published and up to now no pregnancy was recorded in human.[5] Therefore, the improvement of *in vitro* culture of ovarian tissue is challenging because it isn't defined the optimal maturation condition (physical and biochemical condition) for IVC of mouse ovarian tissue.[6,7]

Many researchers have also been committed to trigger the activation of primordial follicles and oocyte maturation by adding some supplements to culture media such as growth factors, antioxidants, gonadotropins, nutrients.[5,8-10]

Lysophosphatidic acid (LPA) is a phospholipid with 430-480 Da molecular weight[11] that was detected in the blood serum. It is produced by cell components of follicle in all stages of follicular development.[12] It is known as a serum-derived growth factor, involving in several physiological functions of the cells in the female reproductive system[13-16] including: oocyte maturation, *in vitro* fertilization and embryo implantation.[17-20] LPA mediates these functions by transmembrane G protein-coupled receptors.[21] It has been shown that following LPA binding to its receptors, multiple signaling pathways can be activated via variety heterotrimeric G proteins subtypes.[20,22-25]

Previous studies demonstrated that the specific cell surface receptors of LPA (LPARs) are expressed in oocyte, cumulus cells, endometrial cells and mast cells.[18,25-29] Available data regarding LPA receptor in the ovarian tissues and cells demonstrated that mRNA of LPAR1, LPAR2 and LPAR4 was detected in mouse ovarian tissues,[30,31] while the expression of LPAR3 is not demonstrated in mouse ovary.[31,32] It may be happened because LPAR3 participates in the initiation of downstream signaling cascades though main cellular mechanisms.[27,33-40]

To the best of our knowledge, there is not sufficient information regarding to the effects of different concentrations of LPA on the follicular development of mouse ovarian tissue. Since the biological effects of LPA may be concentration dependent, therefore, in the present study, the survival and development of mouse ovarian follicles were investigated using different concentrations of LPA.

The results of this work can be used to provide better culture conditions for *in vitro* culture of human primordial follicles. Furthermore, the present study updates our knowledge on the effect of LPA on the expression of LPA receptor genes in neonatal and postnatal mouse ovaries.

Materials and Methods

Chemicals. All supplements were acquired from Sigma-Aldrich (Dusseldorf, Germany) except otherwise indicated.

Animals and ovarian tissue. In this experimental study, the ovaries were collected from neonatal (7-day-old) National Medical Research Institute (NMRI) mice that were kept under a controlled condition (22.00 ± 2.00 ˚C, 12 hr light: 12 hr dark and 40 to 50% humidity) in the animal house of Tarbiat Modares University. These experiments were performed according to the ethical guidelines for the Care and Use of Laboratory Animals in Tarbiat Modares University (Ref No: 52.8188). The mice (n = 45) were sacrificed by cervical dislocation, and their ovaries were removed and dissected mechanically and washed in alpha-minimal essential medium (α-MEM; Invitrogen, Paifley, UK) supplemented with 5.00% fetal bovine serum (Invitrogen), 100 IU mL^{-1} penicillin (Invitrogen), 100 µg mL^{-1} streptomycin (Invitrogen), 0.23 mMol sodium pyruvate (Sigma-Aldrich) and sodium bicarbonate (Sigma-Aldrich).

Experimental design. The collected ovaries were randomly divided into six groups (n = 15 in each group and in three repeats). The ovaries were cultured in α-MEM supplemented with different concentrations of LPA (0, 5, 10, 20 and 40 µM).[19] One week after *in vitro* culture of mouse ovaries, the morphology of cultured ovaries were studied using hematoxylin and eosin staining and the expression of genes related to folliculogenesis and LPA receptors were evaluated by real time RT-PCR.

***In vitro* culture of ovarian tissue.** The whole ovaries in all studied groups (n= 15 in each group) were cultured on inserts (Millicell-CM, 0.4-m pore size; Millipore Corp, Billerica, USA) in 24-well plates including 400 µL of basic culture medium consisted of α-MEM supplemented with 1% insulin, transferrin and selenium (ITS; Gibco), 5.00% fetal bovine serum, 100 mIU mL^{-1} recombinant FSH (rFSH Serono. Laboratories, Aubonne, Switzerland) and 5, 10, 20 and 40 µM LPA at 37 ˚C in a 5.00% CO_2 for one week. Half of the culture medium (200 µL) was renewed with fresh medium every other day and the collected media were reserved at −20 ˚C for evaluation of 17-β-estradiol hormone.

Histological evaluation. Histological features of the ovarian tissues (n = 5 in each group) were evaluated by hematoxylin and eosin staining. The ovaries were fixed in Bouin's fixative for eight hr. After fixation, the ovaries were embedded in paraffin and the tissue sections were prepared serially at 5 µm-thickness and subsequently mounted on slides with 5^{th} intervals and stained with routine hematoxylin and eosin method. The morphology of ovarian tissues and the number of ovarian follicles were assessed under light microscope field by field. Classification of ovarian follicles (primordial, primary,

preantral and antral) was performed according to the previously described method.[41] The follicles containing germinal vesicle in the oocyte were counted before and after seven days *in vitro* culturing. The normal follicles had intact oocyte and granulosa cells and the degenerated follicles had pyknotic oocyte nuclei and disarranged granulosa cells.

Ovarian surface area. The micrographs of each ovary at the same magnification during culture period (on days 0, 3, 5 and 7 of culture) were captured and the area of each ovary was analyzed and determined in cubic micrometers (μm^2) by Digimizer software (MedCalc Software bvba, Acacialaan, Belgium).

RNA extraction and cDNA synthesis. Total RNA was extracted from 0 and seven days cultured ovaries (n = 3 in each group in three repeats) using the trizol reagent (Invitrogen) according to procedure described by the manufacturer's instructions RNeasy Mini Kit (Qiagen, Hilden, Germany). Briefly, the ovaries were homogenized in 0.5 mL of trizol reagent then, 0.2 mL of chloroform was added per one mL of trizol and the samples were centrifuged at 12000 *g* for 10 min. The upper colorless aqueous phase was transferred to a fresh two mL microtube and 700 µL of 100% isopropanol (Sigma-Aldrich) were added and centrifuged at 12000 *g* for 10 min. Then 1.5 mL 70% ethanol (Merck, Dusseldorf, Germany) per one mL of trizol reagent was added. RNA concentration was determined using spectrophotometry (Eppendorf, Hamburg, Germany).

The cDNA was synthesized using cDNA synthesis kit (ThermoFisher Scientific, Lithuania, USA) according to the manufacturer's instructions. Oligo(dT) was used for cDNA synthesis and reverse transcriptase reaction was incubated at 42 ˚C for 60 min. After inactivation of the reverse transcriptase enzyme, the resultant product was stored at – 20 ˚C.

Real-time RT-PCR. The primers for LPAR1-4 genes were designed using Primer-BLAST tool in NCBI (Table 1). The designed primers were analyzed using Oligo-Analyser.[42] In the present study β-actin gene was considered as housekeeping gene. Real-time RT-PCR was performed using QuantiTect SYBR Green RT-PCR kit (Qiagen). Thermal program of the real-time RT-PCR was set with the following parameters: the initial holding stage for 5 min at 95 ˚C, 40 cycles cycling stage of 15 sec at 95 ˚C, 58 ˚C for 30 sec, and 72 ˚C for 15 sec, that was continued by a melt curve stage at 95 ˚C for 15 sec, 60 ˚C for 1 min, and 95 ˚C for 15 sec. After completing the PCR run, the melt curve was obtained using the amplicon. For determining of relative quantitation of target genes the Pfaffl method was used.[43]

Hormonal assay. For the evaluation of the endocrine function of ovaries, the concentration of 17-β-estradiol (E_2), was measured in the collected media obtained from cultured ovaries on days 3 and 7 (n = 15 per groups in three repeats) using microplate enzyme immunoassay kit (Monobind Inc., Lake Forest, USA) with sensitivity = 6.5 pg mL^{-1}).[44]

Statistical analysis. Statistical analysis was performed using SPSS (version 21.0; IBM Corp., Armonk, USA). Values are given as mean ± SE. One-way ANOVA and post hoc Tukey was used to compare differences in follicular count, ovarian area and mRNA expression. The data of hormone level was analyzed with Student's t-test. A *p* value less than 0.05 was considered as statistically significant at the 95.00% confidence level.

Results

Morphology of ovaries and follicular count. The morphological observations of cultured ovaries under invert microscope in different groups of study were shown in Figure 1. Our results demonstrated that the follicles were grown more on the periphery of ovarian tissues and they had a large and clear antral cavity. The growth of follicles was parallel with increasing in the size of ovaries during the culture period. However, in the central parts of the ovaries the density of the tissue was higher than marginal and central parts of tissue was dark.

Light microscopy. The light microscopic observations of examined groups using H&E staining are shown in Figure 2. Before *in vitro* culturing, the primordial follicles were mainly seen and located in the peripheral part of the ovaries. After seven days of ovaries culture, the primordial, primary and antral follicles were prominent within the ovarian tissue (Fig. 2). The healthy follicles with normal structure were visible in all examined groups especially

Table 1. The sequences of the designed primers used for evaluating the expression of LPAR 1-4 genes.

Accession numbers	Gene	Primer Sequence	PCR product size (bp)
XM_011249935.1	LPAR1	Forward primer: CTGCCTCTACTTCCAGCCCTG Reverse primer: GCTCACTGTGTTCCATTCTGTG	141
NM_020028.3	LPAR2	Forward primer: GACCACACTCAGCCTAGTCAAGAC Reverse primer: CTTACAGTCCAGGCCATCCA	106
NM_022983.4	LPAR3	Forward primer: CCACTTTCCCTTCTACTACCTGCT Reverse primer: GACGGTCAACGATTTCGACACC	115
NM_175271.4	LPAR4	Forward primer: GCCAGTTGCCAGTTTACACG Reverse primer: TGGACGCAGACGATCAGA GA	118
NM_031144.2	β-actin	Forward primer: CTATGTTGCCCTAGACTTCG Reverse primer: AGGTCTTTACGGATGTCAAC	228

in 20 µM LPA supplemented group (Fig. 2: D1-D3). The granulosa cells of follicles arranged well around the oocyte. The damage follicles (pyknotic nuclei oocytes and disarranged granulosa cells) were prominent (Fig. 2: A1-A3) especially in the central area of ovaries that were cultured in the absence of LPA (control group; Fig. 2). In addition, in 40 µM LPA-treated group some premature antral follicles were seen (Fig. 2: E1-E3).

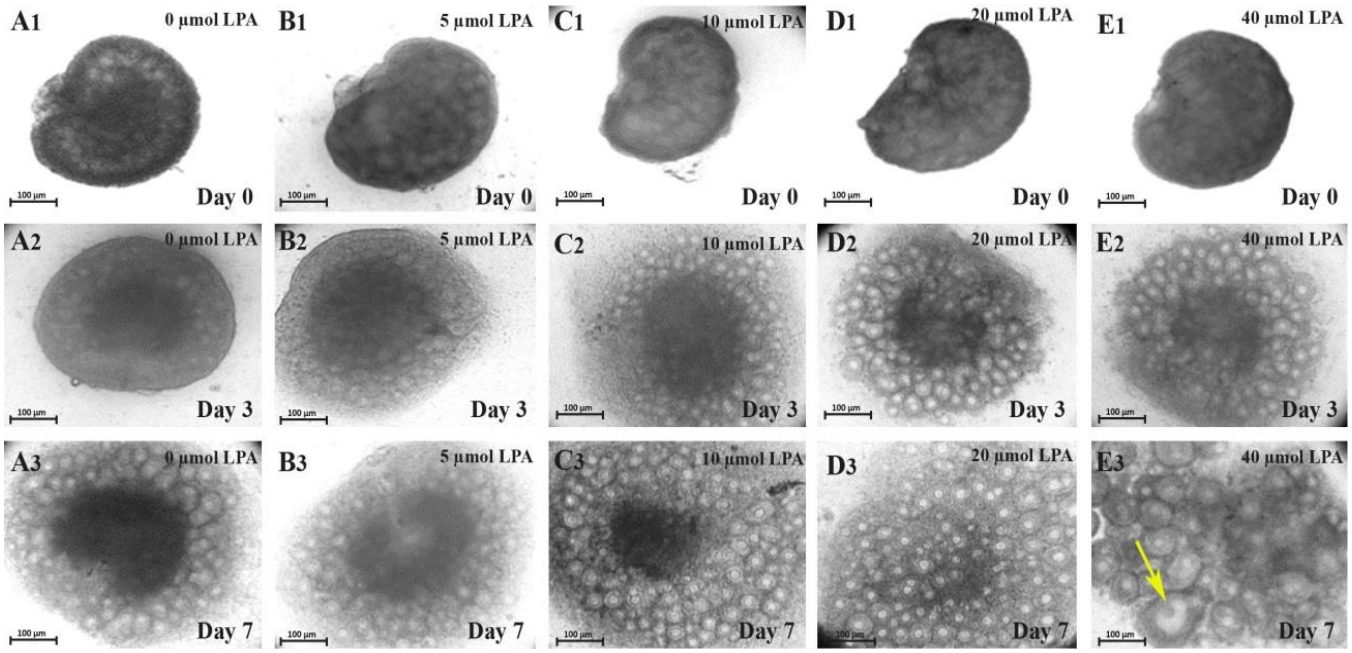

Fig. 1. Photomicrographs of mouse ovaries imaged under an inverted microscope on days 0, 5 and 7. A_1-A_3: cultured group (0 µM LPA) (Control); B_1-B_3: 5 µM LPA treated group; C_1-C_3:10 µM LPA treated group; D_1-D_3: 20 µM LPA treated group; E_1-E_3: 40 µM LPA treated group. The central area of cultured ovaries was observed dense and dark in some groups. The growth of follicles in the marginal parts caused to increase in the size of ovaries during the culture period.

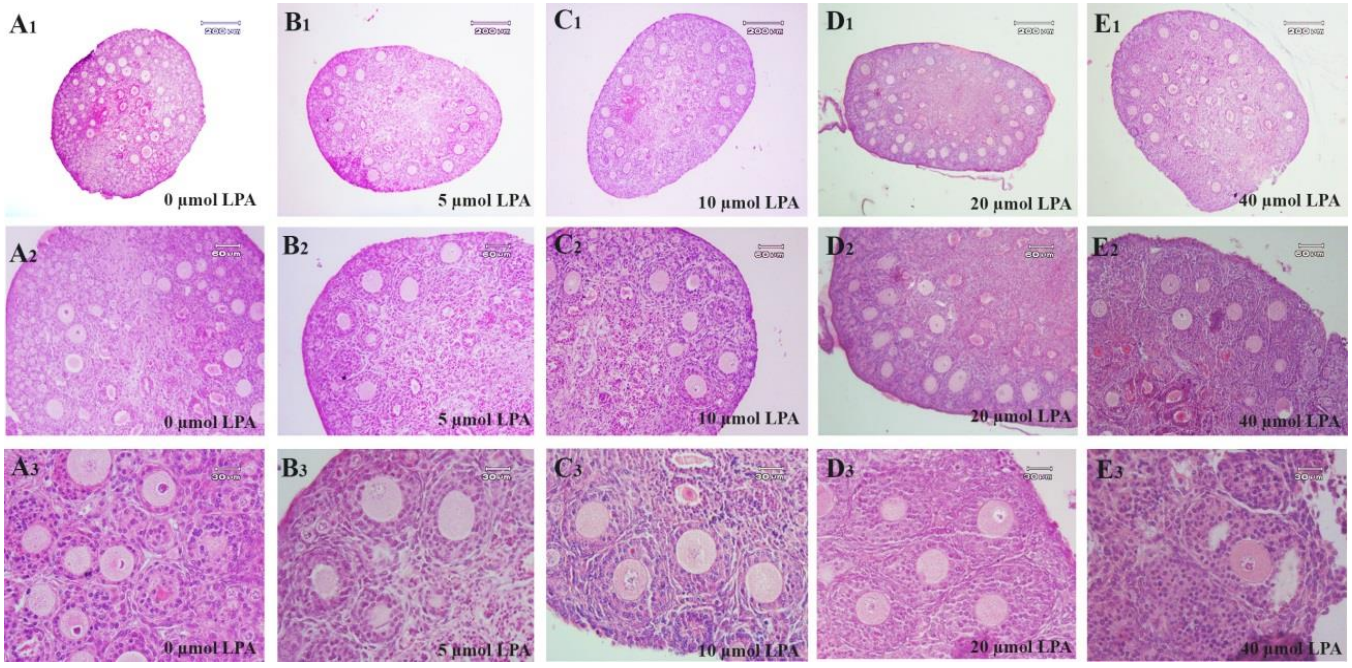

Fig. 2. Photomicrographs of mouse ovarian tissues imaged under the light microscope after seven-day culture using hematoxylin and eosin staining: A_1-A_3: cultured group (0 µM LPA) (control); B_1-B_3: 5 µM LPA treated group; C_1-C_3: 10 µM LPA treated group; D_1-D_3: 20 µM LPA treated group; E_1-E_3: 40 µM LPA treated group. Note: the follicles that were at advanced stages of development with normal and atretic morphology, were seen on day 7 of culture in all examined groups, atretic follicles especially in low concentration of LPA treated groups (less than 20 µM) were prominent than the other groups in central area of ovaries.

The number of normal follicles in the examined groups. The percentages of normal and atretic follicles at different developmental stages at the end of seven days of culture were summarized in Table 2. Before *in vitro* culturing, the proportion of primordial follicles were higher than other stages of follicles but after seven days culture, the percentage of primordial and primary follicles were decreased and the percentages of preantral follicles were significantly increased in all groups ($p < 0.05$). The percent of preantral follicles was significantly higher in the concentration of 20 µM LPA than other groups ($p < 0.05$; Table 2).

Ovarian surface area. The analysis of ovarian surface area data in all groups of study during *in vitro* culture was shown in Figure 3. The mean surface area was significantly increased during seven days culture (Fig. 3) and the surface area was markedly higher in the 20 µM LPA-treated group than those of other groups ($p < 0.05$).

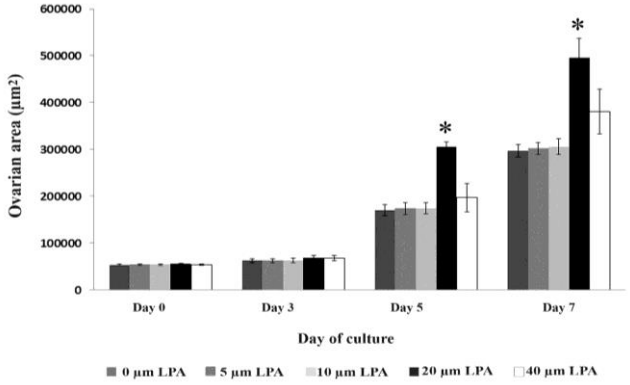

Fig. 3. The mean area of mouse cultured ovaries on days 0, 5 and 7 of culture period. * indicates significant difference with other groups after culturing (using 20 µM of LPA).

Real time RT-PCR results. The expression ratio of the LPA receptors genes to β-actin gene in non-cultured, 20 µM LPA-treated and without LPA cultured (control) groups were demonstrated and compared in Figure 4A to 4D. As the results shown, the expression ratio of LPAR1-4 genes in non-cultured group were 0.27 ± 0.04, 0.21 ± 0.04, 0.21 ± 0.03, 0.15 ± 0.01 in control cultured groups were 0.20 ± 0.03, 0.21 ± 0.01, 0.31 ± 0.01, 0.22 ± 0.01 and in 20 µM LPA-treated cultured group were 0.35 ± 0.02, 0.26 ± 0.01, 0.43 ± 0.01, and 0.35 ± 0.02, respectively ($p < 0.05$). There was no significant difference between control cultured group and non-cultured group. However, there was a significant difference between 20 µM LPA treated cultured group and other two groups ($p < 0.05$). The results demonstrated that the expression of LPAR1 gene was higher than other genes in all examined groups ($p < 0.05$).

Hormonal assay. The concentrations of E_2 hormone obtained from collected media in different groups on days three and seven of culturing period are presented in Table 3. There was a significant difference in the level of E2 hormone on day three of culture between two groups ($p < 0.05$). The level of E_2 hormone significantly increased in 20 µM LPA-treated group in comparison with control group ($p < 0.05$).

Table 3. Concentration of 17-β-estradiol (pg mL^{-1}) in collected media during culture period. Data are presented as mean ± SD.

Groups	Day 3 of culture	Day 7 of culture
Control	2341.47 ± 124.87[a]	8155.48 ± 439.22[a]
Cultured (20 µM LPA)	3095.29 ± 126.60[b]	12120.14 ± 300.86[b]

[ab] values with different letters in each column indicate significant differences $p < 0.05$.

Discussion

The findings of the present study confirmed that the supplementation of the mouse ovarian organ culture medium with 20 µM LPA could lead to achieve the higher percentages of follicular development (up to preantral stage), increase the mean surface area of ovaries and enhance the function of granulosa and theca cells by production of higher levels of 17-β-estradiol in comparison with the other groups. These results revealed that the LPA could stimulate the growth of primordial and primary follicles to preantral follicle stage and it seems these effects of LPA are dose dependent. The follicular growth rate was gradually improved during *in vitro* culture up to 20 µM concentration of LPA then this rate was decreased at concentrations of 40 µM of LPA. The previous studies postulated that LPA involved in the cell survival, activation of entire primordial follicle pool and promotion nuclear and cytoplasmic maturation of mouse oocytes by binding to its receptors and involving in the MAPK (mitogen-activated protein kinase)/p38 and PI3K (phosphoinositol 3-kinase)/Akt pathways.[16,45] Similarly, Jo *et al*. revealed

Table 2. Number of (percentage ± SD) of follicles at different developmental stages in all studied groups.

Groups	Normal follicles	Primordial follicles	Primary follicles	Preantral follicles	Degenerated follicles	Antral follicles
Before culture	3248	2987 (91.96 ± 2.28)	184 (5.67 ± 1.88)	77 (2.37 ± 1.04)	490 (13.10 ± 2.53)	0
Cultured 0 µM LPA	1351	903 (66.84 ± 3.28)[a]	152 (11.25 ± 0.70)[a]	296 (21.91 ± 1.60)[a]	486 (25.36 ± 2.54)[a]	0
Cultured 5 µM LPA	1375	907 (65.97 ± 0.99)[a]	152 (11.12 ± 1.59)[a]	315 (22.91 ± 1.85)[a]	461 (25.92 ± 3.00)[a]	0
Cultured 10 µM LPA	1329	849 (63.93 ± 1.53)[a]	172 (12.95 ± 1.32)[a]	308 (23.19 ± 1.48)[a]	444 (25.04 ± 2.09)[a]	0
Cultured 20 µM LPA	1317	674 (51.17 ± 1.18)[ab]	152 (11.55 ± 0.52)[ab]	491 (37.28 ± 1.23)[ab]	255 (16.22 ± 0.63)[ab]	0
Cultured 40 µM LPA	1446	802 (55.43 ± 1.73)[a]	200 (13.81 ± 1.03)[a]	313 (21.64 ± 0.85)[a]	407 (21.96 ± 2.89)[a]	131 (9.12 ± 1.23)

The percentage of follicles at different developmental stages ovaries before and after culturing in the presence of different doses of LPA.
[a] indicates significant differences with before cultured ovaries in different developmental stage ($p < 0.05$) and [b] indicates significant with other groups after culture ($p < 0.05$).

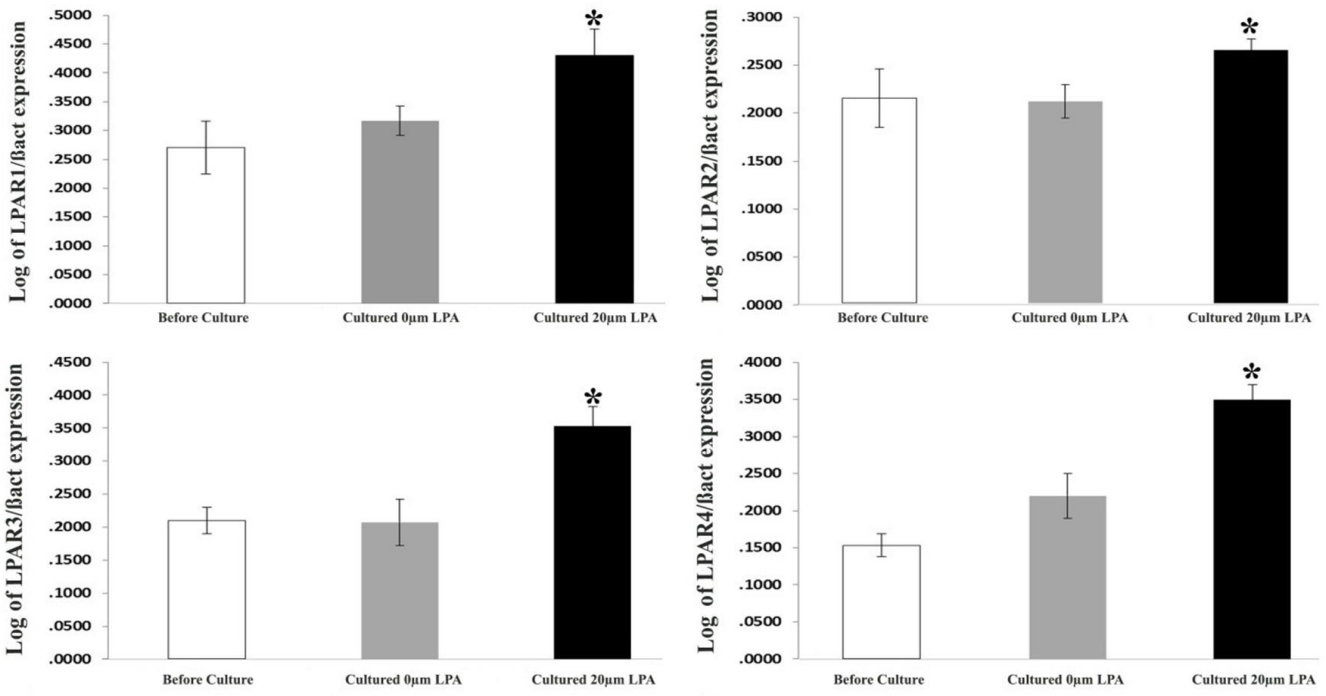

Fig. 4. A, B, C, and **D)** The mRNA expression of LPA receptor genes (LPAR 1, LPAR 2, LPAR 3, LPAR 4) before and after *in-vitro* culture (0 and 20 µM LPA-treated groups) of mouse ovarian tissues. * indicates a significant difference with non-cultured group.

that treatment of mouse oocyte culture media with 30 µM LPA, could significantly enhances the maturation rate of oocyte, the fertilization rate and subsequently the developmental rate of blastocyst.[19] According to Boruszewska *et al*., the supplementation of bovine oocyte maturation medium with LPA increases mRNA abundances of genes involved in bovine oocyte maturation (FST and GDF9).[46] They confirmed that LPA plays a potential autocrine and/or paracrine role between the bovine oocytes and granulosa cells, via several LPARs. However, they did not see any significant influence of LPA on maturation rate of oocytes. They concluded that mRNA level the usage of low concentration of LPA can affect the expression of gene related to maturation of oocytes.[46]

In this study, we examined the expression of several LPA receptors genes in cultured mouse ovaries. Our results showed that in the ovarian tissues of this strain of NMRI mouse, all four types of LPARs (1-4) were expressed at the mRNA level. But there are controversial reports related to the expression of LPA receptors in mouse ovarian tissue.[32,47] Huang *et al*. detected the expression of LPAR1-4 in C57BL/6 mouse ovarian tissue[47] and Ye *et al*. demonstrated that mouse ovary expressed LPAR1, LPAR2 and LPAR4 but there was no evidence about the presence of LPAR3.[32] While our study is the first to demonstrate that the four types of LPA receptors were expressed before and after *in vitro* culture (in the presence and absence of LPA) in mouse ovarian tissue. These different observations may be due to the mouse strain which have been used.

The LPA-LPARs binding activates various types of G-proteins and thereby induces signal intracellular transduction cascade and stimulates many vital pathways.[23] These signaling key pathways regulate many essential processes including cell proliferation, survival and growth, cytoskeletal rearrangement and primordial follicles activation through multiple downstream molecules.[24,27,48,49]

In addition, in this study it was demonstrated that LPA stimulated granulosa and theca cell to produce 17-β-estradiol (functional marker) in mouse ovarian tissues that may promotes ovarian follicular development. Moreover, the levels of 17-β-estradiol was significantly enhanced in 20 µM LPA supplemented group. Boruszewska *et al*. postulated that LPA stimulated 17-β-estradiol synthesis in bovine granulosa cells that correlated with increasing in the follicle stimulating hormone receptor and 17-β-hydroxy steroid dehydrogenase mRNA expression. In addition, they documented this stimulatory effect of LPA enhanced in the presence of follicle stimulating hormone and thereby, induced the expression of cytochrome P450 aromatase genes that convert the androgens to 17-β-estradiol in thecal cells.[50]

In conclusion, this study demonstrated that the addition of LPA to the IVC media, especially at 20 µM, could improve the survival and developmental potential of follicles in mouse whole ovarian tissues after 7 days of culture. Furthermore, the present study updates our knowledge on gene expression of LPA receptors in neonatal and postnatal mouse whole ovaries that may

have positive effects on cell function and stimulation of 17-β-estradiol synthesis.

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Conflict of Interest

The authors declare that there are no conflicts of interest related to this article.

References

1. Adhikari D. *In vitro* activation of dormant follicles for fertility preservation. Adv Exp Med Biol 2013; 761: 29-42.
2. Jin SY, Lei L, Shikanov A, et al. A novel two-step strategy for *in vitro* culture of early-stage ovarian follicles in the mouse. Fertil Steril. 2010; 93(8): 2633-2639.
3. Hirao Y. Isolation of ovarian components essential for growth and development of mammalian oocytes *in vitro*. J Reprod Dev 2012; 58(2): 167-174.
4. Filatov MA, Khramova YV, Kiseleva MV, et al. Female fertility preservation strategies: cryopreservation and ovarian tissue *in vitro* culture, current state of the art and future perspectives. Zygote 2016; 24(5): 635-653.
5. Silva JR, van den Hurk R, Figueiredo JR. Ovarian follicle development *in vitro* and oocyte competence: Advances and challenges for farm animals. Domest Anim Endocrinol 2016; 55: 123-135.
6. Eppig JJ, O'Brien MJ. Development *in vitro* of mouse oocytes from primordial follicles. Biol Reprod 1996; 54(1): 197-207.
7. Devine PJ, Rajapaksa KS, Hoyer PB. *In vitro* ovarian tissue and organ culture: A review. Front Biosci 2002; 7: 1979-1989.
8. Knight PG, Glister C. TGF-beta superfamily members and ovarian follicle development. Reproduction 2006; 132(2): 191-206.
9. Fortune JE, Yang MY, Muruvi W. *In vitro* and *in vivo* regulation of follicular formation and activation in cattle. Reprod Fertil Dev 2011; 23(1): 15-22.
10. Gupta PS, Nandi S. Isolation and culture of preantral follicles for retrieving oocytes for the embryo production: Present status in domestic animals. Reprod Domest Anim 2012; 47(3): 513-519.
11. Yutong Z, Viswanathan N. Lysophosphatidic acid (LPA) and its receptors: Role in airway inflammation and remodeling. Biochim Biophys Acta 2013; 831(1): 86-92.
12. Boruszewska D, Sinderewicz E, Kowalczyk-Zieba I, et al. The effect of lysophosphatidic acid during *in vitro* maturation of bovine cumulus–oocyte complexes: Cumulus expansion, glucose metabolism and expression of genes involved in the ovulatory cascade, oocyte and blastocyst competence. Reprod Biol Endocrinol 2015; 13: 44.
13. McIntyre TM, Pontsler AV, Silva AR, et al. Identification of an intracellular receptor for lysophosphatidic acid (LPA): LPA is a transcellular PPAR gamma agonist. Proc Natl Acad Sci USA 2003; 100(1): 131-136.
14. Waters CM, Saatian B, Moughal NA, et al. Integrin signaling regulates the nuclear localization and function of the lysophosphatidic acid receptor-1 (LPA1) in mammalian cells. Biochem J 2006; 398: 55-62.
15. Murch O, Collin M, Thiemermann C. Lysophosphatidic acid reduces the organ injury caused by endotoxemia-a role for G-protein-coupled receptors and peroxisome proliferator activated receptor-gamma. Shock 2007; 27(1): 48-54.
16. Boruszewska D, Kowalczyk-Zieba I, Sinderewicz E, et al. The effect of lysophosphatidic acid together with interferon tau on the global transcriptomic profile in bovine endometrial cells. Theriogenology 2017; 92: 111-120.
17. Hinokio K, Yamano S, Nakagawa K, et al. Lysophosphatidic acid stimulates nuclear and cytoplasmic maturation of golden hamster immature oocytes *in vitro* via cumulus cells. Life Sci 2002; 70(7): 759-767.
18. Wocławek-Potocka I, Rawińska P, Kowalczyk-Zieba I, et al. Lysophosphatidic acid (LPA) signaling in human and ruminant reproductive tract. Mediators Inflamm 2014; 2014: http://dx.doi.org/10.1155/2014/649702.
19. Jo JW, Jee BC, Suh CS, et al. Addition of lysophosphatidic acid to mouse oocyte maturation media can enhance fertilization and developmental competence. Hum Reprod 2014; 29(2): 234-241.
20. Sheng X, Yung YC, Chen A, Chun J. Lysophosphatidic acid signaling in development. Development 2015; 142(8): 1390-1395.
21. Contos JJ, Fukushima N, Weiner JA, et al. Requirement for the lpA1 lysophosphatidic acid receptor gene in normal suckling behavior. Proc Natl Acad Sci USA 2000; 97(24): 13384-13389.
22. Onno K, Wouter HM. Ras-MAP kinase signaling by lysophosphatidic acid and other G protein coupled receptor agonists. Oncogene 2001; 20: 1540-1546.
23. Reddy VS, Singh AK, Rajasekharan R. The Saccharomyces cerevisiae PHM8 gene encodes a soluble magnesium-dependent lysophosphatidic acid phosphatase. J Biol Chem 2008; 283(14): 8846-8854.
24. Koh JS, Lieberthal W, Heydrick S, et al. Lysophosphatidic acid is a major serum noncytokine survival factor for murine macrophages which acts via the phosphatidylinositol 3-kinase signaling pathway. J Clin Invest 1998; 102(4): 716-727.

25. Anliker B, Choi JW, Lin ME, et al. Lysophosphatidic acid (LPA) and its receptor, LPA1, influence embryonic Schwann cell migration, myelination, and cell-to-axon segregation. Glia 2013; 61(12): 2009-2022.
26. Mori K, Kitayama J, Aoki J, et al. Submucosal connective tissue-type mast cells contribute to the production of lysophosphatidic acid (LPA) in the gastrointestinal tract through the secretion of autotaxin (ATX)/lysophospholipase D (lysoPLD). Virchows Arch 2007; 451(1): 47-56.
27. Ye X. Lysophospholipid signaling in the function and pathology of the reproductive system. Hum Reprod Update 2008; 14(5): 519-536.
28. Woclawek-Potocka I, Kondraciuk K, Skarzynski DJ. Lysophosphatidic acid stimulates prostaglandin E2 production in cultured stromal endometrial cells through LPA1 receptor. Exp Biol Med (Maywood) 2009; 234(8): 986-993.
29. Kowalczyk-Zieba I, Boruszewska D, Saulnier-Blache JS, et al. Lysophosphatidic acid action in the bovine corpus luteum: An *in vitro* study. J Reprod Dev 2012; 58(6): 661-671.
30. Lee Z, Cheng CT, Zhang H, et al. Role of LPA4/p2y9/GPR23 in negative regulation of cell motility. Mol Biol Cell 2008; 19(12): 5435-5445.
31. Ye X, Chun J. Lysophosphatidic acid (LPA) signaling in vertebrate reproduction. Trends Endocrinol Metab 2010; 21(1): 17-24.
32. Ye X, Hama K, Contos JJ, et al. LPA3-mediated lysophosphatidic acid signaling in embryo implantation and spacing. Nature 2005; 435(7038): 104-108.
33. Zhao Y, Natarajan V. Lysophosphatidic acid (LPA) and its receptors: Role in airway inflammation and remodeling. Biochim Biophys Acta 2013; 1831(1): 86-92.
34. Noguchi K, Ishii S, Shimizu T. Identification of p2y9/GPR23 as a novel G protein-coupled receptor for lysophosphatidic acid, structurally distant from the Edg family. J Biol Chem 2003; 278(28): 25600-25606.
35. Kitayama J, Shida D, Sako A, et al. Over-expression of lysophosphatidic acid receptor-2 in human invasive ductal carcinoma. Breast Cancer Res 2004; 6(6): R640-646.
36. Liu Z, Armant DR. Lysophosphatidic acid regulates murine blastocyst development by transactivation of receptors for heparin-binding EGF-like growth factor. Exp Cell Res 2004; 296(2): 317-326.
37. Hinckley M, Vaccari S, Horner K, et al. The G-protein-coupled receptors GPR3 and GPR12 are involved in cAMP signaling and maintenance of meiotic arrest in rodent oocytes. Dev Biol 2005; 287(2): 249-261.
38. Nakamoto T, Yasuda K, Yasuhara M, et al. Expression of the endothelial cell differentiation gene 7 (EDG-7), a lysophosphatidic acid receptor, in ovarian tumor. J Obstet Gynaecol Res 2005; 31(4): 344-351.
39. Kotarsky K, Boketoft A, Bristulf J, et al. Lysophosphatidic acid binds to and activates GPR92, a G protein-coupled receptor highly expressed in gastrointestinal lymphocytes. J Pharmacol Exp Ther 2006; 318(2): 619-628.
40. Kamińska K, Wasielak M, Bogacka I, et al. Quantitative expression of lysophosphatidic acid receptor 3 gene in porcine endometrium during the periimplantation period and estrous cycle. Prostaglandins Other Lipid Mediat 2008; 85(1-2): 26-32.
41. Lee J, Lee JR, Youm HW, et al. Effect of preoperative simvastatin treatment on transplantation of cryopreserved-warmed mouse ovarian tissue quality. Theriogenology 2015; 83: 285- 293.
42. OligoAnalyzer 3.1. Available at: https://eu.idtdna.com/calc/analyzer. Accessed. May 13, 2017.
43. Amoushahi M, Salehnia M, Mowla SJ, Ghorbanmehr N. Morphological and molecular aspects of *in vitro* culture of preantral follicles derived from vitrified ovarian. Cell J 2017; 19(3): 332-342.
44. Abdi S, Salehnia M, Hosseinkhani S. Kit ligand decreases the incidence of apoptosis in cultured vitrified whole mouse ovaries. Reproduction Biomed Online 2015; 30(5): 493-503.
45. Makker A, Goel MM, Mahdi AA. PI3K/PTEN/Akt and TSC/mTOR signaling pathways, ovarian dysfunction, and infertility: An update. J Mol Endocrinol 2014; 53(3): 103-118.
46. Boruszewska D, Torres AC, Kowalczyk-Zieba I, et al. The effect of lysophosphatidic acid during *in vitro* maturation of bovine oocytes: embryonic development and mRNA abundances of genes involved in apoptosis and oocyte competence. Mediators Inflamm 2014; doi: 10.1155/2014/670670.
47. Huang MC, Lee HY, Yeh CC, Kong Y, et al. Induction of protein growth factor systems in the ovaries of transgenic mice overexpressing human type 2 lysophosphatidic acid G protein-coupled receptor (LPA2). Oncogene 2004; 23(1): 122-129.
48. Budnik LT, Brunswig-Spickenheier B, Mukhopadhyay AK. Lysophosphatidic acid signals through mitogen-activated protein kinase-extracellular signal regulated kinase in ovarian theca cells expressing the LPA1/edg2-receptor: Involvement of a nonclassical pathway? Mol Endocrinol 2003; 17 (8): 1593-1606.
49. Zhao Y, Zhang Y, Li J, et al. MAPK3/1 participates in the activation of primordial follicles through mTORC1-KITL signaling. J Cell Physiol 2018; 233(1):226-237.
50. Boruszewska D, Sinderewicz E, Kowalczyk-Zieba I, et al. Influence of lysophosphatidic acid on estradiol production and follicle stimulating hormone action in bovine granulosa cells. Reprod Biol 2013; 13(4): 344-347.