Original Article

Effect of Lysophosphatidic Acid on the Vascular Endothelial Growth Factor Expression in Autotransplanted Mouse Ovaries Encapsulated in Sodium Alginate

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Abstract

Objective: The aim of this study was to evaluate the effect of lysophosphatidic acid (LPA) supplementation during in vitro culture and transplantation of mouse ovaries on the follicular development and expression of vascular endothelial growth factor (VEGF) as an angiogenesis factor at the mRNA and protein levels.

Materials and methods: Three weeks old mice ovaries were cultured in the presence and absence of LPA for 24 hours, then they were capsulated in sodium alginate in the presence and absence of LPA as four experimental groups. After transplantation the vaginal smears were performed daily to evaluate the initiation of the estrous cycle. The morphology and follicular distribution were analyzed at the first and fourth estrous cycles using hematoxylin and eosin staining. Then in the groups that showed higher and lower follicular development the immunohistochemistry assay was conducted to identify VEGF protein expression, and the real time RT-PCR was done to analyze the expression of *Vegf* gene at the first estrus cycle.

Results: The large size follicles and also the corpus luteum were prominent in all transplanted groups at fourth estrus cycle in comparison with intact control groups. The statistically lowest percentage of small size follicles and the highest percentages of large size follicles were seen in LPA^+/LPA^- group ($p<0.05$). The expression ratio of *Vegf* to *β-actin* was significantly higher in this group in comparison with non-LPA treated and intact control groups ($p <0.05$).

Conclusion: LPA as an angiogenesis factor increases the follicular development in transplanted ovaries but it causes early discharge of ovarian reserve.

Keywords: Angiogenesis Inducing Agents; Autologous Transplantation; Lysophosphatidic Acid; Ovary; Vascular Endothelial Growth Factors

Introduction

Ovarian transplantation can be performed by fresh or cryopreserved tissue to preserve fertility potential in women with early ovarian failure (1-3). The hypoxia and free radical production are the main causes of follicular degradation after ovarian transplantation (4-6). A number of important factors such as prostaglandins and vascular endothelial growth factor (VEGF) that secreted by ovarian cells are suggested

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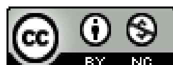

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for regulation of follicular survival, growth and development (6-10). VEGF plays an important role in the post-transplantation angiogenesis of ovarian tissue (10-14). Demeestere et al. indicated that VEGF, as a growth factor, to be able to induce angiogenesis in grafted tissue (15). Zand-Vakili et al. revealed that VEGF influences follicular growth and also corpus luteum development in relation to the formation of new vessels (13). Moreover, usage of VEGF inhibitor caused depletion in follicular development from early stages to antral stage (15).

Administration of antioxidant and factors during ovarian tissue transplantation could prevent ischemia by inducing angiogenesis in order to improve the quality of the grafted ovaries (7-10, 12). Lysophosphatidic acid (LPA) is a small phospholipid (430–480 Dalton) that was detected in several biological fluids such as serum and follicular fluid (16-19).

It is produced from the cell membrane phospholipids by two enzymes including phospholipase A and autotaxin in several cell types (17). LPA in several cell types acts as proliferation and survival factors and prevents cell apoptosis (16, 19-22). LPA has physiological functions in reproductive system including ovary, follicular and early embryo development, fertilization, implantation, pregnancy maintenance and parturition (16, 19, 22-24). It acts via its receptors (LPAR1–6) through several subunits of G-protein (25-27).

In addition, it was shown that LPA contributes to the processes of endothelial cell proliferation, migration and differentiation that all essential for angiogenesis by production of angiogenic factors (17, 28-32). Wasniewski et al. reported that LPA induced angiogenesis by VEGF expression through the activation of hypoxia inducible factor-1α (HIF-1α) (33). Rivera-Lopez et al. revealed that LPA causes angiogenesis in vivo (34). Hisano et al. demonstrated that LPA is a mediator in regulation of angiogenesis and cell migration in tissue growth and development (35). Previous studies showed LPA through the regulation of IL- 8 and IL-6 stimulated the processes of new vessel formation in the corpus luteum, endometrium and placenta (36).

Recently increase attention was done to improve the follicular survival and development in grafted ovaries by an increase in angiogenesis (7-10, 12). Based on our knowledge there is no report regarding to evaluate the effects of LPA on the angiogenesis of transplanted ovaries. Thus in this study after supplementation of LPA during in vitro culture and transplantation of mouse ovaries the follicular development was evaluated in a short time (first estrus cycle) and a long time (fourth estrus cycle). Then the expression of VEGF as an angiogenesis related factor was assessed at the mRNA and protein levels on the first estrus cycle in the groups with high and low follicular development.

Materials and methods

All materials were obtained from Sigma Aldrich (Dusseldorf, Germany) except otherwise indicated.

Animals: In this experimental study, three weeks old NMRI mice (n=52) were kept under a controlled condition (20-24°C, 12/12h light: dark cycles and 40-50% humidity) in the animal house of Tarbiat Modares University. In addition, 6 (n=11) and 9 (n=5) weeks old adult female mice were used as intact control groups. Approval for this study was obtained from the Ethics Committee for Animal Research of the Tarbiat Modares University (Ref No: 1395.530).

In vitro culture and encapsulation of ovaries: Animals in experimental groups (n=52) were anesthetized with intraperitoneal (IP) injection of ketamine (50 mg/kg) and xylazine (5 mg/kg). The right ovary of each mouse was removed and cultured for 24 hours in the α-MEM medium that supplemented with 5% fetal bovine serum, 1% insulin, transferrin, and selenium (Invitrogen, Paifley, UK) and 100 mIU/ml recombinant follicle stimulating hormone (Serono, Switzerland) in the presence and absence of 20 µM LPA. (27) Then, the cultured ovaries were encapsulated into sodium alginate at a concentration of 0.5% (w/v) in phosphate buffered saline (PBS) without or with 20 µM LPA (37).

Autotransplantation of capsulated ovaries: For autotransplantation of ovaries the mice (n=52) were anesthetized again as described earlier, then the cultured-encapsulated ovaries as 4 experimental groups (LPA^-/LPA^-; LPA^-/LPA^+; LPA^+/LPA^- and LPA^+/LPA^+) were inserted under the right kidney capsule. The left ovary of each mice was ignored intact then the mice were kept under aseptic condition.

Collection of transplanted ovaries: Three and six weeks after transplantation, for confirmation of starting the estrus cycle and also detection of the stages of mice estrous cycle the vaginal cytology was done every day. The stage of the estrous cycle was identified under a light microscope at×400 magnification by the presence or absence of the nucleated and cornified epithelial cells, and leukocytes (38).

The animals were sacrificed by cervical dislocation

at proestrus phase of first and fourth estrous cycles and their transplanted ovaries were collected.

Histological evaluation: For morphological analysis the recovered grafted ovaries at proestrus phase of first and fourth estrus cycles (n=5 ovaries in each group in each cycle) were fixed in Bouin's solution for 8 hours, embedded in paraffin wax, serially sectioned at 5µm and mounted on slides with 5th intervals and stained with hematoxylin and eosin method. The same procedure was done for ovaries obtained from mice at proestrus phase of first and fourth estrus cycles as intact control groups (n=5 in each control group) to compare the morphology of ovaries.

The tissue sections were studied under a light microscope for determining the normal and degenerated follicles at different developmental stages. The ovarian follicles were classified as small follicles (primordial and primary) and large follicles (preantral and antral follicles) and corpus lutetium (5).

Immunohistochemistry: Another set of tissue sections from intact mice ovaries (as control group) and recovered grafted ovaries at the first estrous cycle that showed lower (LPA^-/LPA^-) and higher (LPA^+/LPA^-) follicular developmental rates were put on coated slides and used for immunohistochemistry. The tissue sections were deparaffinized, rehydrated and washed in PBS. The cells were permeable by putting the sections in triton X100 (0.3 % for 30 min), washed in PBS and blocked with goat serum (30 min) then, they were incubated with the primary anti-VEGF polyclonal antibody (1:100, Elabscience Biotechnology Co, Wuhan, China) overnight. Then they were incubated with polyclonal goat anti rabbit antibody conjugated with FITC (1: 20Elabscience Biotechnology Co, Wuhan, China) for 30 min and washed in PBS. Then the tissue slides were visualized under a florescent microscope at ×400 magnification. These experiments were done in three repeats.

RNA extraction and cDNA synthesis: According to the obtained morphological data the experimental group that showed lower (LPA^-/LPA^-) and higher (LPA^+/LPA^-) follicular development and intact control group at first estrous cycle were considered for analysis of *Vegf* gene expression (n=3 ovary in each group in 3 repeats). Total RNA was extracted using trizol reagent according to the procedure described by the manufacturer's instructions RNeasy Mini Kit (Qiagen, Hilden, Germany). Then the RNA concentration was determined using spectrophotometry.

The cDNA was synthesized using cDNA synthesis kit (Thermo Fisher Scientific, Bremen, Germany) according to the manufacturer's instructions. Oligo dT was used for cDNA synthesis and reverse transcriptase reaction was incubated at 65˚C for 5 min and 42˚C for 60 min. The reverse transcriptase enzyme was inactivated at 70˚C for 5 min, then the product was stored at – 20˚C until real time RT-PCR.

Real-time RT-PCR: The primers for *Vegf* and *β-actin* as housekeeping genes were designed using Primer-BLAST tool in NCBI and presented in Table 1. Real-time RT-PCR was performed using QuantiTect SYBR Green RT-PCR kit (Qiagen, Hilden, Germany).

Thermal program of the real-time RT-PCR was set with the following parameters: the initial denaturation stage for 5 min at 95ºC, cycling stage (40 cycles) of denaturation for 15 sec at 95ºC, annealing stage 58ºC for 30 sec, and melting stage 72ºC for 15 sec, that was followed by a melt curve stage at 95ºC for 15 sec, 60ºC for 1 min, and 95ºC for 15 sec. After completing the PCR run, the melt curve was obtained using the amplicon. For determining of relative quantization of target genes the Pfaffl method was used.

Statistical analysis: Statistical analysis was performed using SPSS software. Values are given as mean ± SD. One-way ANOVA and post hoc Tukey were used to compare differences in follicular count and mRNA expression. A p value less than 0.05 was considered statistically significant.

Results

The morphology and distribution of follicles of transplanted ovaries at first and fourth estrus cycles: The light microscopy observations of cultured-transplanted ovaries sections stained by H&E are presented in Figures 1 and 2.

Table 1: The sequences of the designed primers

Gene	Primer Sequence	Accession numbers	PCR product size (Bp)
Vegf	F: CTGCACCCACGACAGAAGG R:AGCTTCGCTGGTAGACATCC	NM-001025257.3	72
β-actin	F: AGTCATAGTCCGCCTAGAAGC R: TGAAGATCAAGATCATTGCTCCC	NM-021163894.1	168

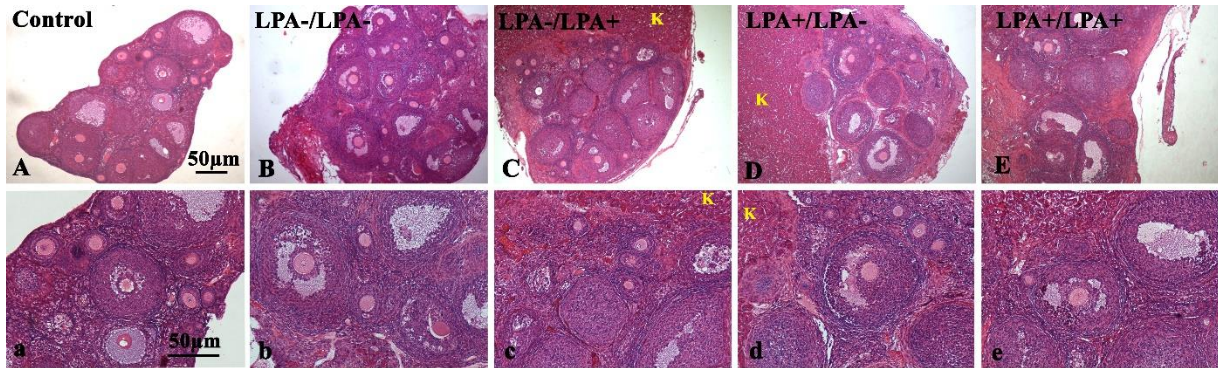

Figure 1: Light microscopic observation of transplanted ovaries at first estrus cycle using hematoxylin and eosin. The morphology of ovarian tissue sections with low magnification are demonstrated in the first row and with higher magnification of the same groups are shown in second row. A,a: LPA-/LPA-; B,b: LPA-/LPA+; C,c: LPA+/LPA-and D,d: LPA+/LPA+; K: kidney tissue. The scale bar is 50µm and is indicated for the first row in part A and for the second row in part a.

As these figures showed with low and higher magnifications in all examined groups at first estrus cycle (Figure 1) the follicles in several growing stages were seen. However, the large size follicles (preantral and antral follicles) and also the corpus luteum was prominent in all transplanted groups at fourth estrus cycle (Figure 2) in comparison with intact control groups.

The higher percentages of normal follicles at first and fourth estrus cycle were significantly seen $LPA^+/LPA-$ group ($p<0.05$; Figure 3).

The follicular distribution in studied groups at first and fourth estrus cycles was presented and compared in Figure 4. The higher percentage and lower percentage of small size follicles at both fourth estrus cycles were seen in intact control and $LPA^+/LPA-$ group respectively ($p<0.05$). In parallel regarding to the large size follicles at both fourth estrus cycles, the higher percentage and lower percentage of these follicles were seen in $LPA^+/LPA-$ and intact control group respectively ($p<0.05$).

Immunohistochemistry for VEGF: The representative photomicrographs of immunostaining tissue sections of transplanted ovaries related to LPA^-/LPA^- and LPA^-/LPA^+ groups and 6 weeks old mice ovaries as intact control were presented in Figure 5 A-C. As these figures showed the prominent positive reactions were shown in the blood vessels with small and large size (white arrow) within the ovarian stroma. As in Figure 5-part C showed these reactions were abundant in LPA^+/LPA^- group.

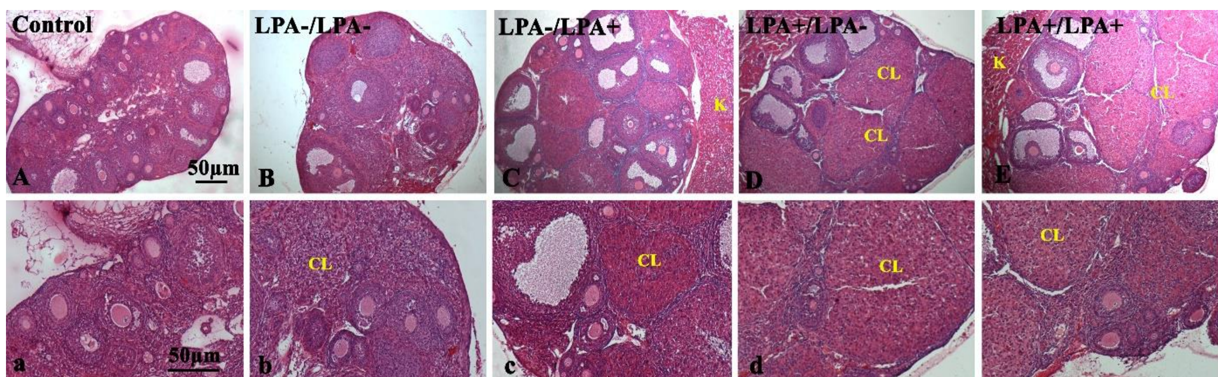

Figure 2: Representative micrograph of transplanted ovaries at fourth estrus cycle using hematoxylin and eosin. The morphology of ovarian tissue sections are presented with low magnification in first row and with the higher magnification of the same groups in the second row. As the figure shows several corpus luteum were prominent in the transplanted tissues (CL). A,a: LPA-/LPA-; B,b: LPA-/LPA+; C,c: LPA+/LPA-and D,d: LPA+/LPA+; K: kidney tissue. The scale bar is 50µm and is indicated for the first row in part A and for the second row in part a.

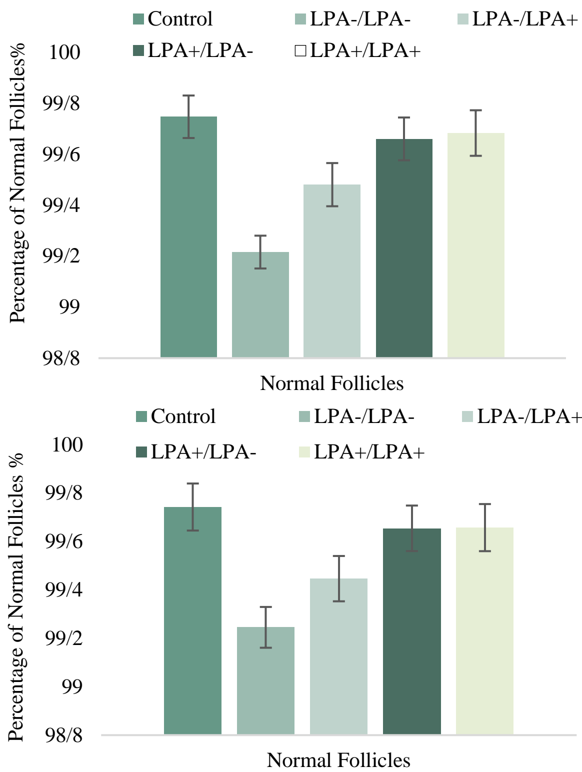

Figure 3: The percentages of normal follicles in transplanted ovaries at first and fourth estrous cycles are presented and compared between studied groups. *Significant difference with other groups (p<0.05).

Real time RT-PCR: The relative expression ratio of *Vegf* and *β-actin* in intact control, LPA^-/LPA^- group and LPA^+/LPA^- group were 0.044 ± 0.002, 0.092 ± 0.007 and 0.04 ± 0.003 respectively (Figure 5 D). The expression ratio of *VEGF* was significantly higher in LPA^+/LPA^- group in comparison with non-treated and intact control groups ($p < 0.05$). The gel electrophoresis of PCR product related to these genes is presented in Figure 5 E.

Discussion

A major concern in ovarian transplants is follicular loss during the initial period of transplantation due to hypoxia. In the present study, LPA has been employed to improve angiogenesis in transplanted ovary under kidney capsule.

Our results demonstrated that the rate of follicular growth and development in all transplanted ovaries was significantly faster than the intact control once. These differences were prominent especially in fourth estrus cycle. This phenomenon demonstrated the rapid discharge of ovarian reserve in all transplanted groups, and it could affect the longevity of transplanted tissue. Similar observations have also been observed in other mammalian species (38-40).

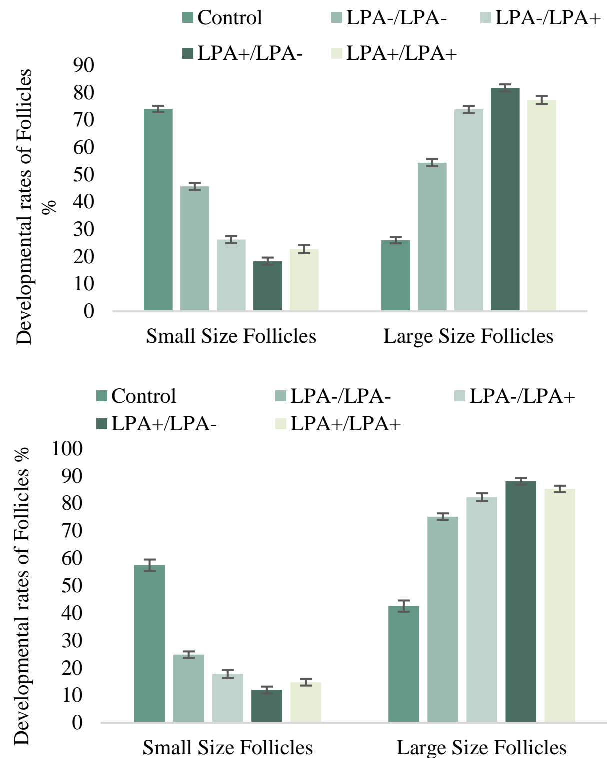

Figure 4: The percentages of follicles with different size in transplanted ovaries at first and fourth estrous cycles are presented and compared between studied groups. *Significant difference with other groups (p<0.05).

Moreover, the high percentage of large size follicles and corpus luteum in LPA^+/ LPA^- group indicated that LPA stimulates the growth of primordial and primary follicles to subsequent stages by expansion of follicular cells. Similarly, it was shown that LPA induced the proliferation and expansion of several cell types by binding to its receptors and involving in the phosphoinositide 3-kinase (25-27). Abedpour et al. showed that four receptors related to LPA are expressed in the mouse ovary and it seems that the same signaling pathway involves during the addition of LPA to the culture medium (27). Sinderewicz et al., postulated that LPA enhanced the growth and development of follicles via expression of LPARs and AX genes (20, 25).

Boruszewska et al. reported that the addition of LPA to the culture medium induced growth factors and stimulates cell proliferation and improves oocyte maturation (22, 41).

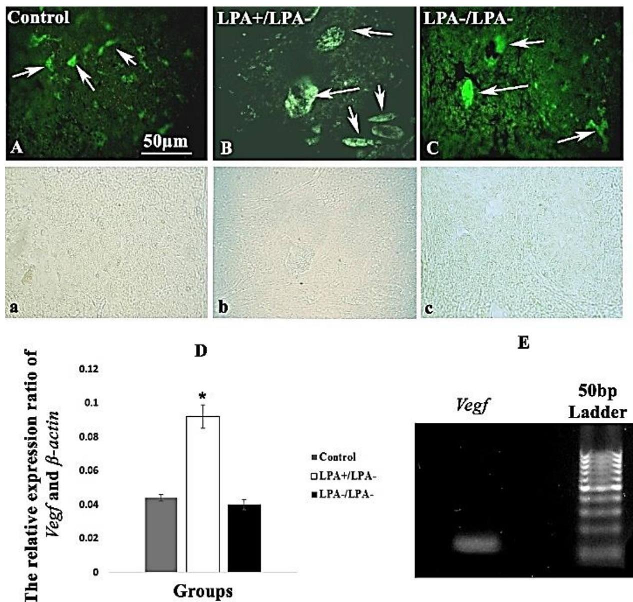

Figure 5: The fluorescent microscopy observations of transplanted mouse ovarian tissue sections immunostained for VEGF antibody (first row) and phase contrast of the same groups in second row. A and a: Intact control group; B and b: LPA^{+}/LPA^{-}; C and c: LPA^{-}/LPA^{-}. Green color shows the positive cell reaction (white arrow) for VEGF antibody (Bar=50µm). The comparison of the relative expression ratio of *Vegf* gene to *β- actin* in transplanted mouse ovaries and intact control group are shown in parts D.
*Significant difference with other groups ($p<0.05$). Agarose gel electrophoresis of the PCR products is shown in part E (ladder 50 bp).

Despite the high level of follicular growth in $LPA^{+}/$ LPA^{-} group in comparison with other groups but the ovarian reserve was declined in this group at the 4th estrus cycle. This process could have harmful effect on the function of transplanted ovaries in long term. Moreover, our results indicated that supplementation of ovarian culture media with LPA is more effective than the addition of LPA to sodium alginate for encapsulation of ovaries to increase follicular growth and development.

In other parts of the present study, our results confirmed that LPA significantly increased the expression of *Vegf* gene, whereas, we have not quantified the level of VEGF at the protein level but the positive reaction for these protein was prominent in LPA treated group.

These resultss suggested that LPA could act as an angiogenesis factor directly or indirectly in cultured-transplanted ovaries. This conclusion agreed with the findings of Park et al., who reported before, LPA induced angiogenesis by Vegf expression through the activation of hypoxia inducible factor-1α (42). Hisano et al. demonstrated that LPA is a mediator in regulation of angiogenesis and cell migration (35). Also, it was shown that LPA signaling has effects on endothelial cell proliferation, migration, adhesion and morphology during vascular formation (28, 29, 32). In agreement with these results in mice, showed that if the level of LPA decreased the early blood vessel formation from angioblasts, failed to develop into mature and stable vessels (30, 31).

Conclusion

LPA as an angiogenesis factor increases the follicular development in transplanted ovaries through enhancement in the expression of *Vegf* gene but it causes early discharge of ovarian reserve.

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