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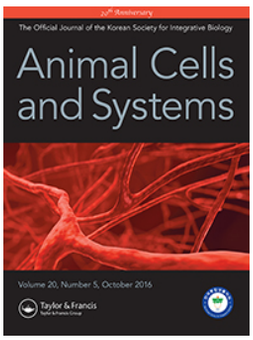

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Histological evaluation of the effect of VEGF on auto-transplanted mouse ovaries

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ABSTRACT

One of the most important factors affecting survival rate of ovarian follicles during transplantation period is proper vascular development. The objective of the study was to evaluate the effect of vascular endothelial growth factor (VEGF) on auto-transplanted ovarian tissue. Twenty-one-day-old female mice ($n = 30$) transplanted as control group and 21-day-old female mice ($n = 40$) were divided into 4 groups that were treated with 0.5, 1, 2 and 4 µg/mL of VEGF directly injected to auto-transplanted ovarian tissue. Twenty-one days after transplantation, mice were treated with 7.5 IU pregnant mare serum gonadotropin and human chorionic gonadotropin. Transplanted ovaries were removed and sections were prepared from transplanted tissues for staining. The most effective dosage of VEGF on transplanted tissue was determined over H&E (hematoxylin and eosin) staining results. Slides were compared using TUNEL staining and CD31 assay for the most effective dosage. The percentages of preantral and antral follicles were not significantly different between transplanted group with 4 µg/mL VEGF and non-transplanted group. Lower apoptotic areas and higher CD31 expression were observed in transplanted ovaries treated with 4 µg/mL VEGF when compared to transplanted ovaries without VEGF treatment. VEGF positively affects the quality of ovarian tissue during transplantation. Survival rate of follicles and follicular development has improved with the effect of VEGF.

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Introduction

Chemotherapy, radiotherapy and bone marrow transplantation cure more than 90% of young women undergoing cancer treatment. Ovaries are very sensitive to chemotherapy drugs and radiotherapy (Wallace et al. 1989; Adriaens et al. 2006; Anderson & Wallace 2013). Premature ovarian failure is a well-known consequence of chemotherapy drugs on females (Rivkees & Crawford 1988). The options for fertility preservation in patients receiving chemotherapy drugs are collection and storage of oocyte, embryo, primordial follicles and ovarian tissue (Torrents et al. 2003). It is fortunate that ovarian tissue is tolerant to cryopreservation, and follicular population is mainly in primordial stage in which oocytes are smaller, less differentiated and more resistant to cryoinjury (Meirow 2000). Therefore, cryopreservation and auto-transplantation of ovaries have been developed (Oktay & Karlikaya 2000) for the recovery of fertility and natural hormone activity (Donnez et al. 2004). Fertility restoring after auto-transplantation of cryopreserved ovarian tissue has been achieved previously (Demeestere et al. 2007). Therefore, this technique can be promising for fertility preservation in cancer patients. However, one of the most important concerns is the ischemic injury occurring during the time necessary for the revascularization of transplanted ovarian tissue. Ischemic injury can adversely affect follicular survival and life span of ovarian tissue after transplantation (Demeestere et al. 2009). Ischemic injuries during auto-graft process induce depletion of 60–95% follicular reserve (Liu et al. 2008). This follicular depletion observed after ovarian tissue transplantation is a main concern, especially in humans and large animal species that have a dense ovarian cortex, as it may affect the follicular growth dynamic, the hormonal environment and the fertility restoration potential (Demeestere et al. 2009). Grafting whole ovaries cryopreserved with vascular anastomosis may prevent follicle loss through ischemia (Nichols-Burns et al. 2014). Ischemia-reperfusion injuries mostly occur within 24–48 h after the grafting. During this stage, the graft must receive adequate blood supply (Israely et al. 2003) and vascularization of grafted tissue resulted from anastomosis between ovarian vessels and host vessels (Van Eyck et al. 2010).

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It has been shown that early graft vascularization resulted in improved graft perfusion and follicular survival (Israely et al. 2006). Previous researches indicated that growth factors such as vascular endothelial growth factor (VEGF) promote biomaterial vascularization (Patel et al. 2008). It is the main regulator of blood vessels growth during vasculogenesis and angiogenic sprouting (Otani et al. 1999). It has been shown that VEGF has beneficial effects on xenografted ewe ovarian tissue on SCID mice (Mineur et al. 2007). Furthermore, Labeid et al. showed that VEGF can accelerate angiogenesis and improve the viability of ovarian cortex by limiting ischemia in xenografted sheep ovarian cortex (Labied et al. 2013). In fact ischemia can cause primordial follicle death more than freezing and thawing. Survival of primordial follicles after transplantation is between 5% and 50% (Aubard et al. 1999). Therefore, the objective of the study was to evaluate the incidence of apoptosis in VEGF-treated auto-transplanted ovaries.

Materials and methods

All chemicals were purchased from Sigma (Germany), except those mentioned below.

Animals

Animal experiments were carried out according to the declaration of Helsinki and the guiding principles in the care and use of animals (DHEW publication, NIH, 80–23).

21-day-old SW (Swiss Webster) female mice (Pasteur Institute, Tehran, Iran) were housed and bred at Royan Institute Resource and they were kept at the temperature of 20–25°C and 50% humidity in light-controlled condition (12L: 12D photocycle) and provided with sterile food and water.

Auto-transplantation

Female mice were anesthetized by i.p. (intraperitoneal) injection of Ketamine–Xylazine mixture (100 mg/kg ketamine and 10 mg/kg Xylazine), and left ovaries were taken by a surgical incision on the skin and peritoneum over the ovary site (Ethicon, Belgium). Afterward the envelop tissue surrounded the ovary dissected in α MEM (GIBCO, USA) medium by insulin needles. Incisions in peritoneum and skin were sutured separately by 6-0 absorbable suture (Ethicon, Belgium). Ovaries were immediately auto-grafted into the gluteal muscle. A single surgical incision of the skin gave access to the gluteal muscle. By making about 2-mm-deep incision, the ovary was placed in the muscular site.

Experimental groups and treatments

VEGF was diluted in phosphate buffered saline (PBS) to 0.5, 1, 2 and 4 µg/mL. Each dosage was considered as an experimental group. From each experimental group, 10 female mice were auto-transplanted and determined dosages of VEGF for each group were injected around transplanted ovarian tissue. A group of 10 auto-transplanted mice were selected as group with no injection of VEGF factor. A group of 10 intact female mice were considered as control group which were non-transplanted. In all groups after the end of transplantation process, muscle and skin incisions were sutured (5-0 non-absorbable for muscle and 5-0 absorbable for skin, Ethicon, Belgium). About 21 days after auto-transplantation, female mice in all groups were treated for superovulation. All groups were treated with peritoneal injections of 7.5 IU pregnant mare serum gonadotropin (PMSG) (Folligon, Intervet) and after 48 h they were treated with 7.5 IU human chorionic gonadotropin (hCG) (Pregnenlone, Intervet). About 14 h after HCG injection, all female mice were killed by cervical dislocation. Transplanted and opposite ovaries of each mouse were taken and fixed for histological evaluations.

Preparation of sections

Ovaries harvested from each group were fixed in Bouin's fixative overnight. Fixed ovaries were dehydrated in ascending concentrations of ethanol (70–100%) by an automated tissue processor. Afterward tissues were embedded in paraffin, and serially sectioned at 6 µm serial sections and used for staining.

H&E staining

The sections were stained by H&E (hematoxylin and eosin) and follicles were classified according to developmental stage as primordial (with one layer of flat granulosa cells), primary (with one layer of cuboidal granulosa cells), preantral (two or more layers of granulosa cells with no cavity within) and antral (within cavity of antrum forming). To avoid double counting of the same follicle and to ensure inclusion of the largest cross-sections of follicles, counts were performed based on visible nucleolus within the oocyte. Follicle population was counted in sections on each H&E slide using light microscope (Nikon). Primordial follicles were counted only when the oocyte had a definite nuclear membrane (Eimani et al. 2009). The optimal dosage of VEGF was determined with counting population of follicles in ovaries. Figures 1–2 show transplanted and non-transplanted tissue sections with H&E staining.

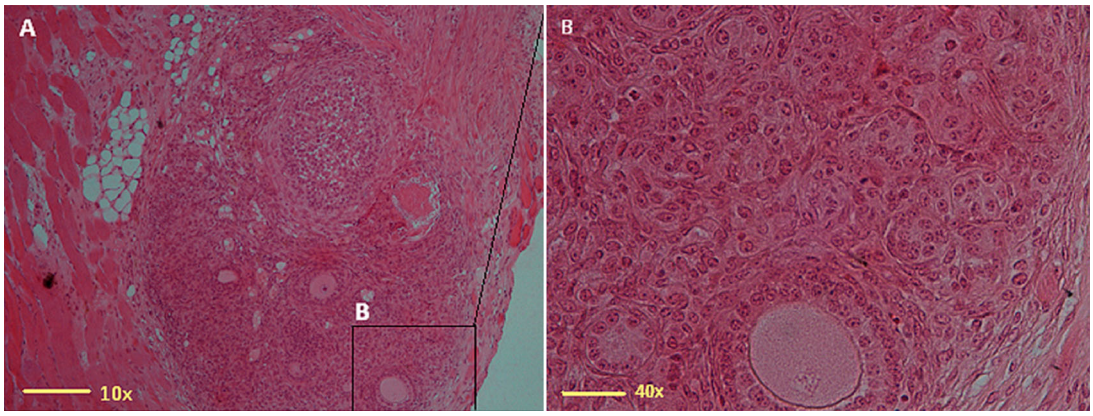

Figure 1. (a) A sample slide of H&E-stained transplanted ovary (10X). (b) A part of slide shown with higher magnification (40X). High number of primordial follicles with one primary follicle is visible.

TUNEL assay

Sections of transplanted ovaries with the best dosage of VEGF and non-transplanted ovaries were randomly selected for TUNEL test. DNA fragmentation was assessed by terminal-deoxynucleotidyl-transferase (TdT)-mediated deoxyuridine triphosphate–biotin nick-end labeling, according to the kit manufacturer's instructions (In Situ Cell Death Detection Kit, Roche, Mannheim, Germany). The 6-µm-thick prepared sections were incubated in 3% hydrogen peroxide (H_2O_2) (Merck, Germany) to neutralize endogenous peroxidases for 10 min, followed by digestion with 20 Rg/mL proteinase K for 30 min at 37°C. The TUNEL reaction mixture was added, the slides were incubated for 60 min at 37°C in a humidified dark chamber and then incubation with Converter-POD at 37°C for 30 min was carried out. A series of tissue sections were incubated in the reaction buffer without TdT as a negative control. The samples were stained with diamino benzidine substrate (Roche, Germany) for 10 min, followed by counterstaining with hematoxylin mounted in entellan (Merck, Germany) and examined by light microscope (Figure 3). Oocytes and follicular cells containing stained nuclei (dark brown) were considered TUNEL-positive. If more than 10% of the follicular cells were TUNEL-positive, the follicle was considered apoptotic (Figure 4) (Golkar-Narenji et al. 2013).

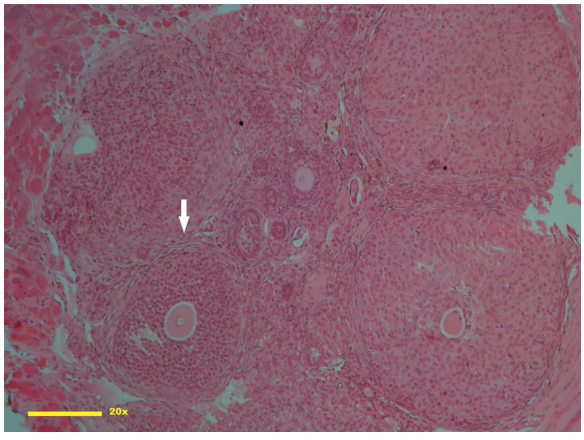

Figure 2. A sample slide of H&E-stained transplanted ovary with all types of follicles (20X). Antral follicle is shown by arrow.

CD31 assay

Immunohistochemical analyses were carried out to assess vascularization in transplanted tissue. Paraffin slides (6-µm thick) of the ovaries were baked, dewaxed, and then rehydrated in xylene, ethanol and water. Rehydrated slides were incubated in 37°C for 15–25 min with 10 µg/mL proteinase K, which is an antigen retrieval agent. This process breaks linkage between proteins and cause realizing superficial antigens to be more accessible for antibody. After washing in deionized water and H_2O_2 (3%), slides were rinsed in PBS-tween20 (0.1%) and incubated with blocking buffer (goat serum 10%) for 10 min in 37°C. Rat *anti*-mouse CD31 as primary antibody prepared 1:100 and slides were incubated at room temperature for 2 h. After washing with PBS-tween20 (0.1%), deionized water-slides were dried, mounted and viewed under fluorescence illumination.

Statistical analysis

Follicle numbers are presented as mean ± SE. Kolmogorov–Smirnov test of normality were performed to choose the appropriate statistical test. Kruskal–Wallis and Mann–Whitney tests was used to compare the significances of difference between mean numbers of primordial, primary, preantral and antral follicles present in all transplanted and non-transplanted groups. When the *P*-value was less than .05, the difference was

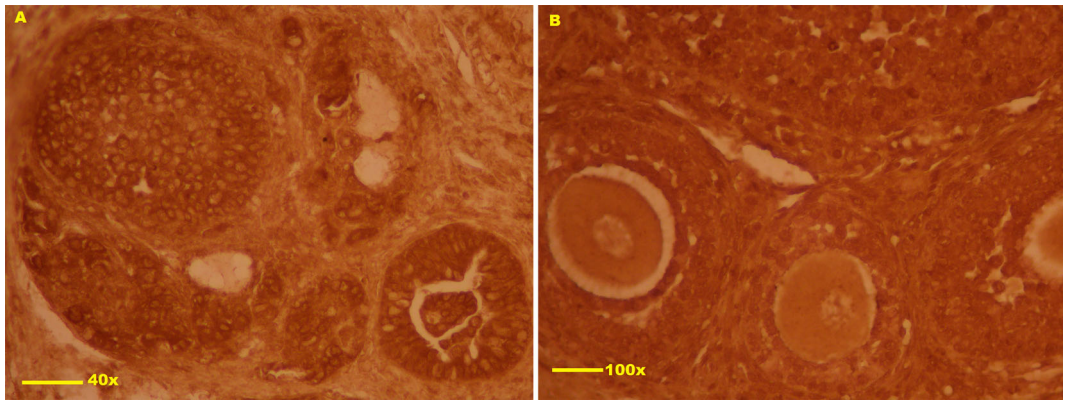

Figure 3. TUNEL stained slides of transplanted ovarian tissue with and without VEGF treatment. (a) Transplanted ovarian tissue without VEGF factor. Apoptotic areas are shown by arrows. (b) Transplanted ovary treated with VEGF.

considered significant. All analysis was performed by SPSS statistical analysis program.

Results

Follicular population

Follicle numbers (including primordial, primary and preantral follicles) were evaluated using H&E staining and expressed as mean ± SEM. The mean percentage of primordial follicles was significantly higher in transplanted group without VEGF and lower dosage of VEGF (0.5 µg/mL) compared to non-transplanted group (Figure 1). However, there was no significant difference between the percentages of primordial follicles in transplanted groups with higher dosages of VEGF (1, 2, 4 µg/mL) and non-transplanted group. The percentages of preantral and antral follicles were significantly lower in transplanted groups without VEGF treatment and with lower dosages of VEGF compared to non-transplanted group ($P < .05$). Higher percentages of preantral and antral follicles were observed in transplanted group with higher dosage of VEGF (2 and 4 µg/mL) compared to lowest dosage of VEGF (0.5 µg/mL). And there were no significant differences in the percentage of all types of follicles between transplanted groups treated with (2 and 4 µg/mL) VEGF compared to non-transplanted group (Table 1). In Figure 2, a section of transplanted tissue with antral follicles is shown.

TUNEL assay

Visual observation of 10 randomly selected stained slides from each experimental group and non-transplanted group showed that there was higher apoptotic area in transplanted ovaries compared to non-transplanted ovaries. There visually appears to be a higher incidence of apoptosis in transplanted tissue without VEGF. With a visual comparison of TUNEL stained slides of transplanted tissue with the effect of 4 µg/mL VEGF on the transplanted ovary, no apoptotic areas were observed. However, in the groups treated with lower dosages of

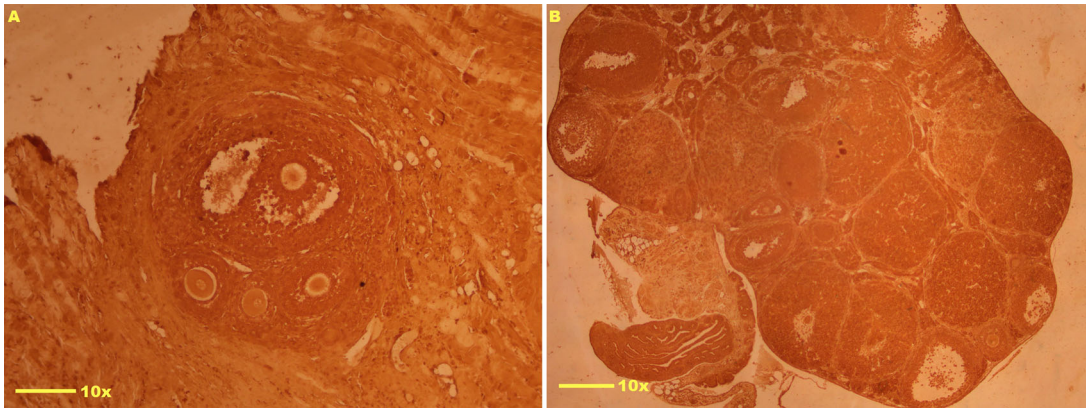

Figure 4. TUNEL stained slides of transplanted and non-transplanted ovarian tissue. (a) Transplanted ovarian tissue with the effect of the best dosage of VEGF (4 µg/mL). (b) Non-transplanted ovarian tissue.

Table 1. Follicular population three weeks after transplantation.

Experimental groups	VEGF (µg/mL)	Ovaries (*n*)	Follicular population			
			Primordial on (%)	Primary (%)	Preantral (%)	Antral (%)
Non-transplanted	0	4	49.81± 1.36^{ab}	26.30± 2.13	16.7± 1.36^{ab}	7.16± 0.76^{a}
Transplanted	0	4	73± 2.1^{a}	18.04± 3.26^{a}	5.9± 0.71^{a}	3.13± 0.76
	0.5	3	74± 5.03^{b}	18.8± 3.3	5.3± 1.1^{b}	2± 1^{a}
	1	3	56.11± 1.34	32.4± 1.2^{a}	8.41± 0.54	3.06± 0.54
	2	3	66.1± 11.42	23.16± 5	7.75± 4.44	3.18± 2.01
	4	3	66.8± 3.64	20.36± 2.35	9.7± 0.39	3.04± 0.94

Note: Different superscript letters in each column indicate significant differences ($p < .05$).

VEGF (0.5, 1, 2 µg/mL) and transplanted ovaries with no VEGF treatment, apoptotic areas were observed. In Figure 3, the difference between transplanted tissue with or without the effect of VEGF is shown. In Figure 4, the difference between transplanted tissue with the effect of VEGF (4 µg/mL) and non-transplanted tissue can be observed, which indicates no apoptotic areas in both groups.

CD31 assay

During visual observation, the visual of CD31 stained slides which have been randomly selected from transplanted ovaries treated with different dosages of VEGF, the highest positive areas (Figure 5(a)) were observed in slides of treated transplanted ovaries with 4 µg/mL of VEGF. However, lower positive CD31 areas (Figure 5 (b)) were observed in selected slides from transplanted ovaries without VEGF treatment.

Discussion

Although ovarian tissue transplantation is a promising technique for fertility preservation, several drawbacks remain. One of the most important disadvantages of ovarian tissue transplantation is related to ischemia, which can cause follicular loss (Schubert et al. 2008). There are many published reports aimed at improving ovarian tissue transplantation efficiency by reducing ischemia in the transplanted tissue (Israely et al. 2006; Behbahanian et al. 2013; Labied et al. 2013; Henry et al. 2015; Youm et al. 2015). One of the factors affecting angiogenesis which prevents ischemia in transplanted tissue is site of transplantation (Behbahanian et al. 2013; Youm et al. 2015). In our previous research, gluteal muscle was shown to be an intriguing site for whole ovarian transplantation (Behbahanian et al. 2013). Therefore, ovaries were transplanted in gluteal muscle and effect of VEGF injection was evaluated on the histology of transplanted ovary. In transplanted ovaries, the survival rate of follicles and their ability to develop to higher stages of development has been reported (Adriaens et al. 2006). In this research, higher percentage of primordial follicles and lower percentage of primary, preantral and antral follicles indicate that the rate of follicular development to higher stages is low in transplanted ovarian tissue compared to non-transplanted ovarian tissue. Injection of different dosages of VEGF (1, 2, 4 µg/mL) to transplanted ovaries decreased primordial follicles in transplanted ovaries and there was no significant difference in the percentage of primordial follicles between those experimental groups and non-transplanted group. Angiogenesis in transplanted ovaries can lead to prolonged graft functionality (Israely et al. 2006). It has been reported that angiogenic factors are effective to prevent follicular

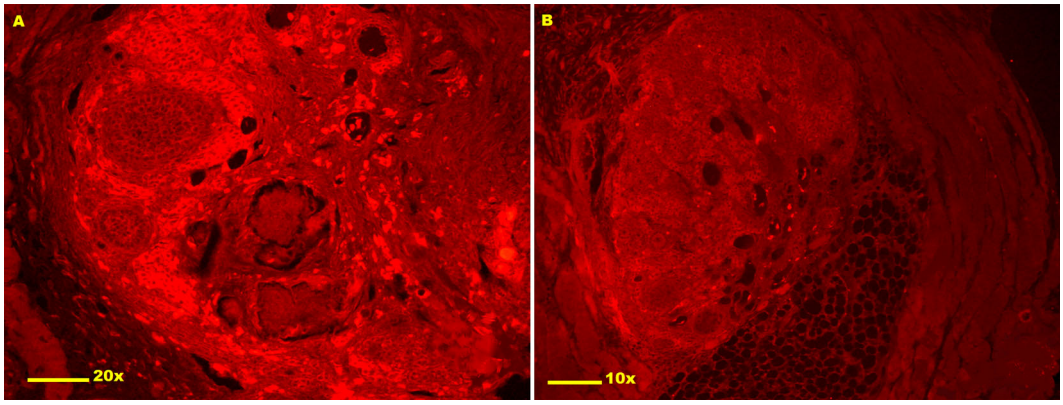

Figure 5. CD31 stained slides of ovarian tissue. (a) Transplanted ovarian tissue with the effect of best dosage of VEGF (4 µg/mL). (b) Transplanted ovarian tissue without VEGF treatment.

loss due to prevention of ischemia. Labied et al. indicated that angiogenesis with the effect of $VEGF_{111}$ increased follicles' viability in sheep transplanted ovarian cortex with reduction of ischemia (Labied et al. 2013). Results of present research show that the population of all types of follicles in transplanted groups treated with higher dosages of VEGF (2, 4 µg/mL) are similar to those in non-transplanted group. Observation of higher CD31 positive areas in transplanted ovaries treated with the highest dosage of VEGF (4 µg/mL) in comparison with other dosages indicated that more angiogenesis has been occurred with the effect of higher dosage of VEGF. There is evidence that ischemia in the early stage after ovarian graft causes massive follicle loss due to induction of ischemia (Labied et al. 2013). Visual observation of TUNEL test showed that the apoptotic area was higher in transplanted ovaries with no VEGF treatment or ovaries that have been treated with lower dosages of VEGF (0.5, 1, 2 µg/mL) compared to non-transplanted ovaries (Figure 3). It has been shown that inhibition of VEGF expression caused apoptosis and atresia in follicles (Abramovich et al. 2006). A visual comparison between ovaries treated with highest dosage of VEGF (4 µg/mL) and non-transplanted ovaries is shown in Figure 4 – that is, showing a sample of no apoptotic areas in both transplanted ovaries. Results of CD31 and TUNEL assays in ovarian tissues treated with VEGF (4 µg/mL) indicate that higher angiogenesis caused the absence of apoptotic areas, which is similar to that of non-transplanted ovaries. One of the main properties of VEGF is prevention of apoptosis and inflammation (Jung Won Jeona et al. 2014). Results of the present research are similar to those of a previous study that has demonstrated VEGF injection to the ovary caused angiogenesis, which reduces ischemia and prevents apoptosis (Shimizu 2006). In this research, a higher dosage of VEGF was shown to be the most effective to induce angiogenesis and to prevent apoptosis in transplanted tissue. Higher number of developed follicles and lower rate of apoptosis with injection of the highest dosage (4 µg/mL) to the transplanted ovary shows that injection appropriate dosage of VEGF to transplanted ovarian tissue helps to preserve follicular development. VEGF as an angiogenic factor decreases ischemia and prolongs the graft functionality that helps to keep normal follicular population in whole transplanted mouse ovary. Results of this research confirm previous researches about positive effects of VEGF on function transplanted ovaries (Shimizu 2006; Labied et al. 2013). However, the effect of VEGF on transplantation of whole mouse ovary on gluteal muscle was evaluated. Also the effect of different dosages of VEGF was studied and appropriate dosage was determined. The main result of this research is to show that 4 µg/mL VEGF saves normal population of follicles, increases angiogenesis and prevents the incidence of apoptosis in transplanted ovarian tissue.

Disclosure statement

No potential conflict of interest was reported by the authors.

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