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Original Article

Effects of silymarin, cabergoline and letrozole on rat model of endometriosis

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ABSTRACT

Objective: Silymarin as an herbal drug has potent antioxidant effects that could make it a good choice for endometriosis therapy. The aim of the current study was to determine the effects of silymarin as an herbal drug on induced endometrial lesion in rat model of endometriosis.

Materials and methods: A total of 32 mature, female Sprague–Dawley rats were allocated into 4 experimental groups. The duration of study was about 6 months. Endometriosis implants were surgically prepared and autografted into 32 rats. Three weeks after endometriosis induction, animals were randomly allocated into four groups: Group 1 received cabergoline (CAB group); Group 2 received letrozole (LET group); Group 3 received silymarin (SIL group) and Group 4 received no medication (CONT group). Experimental groups were treated for 3 weeks and then were sacrificed for volume and histopathological evaluation of implants and biochemical assessment. Serum and peritoneal levels of vascular endothelial growth factor (VEGF), total antioxidant activity (TAC) and tumor necrosis (TNF)-α were measured.

Results: Mean volume of the implants decreased significantly in silymarin ($p < 0.001$), letrozole ($p < 0.001$) and cabergoline ($p < 0.001$) groups compared to the control. Histopathologic score was significantly lower in silymarin ($p: 0.039$), letrozole ($p: 0.017$) and cabergoline ($p < 0.001$) groups compared to the control. Those receiving silymarin had significantly higher serum TAC compared to control after 21 days of therapy ($p < 0.001$).

Conclusion: Silymarin, Letrozole, and Cabergoline administration resulted in decreased size and histopathologic grade of the induced endometrial lesions in a rat model. Silymarin appears to be a virtual novel therapeutic agent for treatment of endometriosis.

Introduction

Endometriosis is among the most common causes of chronic pelvic pain which is defined as the presence of functional endometrial tissue outside the uterine cavity [1]. Endometriosis is usually presented with chronic pelvic pain, infertility, menstrual irregularity, dyspareunia and impaired quality of life [2]. About 5–10% of women of reproductive age suffer from the condition while the prevalence increases to 20–50% in infertile women [3]. The pathophysiology and the etiology of the endometriosis is yet to be identified and exact pathophysiology of this disease is still uncertain; however, it is well known that endometriosis is an estrogen-dependent condition while the symptoms are cyclic parallel to menstrual cycle and resolve after menopause [4]. Several optional etiological theories have been suggested. So a novel treatment for endometriosis is widely quested.

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Aromatase is a key enzyme in pathogenesis of endometriosis while it converts the precursor steroids into estradiol and estrogen enhancing the proliferation of endometrial tissue. In addition, the aromatase enzyme plays an important role in inflammatory response which leads to progression of the disease [5]. Thus, inhibition of aromatase enzyme could be an effective way for treatment and control of the endometrial tissue proliferation. Letrozole is a non-steroid third generation aromatase enzyme inhibitor which is well tolerated and highly potent and is used for treatment of endometriosis. It has been demonstrated that letrozole suppresses the estrogen production both locally and systemically and reduces the symptoms significantly [6–8].

The endometrial tissue requires blood supply for growth and proliferation which is supplied via neoangiogenesis. Thus, angiogenesis growth factors such as vascular endothelial growth factor (VEGF) are required for growth and proliferation of the endometrial lesions [9]. It is postulated that blocking these growth factors can be an effective route of endometriosis treatment [10]. It has been demonstrated that dopamine and its agonists including cabergoline induce the endocytosis of the VEGF receptor-2 in endothelial cells resulting in decreased VEGF-VEGFR-2 binding. This will inhibit the neoangiogenesis in endometrial tissue [11]. Cabergoline has been shown to be effective in decreasing the size of the endometrial tissue [12].

Silymarin is the extract of *Silybum marianum* and is reported to have antioxidant activities and acts as a free radical scavenger which results in increased glutathione levels. Silymarin has also been reported to activate and increase the activity of superoxide dismutase (SOD) and glutathione peroxides [13]. In animal model of rat, it has been shown that silymarin acts as a cell membrane stabilizer which inhibits the cell damage by preventing the lipoperoxidation cascade [14,15]. The effects of silymarin on fertility parameters was previously evaluated. Although previous reports have failed to show beneficial effects of silymarin on folliculogenesis and granolusa cell apoptosis [16], its protective effects on ovarian reserve and response [17] and the sperm motility have been well established [18]. We postulated that the antioxidant and anti-inflammatory effects of silymarin would be effective in treating endometrial lesions. In the current in vivo study, we compared the effects of cabergoline, letrozole and silymarin on surgically induced endometriosis.

Materials and methods

Animals

Thirty-two mature, non-pregnant female Sprague–Dawley rats weighting 230–250 g were used for induction of the experimental endometriosis model. The rats were caged individually in a controlled environment (room temperature of 22 ± 2 °C and humidity of 50 ± 10%) with 12-h light/dark cycles, and were fed *ad libitum*. Before the surgical induction of endometriosis, the rats underwent daily vaginal lavages to detect the phase of estrus cycle. Vaginal secretions were examined under a light microscope to identify the estrus cycle by the dominancy of the anucleate cornified cells. The Avicenna Research Institute committee on the use and care of animals approved the experimental procedures and all investigations were performed in compliance with international guidelines on the ethical use of animals. The study was performed at the Surgical Research Center of Avicenna Research Institute, Tehran, Iran.

Surgical procedure

Step 1: Establishment of the endometriosis model

Ectopic endometrium was inserted surgically in rats by transplanting an autologous fragment of endometrial tissue onto the inner surface of the abdominal wall as described by Vernon and Wilson [19] with minor modifications of Lebovic et al. [20]. Briefly, the left uterine horn was ligated at both the uterotubal junction and the cervical end using 4-0 polyglyconate coated synthetic absorbable sutures and then removed. A 7-mm segment of the excised horn was cut from the anti-mesentric side and placed in sterile phosphate-buffered saline (PBS) at 37.8 °C. The endometrium was separated from the myometrium and trimmed to 5 × 5 mm. This piece of endometrial tissue was transplanted onto the inner surface of the right abdominal wall with the serosal surface apposed, and secured with single synthetic absorbable 4–0 suture. The vertical abdominal incision was closed using two-layer 4–0 synthetic absorbable sutures. The skin incision was closed with a horizontal mattress. The duration of surgery was limited to 15–20 min for each rat to minimize tissue drying. After the first surgery, all rats were caged individually and their body weight and wound healing were observed for 21 days and during this time they did not receive medication. After these 21 days (for establishment of endometrial implantation), the 32 rats were randomly allocated into four groups. The rats in Group 1 were given 0.5 mg/kg/day Cabergoline subcutaneously (Iran hormone, Tehran, Iran; CAB group). The Cabergoline dose was based on a previous study [21]. The rats in Group 2 were given 0.18 mg/kg/day letrozole subcutaneously (Abureihan, Tehran, Iran; LET group). Dose of letrozole was chosen on the base of a previous study [22]. The rats in Group 3 were administered 100 mg/kg/day silymarin subcutaneously (Sabzdaro, Esfahan, Iran; SIL group). Dosage choice were based on previous studies [23,24]. The rats in Group 4 had no medication (CONT. group). All rats in experimental groups were treated for 21 days.

Step 2: Evaluation of the outcome

After 21 days of medication therapy in experimental groups, the rats were euthanized with ketamine 10% and xylazine 2%, and a laparotomy was done. Before excising the endometrial implants, peritoneal lavage with 2 mL saline was performed to assess the inflammatory and oxidative markers in the peritoneal fluid. Ectopic endometrial tissues were isolated and three dimensional measurements were performed (length × width × height) using a digital caliper. The prolate ellipsoid formula was used for calculation of the spherical volume of each ectopic uterine tissue: V (mm3) = 0.52 × length × width × height. For histopathological evaluation, the implants were fixed in 10% formalin. Blood samples were obtained by cardiac puncture using 5 mL syringe for the biochemical assessment in the plasma (TNF-α and TAC in SIL and CONT groups and VEGF in CAB and CONT groups) (Fig. 1).

Biochemical analysis

Blood and peritoneal fluid samples were centrifuged at 3000 rpm for 10 min at room temperature and stored at −80 °C before the biochemical assay. Serum and peritoneal fluid levels of rat TNF-α and VEGF were quantified by enzyme-linked immunosorbent assay (ELISA) using commercially available matched antibodies (for TNF-α: Invitrogen, Camarillo, CA, USA and for VEGF: Sigma Aldrich, Catalog Number RAB0511, USA) according to the manufacturer's instructions. Serum and peritoneal fluid levels of rat TAC were analyzed by calorimetric method using TAC assay kit (ZellBio Inc., Ulm, Germany, cat No: ZB-TAC96, V40227). According to the supplier of the kits, the intra-assay and inter-assay coefficients of variation (CV) for TNF-a were 6% and 8%, respectively, while for VEGF they were <10% and <12%, respectively. The sensitivity was calculated to be <4 pg/mL for TNF-α and <2 pg/mL for VEGF.

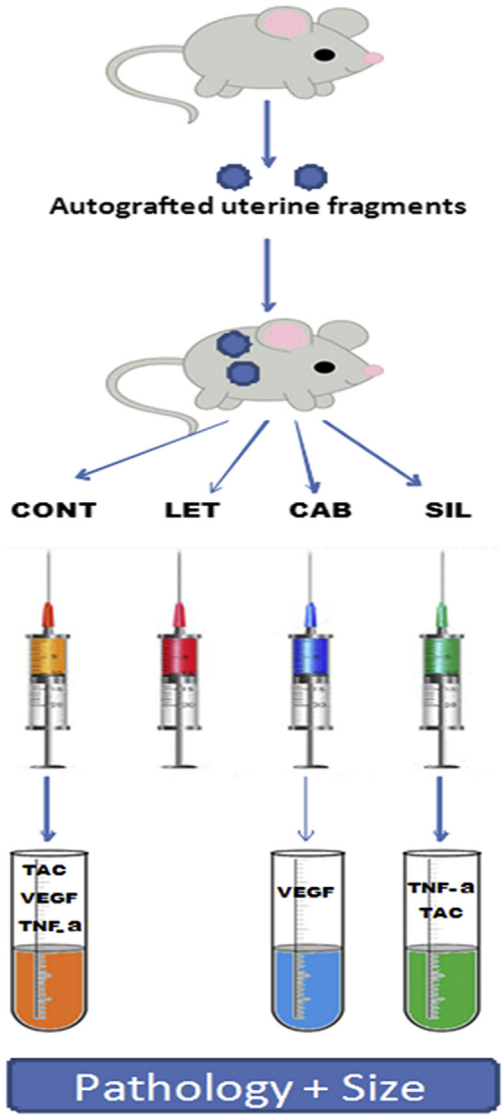

Fig. 1. Illustrative summary of the main methods. CONT: Control group, LET: Letrozol treated group, CAB: Cabergolin treated group, SIL: Silymarin treated group, TAC: Total Anti Oxidant Capacity, VEGF: Vascular Endothelial Growth Factor, TNF-α: Tumor Necrosis Factor α

Histopathological examination

Endometriotic implants were embedded in paraffin blocks after formalin fixation. 5 μm thick tissue sections were obtained, stained in hematoxylin eosin, and evaluated with light microscopy (CX-41, Olympus). The epithelial lining of the endometrial implants was evaluated semi-quantitatively according to the previously described method [25]. Grade 0: No epithelium; Grade 1: Poorly preserved epithelium (occasional epithelial cells only); Grade 2: Moderately preserved epithelium with leukocyte infiltrate; Grade 3: Well preserved epithelial lining. Histological evaluations were made blindly by the same histologist in accordance with a previous study on rat endometriosis [25] and were photographed.

Statistical analysis

All the statistical analyses were performed using statistical package for social sciences (SPSS Inc., Chicago, Illinois, USA) version 11.0. All data are presented as mean ± SEM or proportion as appropriate. The parametric data were compared between study groups using one-way analysis of variance (ANOVA) with post-hoc tests for further analyses. Non-parametric data were compared using Kruskal–Wallis one-way analysis of variance. Independent t-test was used to compare the parametric variables between two study groups. A two-sided p-value of less than 0.05 was considered statistically significant.

Results

Overall 32 rats in 4 study groups (each including 8 rats) were included. All the animals passed the study and none of them experienced drug adverse reactions. There was no significant difference between the four experimental groups regarding to the baseline characteristics.

At the end of the treatment period, the mean volume of implants was smaller in LET Group ($P < 0.001$), CAB Group ($P < 0.001$) and SIL Group ($P < 0.001$) compared to the control group (Table 1). Sample views of the endometriotic implants are shown in Fig. 2.

The mean score of the histopathological evaluation of the implants at the end of the treatment was lower in LET, CAB and SIL groups in comparison with the control group ($P < 0.01$; Table 1). Sample views of the histopathologic scores of endometriotic implants are shown in Fig. 3.

At the end of the treatment period, serum TAC concentration was significantly higher in SIL group compared to the CONT. group, ($P < 0.001$; Table 1). However, there was no significant difference in TAC level in peritoneal fluid (Table 2). Peritoneal level of VEGF, TAC and TNF-α have been shown in Table 2. After the medication therapy in rats, there were no significant differences in VEGF and TNF-α levels in both plasma and peritoneal fluid in all experimental groups.

Discussion

The pathophysiology of the endometriosis is still unknown and thus the treatment options remain controversial. Most of the available therapies for the endometriosis are anti-estrogen agents [6,26–29] and dopamine agonists [30,31]. Some other therapies target the oxidative stress in the endometrial lesions and it has been shown that antioxidants might be beneficial to these patients [11,32–35]. In the present study, for the first time, silymarin, as an antioxidant and anti-inflammatory agent, was used and compared with two other previous therapeutic agents in the rat model of endometriosis.

In this experimental study, administration of silymarin, letrozole and cabergoline was equally and significantly effective in reducing the size and the histopathology grade of the endometrial lesions. It has been well demonstrated that oxidative stress and inflammation play an important role in pathogenesis of endometriosis [36–38]. Several studies have indicated that induction of oxidative stress and inflammation in endometriosis may be related to presence of erythrocytes, apoptotic endometrial tissue, cell debris transplanted into the peritoneal cavity by menstrual reflux and macrophages [38]. It is well established that there is an endometriosis-related imbalance between reactive oxygen species (ROS) and antioxidants [32]. The release of ROS results in cellular damage and dysfunction that affects on gene expression and consequently protein synthesis [32,38,39]. The elevated oxidative stress (OS) in endometriosis may either be a cause or a consequence of the pathophysiology of endometriosis [38]. Therefore, it has been assumed that targeting the OS will result in reduction of size and histopathologic grade of the endometrial implants. With regard to this point, several studies have used antioxidant agents for management of patients with endometriosis [37,40,41].

Table 1
Parameters evaluated in experimental groups after treatment with Letrozole (0.18 mg/kg), Cabergoline (0.5 mg/kg) and Silymarin (100 mg/kg).

Variable	Mean volume of the implants	Histopathological score of the implants	VEGF level in serum	TNF-α level in serum	TAC level in serum
LET	0.69 ± 0.08^{a}	1.2 ± 0.5^{a}	–	–	–
CAB	1.81 ± 0.36^{b}	0.5 ± 0.3^{a}	16.21 ± 3.7^{a}	–	–
SIL	2.04 ± 0.25^{b}	1.3 ± 0.6^{a}	–	10.11 ± 4.4^{a}	0.19 ± 0.06^{a}
CONT	5.04 ± 0.40^{c}	2.6 ± 0.2^{b}	15.96 ± 2.8^{a}	15.1 ± 7.6^{a}	0.07 ± 0^{b}

VEGF: Vascular Endothelial Growth Factor, TNF-α: Tumor Necrosis Factor α, TAC: Total Antioxidant Capacity, LET: Letrozole treated group, CAB: Cabergoline treated group, SIL: Silymarin treated group, CONT: Control group.
[a, b, c]: Numbers with different lowercase superscript letters in the same column differ significantly ($P < 0.05$). Data were expressed as mean ± SEM. Differences among groups were analyzed by one-way ANOVA followed by post -hoc tests for further analyses.

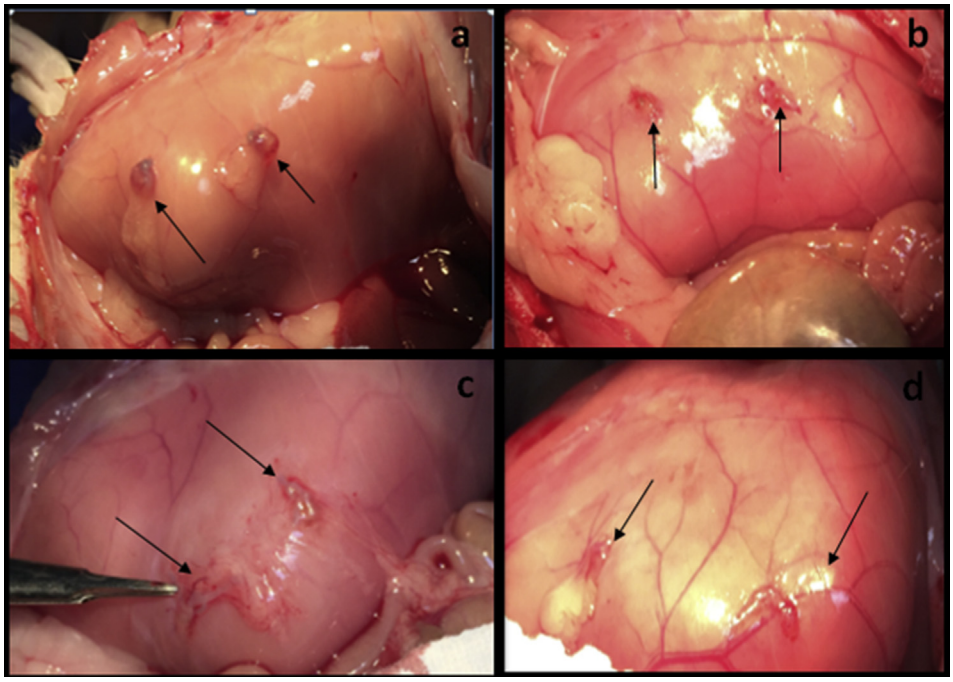

Fig. 2. Macroscopic appearance of endometriotic implants (arrows) in (a) the control group, (b) Cabergoline treated Group, (c) Letrozole treated Group and (d) Silymarin treated Group.

Some antioxidants have been used for management of endometriosis including vitamin C and E. In an animal study, Durak et al. [42] showed that a dose-dependent vitamin C supplementation significantly reduced both volume and weight of the endometriotic cysts and the natural killer cell content. Yavuz et al. [43] demonstrated that in a rat endometriosis model, resveratrol (a natural polyphenolic, non-flavonoid antioxidant) had potential ameliorative effects on endometriotic implants probably due to its potent antioxidative properties.

It has been demonstrated that the number of activated macrophages increases in the peritoneal cavity of those with endometriosis, leading to inflammation and oxidative stress [33,34,44]. In addition, it has been well indicated that suppression of the ROS in the peritoneal fluid of patients with endometriosis is associated with decreased symptoms as well as lesion size [32,45].

In the present study, silymarin significantly reduced volumes and histopathologic scores of the endometriotic lesions, and increased serum levels of TAC. Silymarin has been demonstrated to have antioxidant capacity and activity in different conditions [13,24,46]. Silymarin is probably able to antagonize the depletion of two main detoxifying mechanisms, GSH and superoxide dismutase (SOD), by reducing the free radical load, increasing GSH levels and stimulating SOD activity [47]. Several experimental studies have clearly demonstrated that silymarin has antioxidant properties and acts as free radical scavenger and prevents the lipid peroxidation [24,46,47]. Moosavifar and his colleagues had evaluated the effects of silymarin on the granulosa cells of patients undergoing in vitro fertilization (IVF). They indicated that administration of silymarin along with gonadotropin in IVF patients is associated with reduction of granolusa cell apoptosis but does not have any effect in promotion of follicular development, oocyte retrieval or endometrial thickness [16]. Since silymarin as an approved herbal drug has been used in different human disease for many years [48–50] and as there is not any report of its side effects, it could be considered as a choice for treatment of endometriosis. Regarding the increase in serum level of TAC in silymarin treated group in the present study, it could be suggested that silymarin improves endometriotic lesions as an antioxidant agent.

In the present study, there was not any significant decrease in serum and peritoneal levels of TNF-α in the silymarin treated group. In other studies, silymarin decreased the serum level of TNF-α both in experimental model [51] and in clinical settings [41]. Previously, Dehmlow et al. demonstrated that silibinin (active ingredient of silymarin) concentrations below 100 μmol/L had no influence on the serum levels of TNF-α [52]. Therefore, this might be an explanation as to why the serum levels of TNF-α were not decreased in the current study. Furthermore, it could mean that silymarin does not have anti-inflammatory effects for treatment of endometriosis.

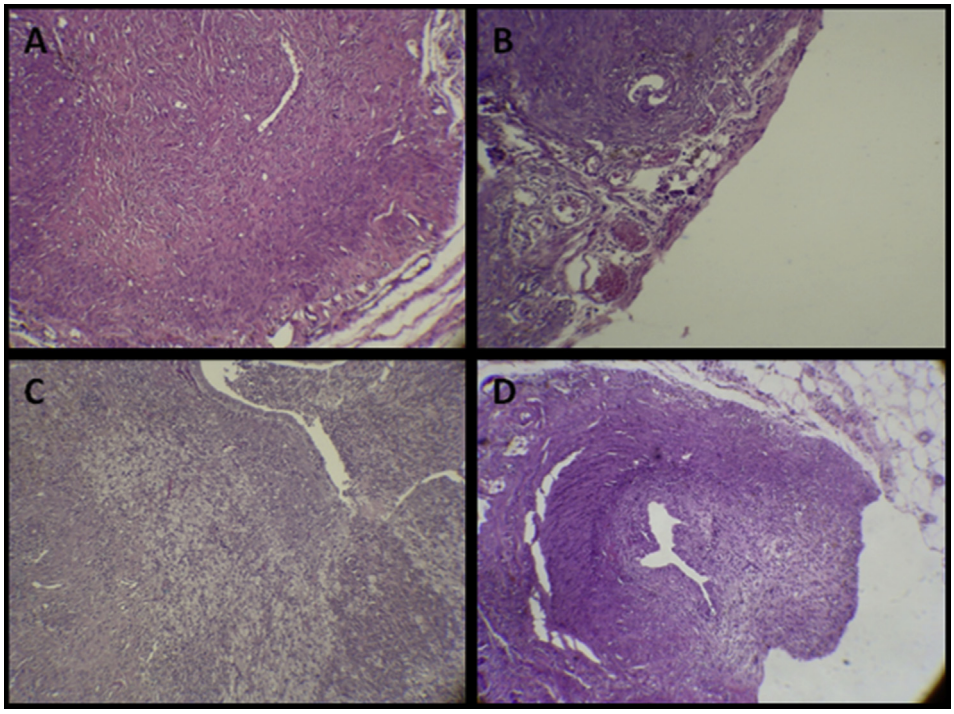

Fig. 3. Histopathologic examination of endometriotic lesions and histopathologic scores (stain: hematoxylin and eosin; magnification: A and C: ×100, B and D: × 50). (A) Score 0: no epithelium; (B) Score 1: poorly preserved epithelium; (C) Score 2: moderately preserved epithelium with leukocyte infiltrates; (D) Score 3: well-preserved epithelial lining.

Table 2
Peritoneal level of TNF-α, TAC and VEGF in experimental groups after treatment with Letrozole (0.18 mg/kg), Cabergoline (0.5 mg/kg) and Silymarin (100 mg/kg).

Variable	TNF-α	TAC	VEGF
LET	–	–	–
CAB	–	–	4.84 ± 1.1
SIL	45.8 ± 17	0.04 ± 0	–
CONT	38.2 ± 11.6	0.05 ± 0.01	5.59 ± 2
P Value	0.718	0.284	0.763

VEGF: Vascular Endothelial Growth Factor, TNF-α: Tumor Necrosis Factor α, TAC: Total Antioxidant Capacity, LET: Letrozole treated group, CAB: Cabergoline treated group, SIL: Silymarin treated group, CONT: Control group. Data were expressed as mean ± SEM. Differences among groups were analyzed by one-way ANOVA followed by post -hoc tests for further analyses.

In the present study, the size and histopathological grade of the endometriotic lesions were significantly decreased in cabergoline and letrozole groups. Novella-Maestre et al. [35] demonstrated that administration of cabergoline resulted in regression of implanted endometrial tissue in nude mice peritoneum. The action occurred through suppression of cell proliferation and VEGF-mediated angiogenesis. The same group further found that VEGF gene and protein expression were significantly reduced in endometriotic lesions treated with cabergoline rather than in controls [11]. There are some other studies which demonstrated positive effects of dopamin agonists and letrozol on size and histopathological grade of endometriotic implants [8,12,22,27,53–57]. Levels of angiogenic growth factor such as VEGF have been shown to be elevated in the peritoneal fluid of women with endometriosis [31,58].

In the current study, there was not any significant difference between experimental groups in the serum and peritoneal levels of VEGF. Some studies have demonstrated that endometriosis is not associated with change in the level of circulating VEGF which corresponds with our results [59,60]. However, some studies found that the peritoneal levels of VEGF were significantly higher in those with endometriosis compared to healthy controls [59,60]. This could be explained according to the previous in vitro findings that indicate increased release of VEGF by peritoneal macrophages in women with endometriosis [61,62].

In conclusion, silymarin administration resulted in decreased size and histopathologic grade of the induced endometrial lesions in the rat model. Although silymarin administration was associated with increased level of serum TAC, the serum and peritoneal levels of TNF-α remained unchanged. Further clinical studies are required to shed light on this issue.

Conflict of interest

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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