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ENDOMETRIOSIS

A potential novel treatment strategy: inhibition of angiogenesis and inflammation by resveratrol for regression of endometriosis in an experimental rat model

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Abstract

The aim of our study was to evaluate the effectiveness of resveratrol in experimentally induced endometrial implants in rats through inhibiting angiogenesis and inflammation. Endometrial implants were surgically induced in 24 female Wistar–Albino rats in the first surgery. After confirmation of endometriotic foci in the second surgery, the rats were divided into resveratrol (seven rats), leuprolide acetate (eight rats), and control (seven rats) groups and medicated for 21 d. In the third surgery, the measurements of mean areas and histopathological analysis of endometriotic lesions, VEGF, and MCP-1 measurements in blood and peritoneal fluid samples, and immunohistochemical staining were evaluated. After treatment, significant reductions in mean areas of implants ($p < 0.01$) and decreased mean histopathological scores of the implants ($p < 0.05$), mean VEGF-staining scores of endometriotic implants ($p = 0.01$), and peritoneal fluid levels of VEGF and MCP-1 ($p < 0.01$, for VEGF and $p < 0.01$, for MCP-1) were found in the resveratrol and leuprolide acetate groups. Serum VEGF ($p = 0.05$) and MCP-1 ($p = 0.01$) levels after treatment were also significantly lower in the resveratrol and leuprolide acetate groups. Resveratrol appears to be a potential novel therapeutic agent in the treatment of endometriosis through inhibiting angiogenesis and inflammation. Further studies are needed to determine the optimum effective dose in humans and to evaluate other effects on reproductive physiology.

Keywords

Endometriosis, MCP-1, resveratrol, VEGF

History

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Introduction

Endometriosis is thought to have a multifactorial etiology including genetic, hormonal, and immunological factors [1,2]. Implantation, proliferation, angiogenesis, and inflammation are important histopathological events that play a crucial role in the establishment and growth of endometriotic lesions [3–5].

Alterations in humoral and cell-mediated immunity are important factors to increase susceptibility to the survival of endometriotic lesions [2]. Peritoneal macrophages and activated lymphocytes seem to be responsible for the secretion of pro-inflammatory/proangiogenic cytokines [vascular endothelial growth factor (VEGF); interleukins, tumor necrosis factor α (TNF α), and monocyte chemotactic protein (MCP)-1] [6–16].

The therapeutic approach has recently been revised by improvement of our understanding concerning the pathogenesis, growth, and maintenance of ectopic endometrium, particularly in terms of angiogenesis, inflammation, and immune response. It is theoretically rational to develop alternative agents by considering other mechanisms that contribute to the pathogenesis of endometriosis. Thus intense research has focused on alternative novel non-hormonal agents for the treatment of endometriosis that are as efficient as hormonal drugs and that have fewer systemic side effects. TNF-α inhibitors, matrix metalloproteinase inhibitors (MMPs), immunomodulators, angiogenesis inhibitors, and reactive oxygen species scavengers have been examined in experimental studies [7,13,17–19].

Resveratrol is a natural polyphenolic compound isolated from the roots and fruits of many plants. It is considered to have anti-atherogenic, anti-oxidative, anti-inflammatory, anti-microbial, and anti-angiogenic effects [20–22]. The anti-inflammatory effects of resveratrol, which might be the major mechanism in the prevention of diseases, have been shown to be through the inhibition of prostaglandin synthesis via the inhibition of COX enzyme synthesis, inhibition of synthesis and release of pro-inflammatory cytokines, and inhibition of activated immune cells [20–22]. Resveratrol has also been shown to exhibit apoptosis-inducing activities in several biological systems [22]. Resveratrol performs its anti-cancer and anti-atherogenic effects by the inhibition of the angiogenic process via inhibition of proangiogenic factors such as VEGF and MMPs, and inhibition of proliferation and migration of vascular endothelial and smooth muscle cells [21,23,24]. Recently, it was demonstrated that

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resveratrol inhibited development of endometriosis in a nude mouse model *in vitro* and further reduced invasiveness of human endometrial stromal cells *in vivo*; a decrease in the proliferative activity and an increase in the apoptotic activity of the endometriotic lesions were also shown [25]. We aimed to evaluate the effectiveness of resveratrol in a rat endometriosis model and to compare the effect of GnRH agonist.

Materials and methods

Animals

Twenty-four Wistar–Albino rats weighing 200–250 g were caged in a controlled environment of 22 °C with 12 h light/dark cycles. Standard rat feed and reverse-osmosis-purified water were provided ad libitum. All rats were allowed to acclimatize to this environment for 1 week before the experiments. The Gazi University Committee on the Use and Care of Animals approved the experiments, and all investigations were complied with the National Academy of Science's Guide for Care and Use of Laboratory Animals (1996).

Surgical procedures

All the rats were anesthetized with an intramuscular administration of 50 mg/kg of ketamine hydrochloric acid (Ketalar; Eczacibasi Warner-Lambert Ilac Sanayi, Levent, Istanbul, Turkey) and 7 mg/kg of xylazine hydrochloric acid (Rompun, Bayer Sisli, Istanbul, Turkey). Using the aseptic technique, a ventral midline incision was made to expose the reproductive organs. All rats underwent three laparotomies as explained below.

First surgery

Endometriotic lesions were induced surgically as described by Rajkumar et al. [26]. The left uterine horn was ligated at both the uterotubal junction and the cervical end and removed. A 7-mm segment of the horn was excised and put in sterile isotonic saline. Only one implant, the trimmed 5×5 mm section of the endometrium (together with the myometrium), was transplanted per animal into the peritoneal cavity with the epithelial surface of the segment opposed to the peritoneum on the right anterior abdominal wall with a rich vascular area; the implant was sutured in the peritoneum adjacent to a large vessel using sterile 4-0 silk suture. After the first surgery, all rats were observed for 21 d in their cages without any medication.

Second and third surgeries

Two rats died during the observation period. After 21 d of the first operation and after 21 d of the second operation, the remaining rats underwent a laparotomy to evaluate endometriotic foci (Figure 1) and to collect peritoneal fluid to assess VEGF and MCP-1 levels. Additionally, during the third surgery, the implants were excised and fixed in 10% formalin for histopathological

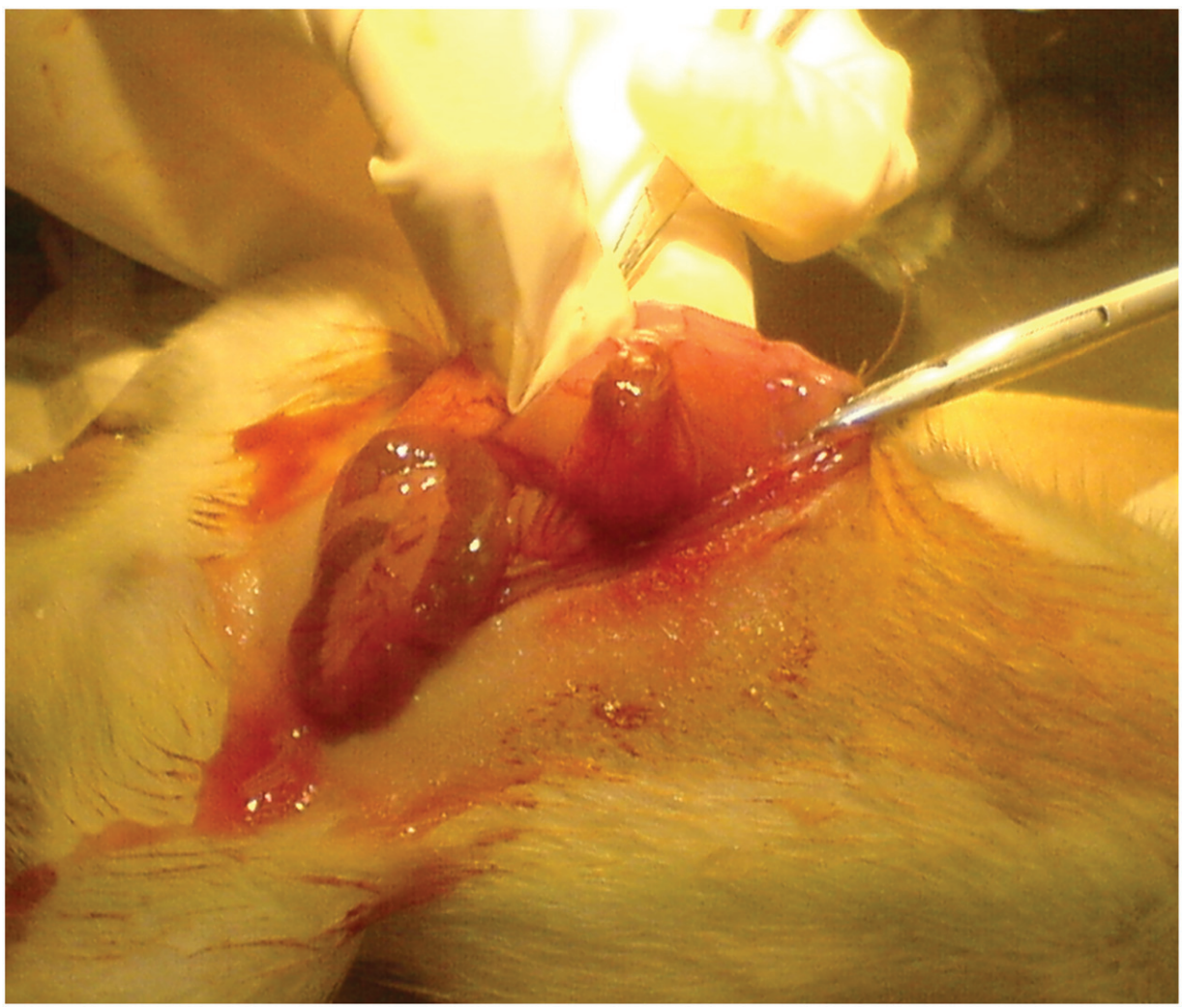

Figure 1. New observed endometritic implants.

examination and arterial blood samples were collected to measure serum VEGF and MCP-1 levels. All the operations and measurements were performed by two physicians blinded to the groups. To collect peritoneal fluid, peritoneal lavage with 3 ml of saline was performed and samples were immediately sent to the laboratory. The length and width (mm) of the implants were measured and the surface areas of the implants were calculated (length × width) and recorded. Following the second surgery, all rats were observed for 3 d. After the second surgery, the rats were randomly divided into three groups. The rats in the first group (resveratrol group, seven rats) were given 60 mg/kg/d resveratrol orally (Resveratrol; Now Foods, Bloomingdale, IL) for 21 d. The rats in the second group (leuprolide acetate group, eight rats) were administered 1 mg/kg leuprolide acetate depot (single s.c. injection, Lucrin; Abbott, Istanbul, Turkey). The leuprolide acetate dose was based on a previous study in which 1 mg/kg was found to be optimal for female rats [27]. In the third group (control group, seven rats), no medication was given. The oral medications were given by gavage via an orogastric tube by the laboratory personnel.

Measurement of VEGF and MCP-1 levels in the peritoneal fluid and arterial blood sampling

The VEGF and MCP-1 levels in the peritoneal fluid and arterial blood sampling were quantitatively assessed using commercially available enzyme-linked immunosorbent assay kits (RayBioR Rat VEGF-A ELISA Kit; Bender MedSystems Inc., Burlingame, CA, for MCP-1) according to the instructions of the manufacturer. The enzyme immunoassay has an intra-assay variability of 10% and 6.7% and inter-assay variability of 12% and 11% for VEGF and MCP-1, respectively.

Histopathological examination and VEGF immunohistochemistry

The histopathological examination was performed by a pathologist blinded to the groups. The formalin-fixed endometriotic foci were embedded in paraffin blocks, sectioned at 5 mm thickness (four sections per sample), stained with hematoxylin and eosin, and examined under a light microscope. The persistence of epithelial cells in endometrial implants was evaluated semi-quantitatively as follows (0–1–2–3): 3 was for a well-preserved epithelial layer, 2 was for moderately preserved epithelium with leukocyte infiltrate, 1 was for poorly preserved epithelium (occasional epithelial cells only), and 0 was for no epithelium. This evaluation was based on a previous rat endometriosis study [28]. The expression of VEGF in endometriotic foci was assessed by immunochemistry. Four-micrometer-thick sections from tissue blocks were stained with VEGF (VEGF Ab-1, Rabbit P Ab IgG, Neomarkers, Fremont, CA) by using the standard streptavidin–biotin indirect method. Incubation with the primary antibody was performed for 2 h at room temperature after blocking endogenous peroxides and proteins. 3-Amino-9-ethylcarbazole (AEC) was used as a chromogen. Breast carcinoma was used as the positive control. Negative controls were incubated with PBS instead of the primary antibody. Cytoplasmic staining was considered positive for VEGF. Immunohistochemical staining for VEGF was evaluated using a scoring system described previously [29]. The staining score was determined according to the sum of the intensity of staining (0: no staining, +1: weak staining, +2: moderate staining, and +3: strong staining) and the percentage of cell staining (0: no staining, +1: positive staining in <25% of cells, 2: positive staining in 26–50% of cells, and 3: positive staining in >50% of cells). Scores between 0 and 2 were regarded as negative, scores of 3 and 4 as weakly positive, and scores and 5 and 6 as strongly positive.

Statistical analysis

The statistical analysis was performed using Statistical Package for the Social Sciences version 13.0 (SPSS, Chicago, IL). Normally distributed (Shapiro–Wilk test) parametric variables were tested by analysis of variance (ANOVA) using the least significant difference test for post-hoc analysis. Non-normally distributed metric variables were analyzed by the Kruskal–Wallis test, the Mann–Whitney *U*-test with the post hoc Bonferroni correction, and the Wilcoxon signed rank test. The mean surface areas of the endometriotic implants and VEGF and MCP-1 levels in the same group (before and after the medical treatment) were analyzed by paired sample *t* test, since they were normally distributed. *p* Values of <0.05 were considered statistically significant. Values were expressed as means ± SD.

Results

The mean surface areas of the endometriotic implants decreased from 44.1 ± 5.0 to 7.1 ± 2.3 in Group I and from 46.0 ± 11.1 to 6.3 ± 2.8 in Group II after the treatment ($p < 0.01$, Table 1). The histopathological evaluation of the endometrial implants revealed significant differences between the mean histopathological scores of the implants among the groups (Table 1 and Figure 2). The post-hoc analysis showed that these differences were statistically significant between Group I and the control group and also between Group II and the control group ($p = 0.05$). The mean immunohistochemical staining scores of the endometriotic implants for VEGF were significantly lower in Groups I and II ($p = 0.01$) (Table 2 and Figure 3). There were significant differences between pre-treatment and post-treatment levels of both VEGF and MCP-1 in the peritoneal fluids in Groups I and II

Table 1. Mean surface area and the histopathological scores of the implants among the groups.

Variables	Group I (resveratrol)	Group II (leuprolideacetate)	Group III (control)	*p* Value*
No. of rats	7	8	7	
Mean surface area of implants at second laparotomy before the medication (mm^2) (mean ± SD)	44.1 ± 5.0	46.0 ± 11.1	47.1 ± 6.7	NS
Mean surface area of implants at third laparotomy after medication (mm^2) (mean ± SD)	7.1 ± 2.3*	6.3 ± 2.8*	43.7 ± 6.4	<0.05
p value†	<0.01	<0.01	NS	
Histopathological score of implants at end of treatment	1.28 ± 1.2*	1.25 ± 0.8*	2.57 ± 0.5	<0.05

NS, not statistically significant.
*Statistically significant difference from the control group.
†Statistically significant difference between mean surface area of implants before and after medication.

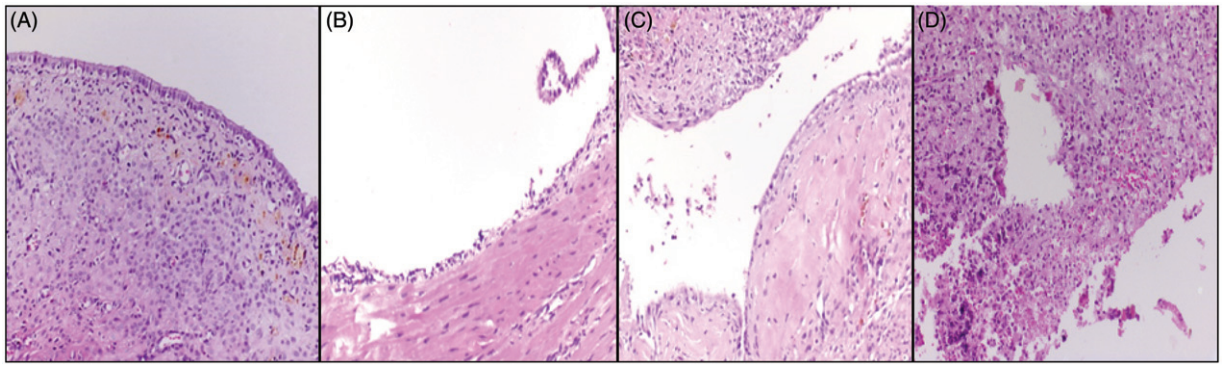

Figure 2. The histopathological scores (A, score 3; B, score 2; C, score 1; D, score 0) of the endometriotic implants.

Table 2. The final staining score of the implants for VEGF among the groups.

Variables	Group I (resveratrol)	Group II (leuprolideacetate)	Group III (control)	*p* Value*
VEGF staining score (mean ± SD)	0.5 ± 0.9*	1 ± 1*	2.5 ± 1.6	=0.01

*Statistically significant difference from the control group.
The staining score = the sum of the intensity of staining (0: no staining, +1: weak staining, +2: moderate staining, and +3: strong staining) and the percentage of cells staining (0: no staining, +1: positive staining in < 25% of cells, +2: positive staining in 26–50% of the cells, and +3: positive staining in>50% of the cells).

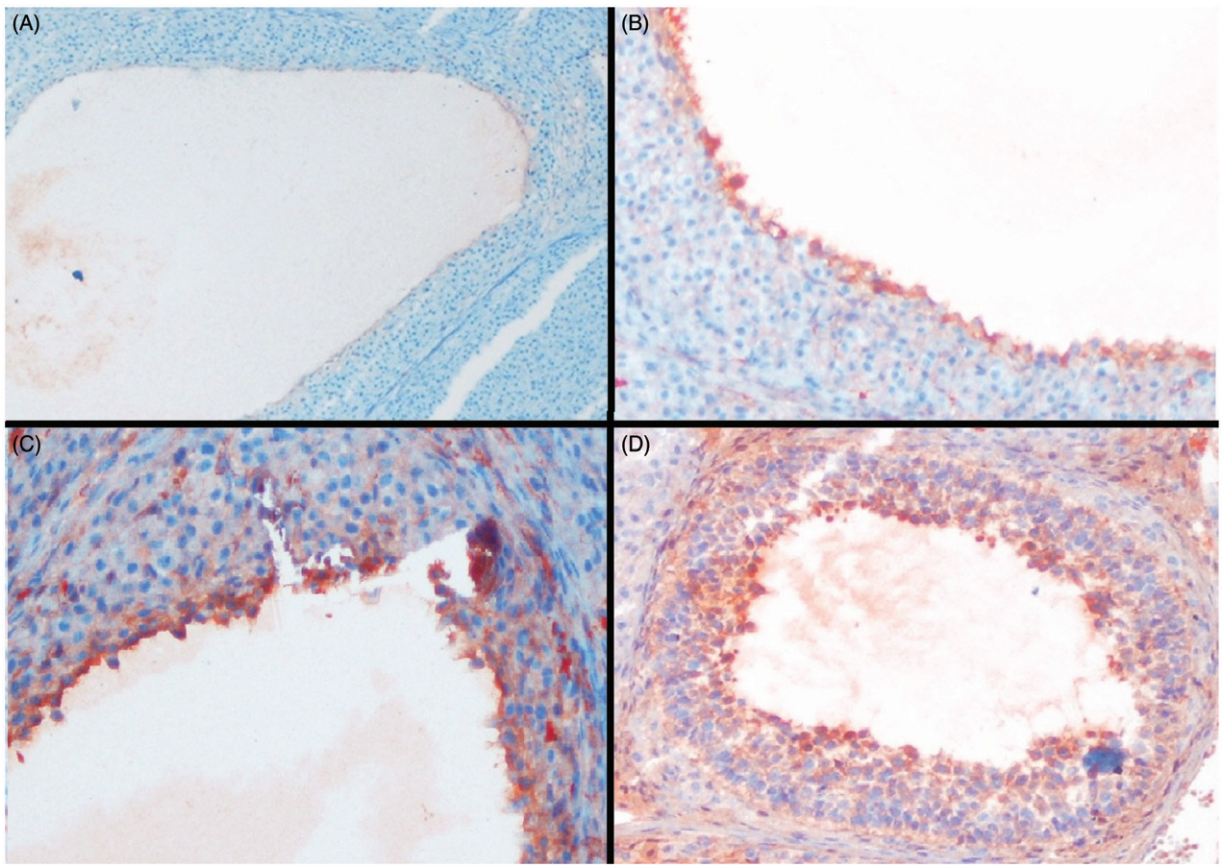

Figure 3. The immunohistochemical staining scores of the endometriotic implants for VEGF (A, score 0; B, score +1; C, score +2; D, score +3).

($p = 0.02$ and $p = 0.03$ for Group I and $p = 0.02$ and $p = 0.02$ for Group II, respectively) (Table 3). There were statistically significant differences between post-treatment VEGF and MCP-1 levels in peritoneal fluids among the groups (Table 3). There were statistically significant differences in post-treatment serum VEGF and MCP-1 between Group I and Group II and the control group for VEGF ($p = 0.05$) and MCP-1 ($p = 0.01$) levels (Table 3).

Table 3. The mean peritoneal fluid, and serum VEGF and MCP-1 levels among the groups.

Variables	Group I (resveratrol), $n=7$	Group II (leuprolide acetate), $n=8$	Group III (control), $n=7$	p Value
M. VEGF in PF pre-Tx (pg/ml) (mean ± SD)	80.95 ± 15.95	69.88 ± 9.64	77.69 ± 9.89	NS
The level of M. VEGF in PF post-treatment (pg/ml) (mean ± SD)	31.36 ± 30.44*	31.64 ± 27.32*	79.48 ± 30.60	=0.03
	$p=0.02$	0.02	NS	
M. MCP-1 in PF pre-Tx (pg/ml) (pg/ml) (mean ± SD)	2.68 ± 1.87	3.74 ± 2.11	5.4 ± 2.5	NS
The level of MCP-1 in peritoneal fluid after treatment (pg/ml) (mean ± SD)	2.07 ± 2.06*	2.00 ± 1.89*	5.17 ± 1.9	=0.05
	$p=0.03$	0.02	NS	
The serum level of VEGF after treatment (pg/ml) (mean ± SD)	4.55 ± 2.102*	4.59 ± 1.66*	10.47 ± 1.78	=0.05
The serum level of MCP-1 after treatment (pg/ml)	106.01 ± 33.8*	80.27 ± 33.60*	224.89 ± 126.66	=0.01†

Tx, treatment; *M*, mean; SD, standard deviation; PF, peritoneal fluid. NS, not statistically significant.
*Statistically significant difference from the control group.
†Statistically significant difference from pretreatment levels

Discussion

Bruner-Tran et al. [25] suggested that the effects of resveratrol to inhibit the development of experimental endometriosis were through increased cell death and decreased proliferation of the endometrial cells. Moreover, Ergenoglu et al. [30] also found significant reductions in implant size, significantly decreased levels of VEGF in the peritoneal fluid and plasma and MCP-1 in the peritoneal fluid, highly significant suppression of VEGF expression in the endometriotic tissue, and considerable histological changes in the endometriotic foci following resveratrol treatment. Likewise, in our study, resveratrol significantly reduced surface areas of the endometriotic lesions, histopathological scores of the endometriotic lesions, VEGF expression in endometriotic implants, and peritoneal fluid and serum levels of VEGF-A and MCP-1 chemokines. We further compared the therapeutic effects of resveratrol with conventional GnRHa treatment and we found that resveratrol was as effective as leuprolide acetate.

It was shown that angiogenesis and alterations in the humoral and cell-mediated inflammatory responses were both linked to the establishment and growth of endometriotic lesions [2,6,10]. It has been shown that resveratrol acts as an anti-angiogenic by the inhibition of VEGF gene expression and synthesis and also inhibits cytokine-induced expression, synthesis, secretion, receptor activity, and chemotactic activity of MCP-1 in various culture tissues and immune cells probably via its anti-inflammatory effects [6,21,23,24,31,32]. Consequently, resveratrol was supposed to change the altered intraperitoneal environment with elevated chemokines inducing inflammatory reactions and angiogenesis based on our and previous study results.

In the recent study, we also found that the use of leuprolide acetate resulted in significant regression in the endometriotic implants and reduction in endometriotic tissue VEGF expression as well as peritoneal fluid VEGF and MCP-1 levels. On one hand, GnRHa was shown *in vivo* to reduce the inflammatory reaction and angiogenesis and to significantly induce apoptosis in tissues derived from women with endometriosis, adenomyosis, and uterine leimyoma [33]. On the other hand, data regarding the direct effects of GnRHa on circulating MCP-1 levels and the expression of MCP-1 in certain tissues are controversial [11,34,35]. The difference between the results of these studies and ours may be explained by the source of the elevated peritoneal and serum MCP-1 levels, which in our study is peritoneal macrophages induced by the altered peritoneal cytokine formation of peritoneal implants. Thus, the regression of the endometriotic implants by the GnRHa therapy lowered the MCP-1 levels indirectly by improving the immunological milieu of the peritoneum.

As the structure of resveratrol has similarities to that of synthetic estrogens, it has been demonstrated to be a phytoestrogen, binding to and activating estrogen receptors. Resveratrol has been shown to have agonist/antagonist effects on estrogen receptors [36–38]. The *in vivo* phytoestrogenic effects of resveratrol on reproductive physiology have been extensively studied before in a rat model [39]. Although resveratrol disrupted estrous cyclicity in gonadally intact female rats as demonstrated by vaginal cytology, it did not demonstrate any estrogenic effects in gonadectomized ones. This finding is in concordance with *in vitro* data in mammary cancer cell lines that resveratrol exerts mixed estrogen agonist/antagonist activities in the absence of estrogen while functioning as an antiestrogen in the presence of estrogen. Direct effects of resveratrol on the growth and functions of granulosa and theca cells other than phytoestrogenic properties via its apoptotic and anti-angiogenic properties might explain in part the effect of resveratrol on ovarian functions. We did not use a gonadectomized rat model as one arm of our study was a GnRH analog group and we wanted to compare the *in vivo* effect of resveratrol on the suppression of endometriotic lesions with a conventional therapy causing hypoestrogenism.

However, there are some limitations of our study. It may also be essential to evaluate the effects of resveratrol in human therapeutic doses on sex steroids, reproductive capacity, and fertility in clinical trials as the phytoestrogenic effects may vary between species. Another limitation of our study was that the effects of only one dose of resveratrol were assessed. The dose of resveratrol and the duration of treatment in a rat endometriosis model should be evaluated using different periods of treatment and different doses of resveratrol in future studies.

In conclusion, resveratrol treatment resulted in a significant regression of experimentally induced endometriotic lesions in a rat model. Furthermore, the effectiveness of resveratrol on the regression of endometriotic lesions was comparable with that observed in the leuprolide acetate group. Further studies are needed to determine the optimum effective dose in humans and to evaluate other effects on reproductive physiology.

Declaration of interest

The authors declare that they have no conflicts of interest.

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