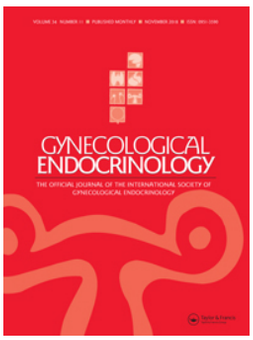

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The beneficial effects of nerolidol and hesperidin on surgically induced endometriosis in a rat model*

Rauf Melekoglu[a], Osman Ciftci[b]‡, Sevil Eraslan[c], Aslı Cetin[d] and Nese Basak[e]

[a]Faculty of Medicine, Department of Obstetrics and Gynecology, University of Inonu, Malatya, Turkey; [b]Faculty of Medicine, Department of Medical Pharmacology, University of Inonu, Malatya, Turkey; [c]Department of Obstetrics and Gynecology, Elbistan State Hospital, Kahramanmaras, Turkey; [d]Faculty of Medicine, Department of Histology, University of Inonu, Malatya, Turkey; [e]Faculty of Pharmacy, Department of Pharmaceutical Toxicology, University of Inonu, Malatya, Turkey

ABSTRACT

The objective of this article is to analyze the effects of nerolidol and hesperidin treatment on surgically induced endometriosis in a rat model. Endometriosis was induced in 24 healthy adult female Wistar albino rats via homologous uterine horn transplantation. Three operations were performed on each rat. After the second operation, the rats were randomized into control, nerolidol, and hesperidin treatment groups, and medications were administered for 2 weeks. The effects of the drugs on the endometriotic foci were evaluated after the third operation. Compared with the endometriosis control group, the average volume of the lesions was significantly lower in rats treated with hesperidin and nerolidol. Malondialdehyde levels were significantly reduced in the nerolidol-treated group, and glutathione levels and superoxide dismutase activity were significantly elevated in the endometriotic foci of both the hesperidin- and nerolidol-treated groups compared with the endometriosis group. Hesperidin and nerolidol treatment also improved histological parameters, such as hemorrhage, vascular congestion, necrosis, and inflammatory cell infiltration in the endometriotic foci. The results of this study demonstrated that treatment with the potent antioxidants nerolidol and hesperidin caused a significant regression of surgically induced endometriotic foci in rats.

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Anti-inflammatory agents; endometriosis; hesperidin; nerolidol; oxidative stress

Introduction

Endometriosis, one of the most common gynecological diseases, is defined by the presence of endometrial stroma and glands outside the uterine cavity [1]. Although the clinical and pathological definition is clear, the etiology and pathophysiology of the disease is not. There is also ongoing debate about the effectiveness of the medical treatment options currently available for endometriosis [2]. A surgically induced endometriosis rat model has been utilized in several studies, and this model offers the opportunity to investigate the effects of new medical therapies on a large group of genetically similar animals at low cost. Additionally, it is suitable for evaluating the mechanisms underlying endometriosis, including the peritoneal attachment of endometrial cells [3,4].

The primary role of free radicals, as signaling molecules mediated by proinflammatory cytokines, in the pathophysiology of endometriosis has been demonstrated [5,6]. Hesperidin is a bioflavonoid that is found in *Citrus* species such as orange and lemon. Nerolidol is a naturally occurring sesquiterpene alcohol that is present in the essential oils of numerous plants with a floral odor. The antioxidant, radical scavenging, and anti-inflammatory effects of hesperidin and nerolidol have been demonstrated in several studies, but no studies have investigated the effects of hesperidin or nerolidol on endometriosis [7,8].

In this study, we investigated the effects of hesperidin and nerolidol treatment in a surgically induced rat endometriosis model by focusing on oxidative and histological changes in endometriotic foci. To our knowledge, this is the first study on the regression of endometriotic lesions due to hesperidin and nerolidol treatment in an experimental animal model of surgically induced endometriosis.

Material and methods

Experiments were performed in accordance with the animal ethics guidelines of the Inonu University Institutional Animal Ethics Committee. A total of 30 healthy adult female Wistar albino rats (aged 3–4 months) were obtained from the Experimental Animal Institute (Malatya, Turkey). Homologous uterine horn transplantation was used to induce endometriosis. Three operations were performed on each rat.

First operation: induction of endometriosis

All surgical interventions were carried out in a controlled laboratory environment under sterile conditions. The endometriosis surgery was performed as reported by Lebovic et al. [9].

A longitudinal incision was made in the uterus to reveal the endometrium. Both uterine horns were excised from the cervix and at a point adjacent to the ovaries. After the parametrial tissues were removed, both uterine horns were separated into two

CONTACT Rauf Melekoglu rmelekoglu@gmail.com The University of Inonu, Faculty of Medicine, Department of Obstetrics and Gynecology, 44280 Malatya, Turkey
*The institute where the work was conducted: The University of Inonu, Faculty of Medicine, The Experimental Animal Institute.
‡Present address: Faculty of Medicine, Department of Medical Pharmacology, University of Pamukkale, Denizli, Turkey.

pieces. Two pieces were sutured to the inner abdominal wall close to the vascular bifurcations by maintaining contact with the endometrial surface and peritoneum. After the procedure, 50 mg/kg cefazolin sodium (daily for 7 days) and 50 mg/kg estradiol depot (twice per week until the second operation) were intramuscularly administered to all of the rats.

Second operation: measurement of the endometriotic foci volumes and randomization of the treatment groups

Two weeks after the inoculation, the second operation was performed (Figure 1). All implants were measured in three dimensions (length, width, and height, in mm). There were no significant volumetric differences among the implants. One of the two implants was removed randomly for histopathological examination. After the second operation, exogenous estrogen administration was discontinued. The rats were randomly divided into three equal groups ($n = 8$ per group), as follows: Group 1, endometriosis (E); Group 2, endometriosis + 100 mg/kg nerolidol (E + N); and Group 3, endometriosis + 50 mg/kg hesperidin (E + H). Nerolidol (100 mg/kg) and hesperidin (50 mg/kg) were orally administered for 14 days by gavage.

Third operation: effects of nerolidol and hesperidin

The animals were euthanized without pain or distress using increasing doses of anesthetic (intraperitoneal lethal doses of pentobarbital; Bioveta, Ankara, Turkey) 14 days after treatment,

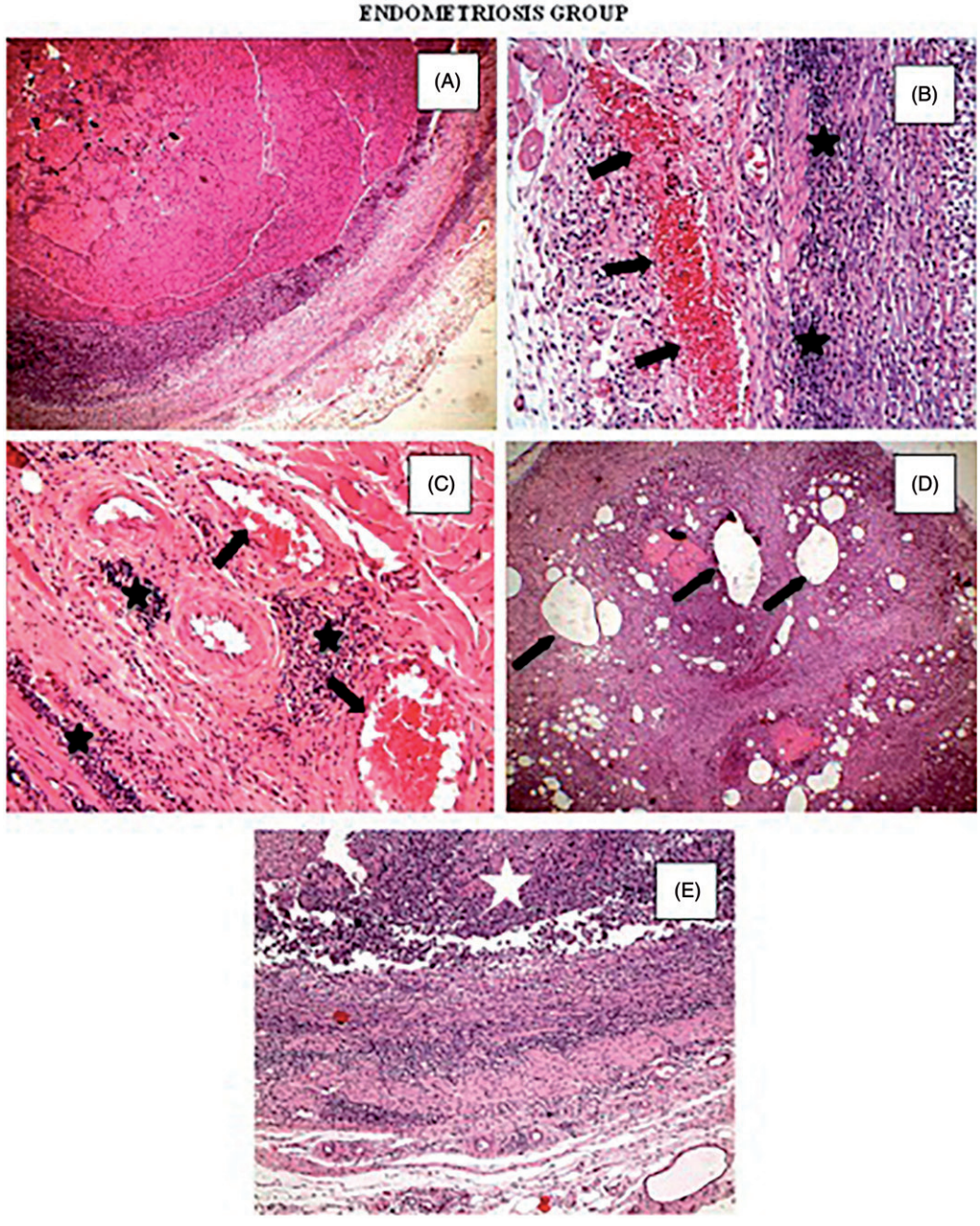

Figure 1. Histopathology of endometriotic lesions in the endometriosis group. Destruction of columnar epithelial cells in the endometrial epithelial layer and glands (A), prominent hemorrhage (B, black arrows), vascular congestion (C, black arrows), necrosis (A, D), inflammatory cell infiltration (B, C, black stars), cystically dilated glands (D, black arrows), and blood cell accumulation in the lumen of endometrial lesions (E, white star). A, D: hematoxylin and eosin staining (H&E), 4× magnification; B, E: H&E, 10×; C: H&E, 20×.

and the endometriotic foci were removed from the abdominal wall. The lesions were measured, and removed for histopathological analysis. Tissue samples were stored at −45 °C until further analysis.

Volume analysis

The volume of each endometriotic focus was calculated using the formula V (mm^3) = 0.52 × length × width × height [10]. The mean volume of the two endometriotic foci was used in comparisons.

Biochemical assays

The tissue homogenates were centrifuged at 18 000 × *g* (4 °C) for 30 min to determine malonaldehyde (MDA) and reduced glutathione (GSH) concentrations, and superoxide dismutase (SOD) and catalase (CAT) activities; and at 25 000 × *g* for 50 min to determine glutathione peroxidase (GSH-Px) activities.

Histopathological examination

Endometriotic foci were fixed in 10% formalin and embedded in paraffin. Paraffin-embedded specimens were cut into 5-µm-thick sections, mounted on slides, and stained with hematoxylin and eosin (H&E). Tissue damage was histopathologically evaluated based on parameters such as destroyed columnar epithelial cells in the endometrial epithelial layer and glands, hemorrhage, vascular congestion, necrosis, inflammatory cell infiltration in endometrial lesion layers, cystically dilated glands, and blood cell accumulation in the lumen of endometrial lesions. At least five microscopic regions were examined to semiquantitatively score the specimens. Each sample was scored according to each criterion using a scale ranging from 0–3 (0, none; 1, mild; 2, moderate; 3, severe).

Statistical analysis

All values are presented as the mean ± standard deviation. Differences were considered significant at $p < .01$. SPSS software (ver. 18.0; SPSS Inc., Chicago, IL, USA) was used for the statistical analyses. The biochemical values were analyzed using one-way analysis of variance (ANOVA) and Tukey's honestly significant difference test was used for *post hoc* comparisons. Histological results were compared using Kruskal–Wallis variance analysis.

Table 1. Mean volume of endometriotic lesions in each group before and after treatment.

Group	Mean volume at end of second week (mm^3)	Mean volume at end of fourth week (mm^3)
Endometriosis	76.6 ± 30.3[a]	81.8 ± 24.9[a]
Endometriosis + N	73.1 ± 28.6[a]	65.7 ± 23.4[b]
Endometriosis + H	70.9 ± 26.7[a]	66.1 ± 30.0[b]

N: nerolidol; H: hesperidin.
Means with different superscripts within the same column were significantly different ($p < .01$).

Results

The mean volume of the endometriotic lesions after the second operation was similar among all samples. The effects of hesperidin and nerolidol were evaluated after the third operation. Compared with the control group, the volume of the lesions was significantly lower in the rats treated with hesperidin and nerolidol. This reduction in lesion volume was more evident in the E + N group than in the E + H group (65.7 ± 23.4 mm^3 and 66.1 ± 30.0 mm^3, respectively) (Table 1).

Biochemical results

The antioxidant (SOD, CAT, GPx, and GSH) and oxidant (MDA) parameters of the endometriotic foci are presented in Table 2. Although there was no significant difference in MDA levels between the E + H and E groups, the MDA level in the E + N group was significantly lower than in the other two groups. GSH levels and SOD activity were significantly higher in both the E + H and E + N groups than in the E group.

Histological results

In the surgically induced endometriosis group, we observed significant changes in endometriotic lesions. Destruction of the endometrium was more evident in the columnar epithelial layer and glands. Prominent hemorrhage, vascular congestion, necrosis, inflammatory cell infiltration, cystically dilated glands and significant blood cell accumulation in the lumen of endometrial lesions were detected in endometrial lesion layers (Figure 1). All histological parameters were significantly improved after hesperidin and nerolidol administration (Figures 2 and 3). Table 3 shows the histopathological scores of the three groups.

Discussion

The medical treatment options currently available for clinical use in endometriosis are unsatisfactory, with no powerful evidence supporting any particular treatment over others [11]. Investigations of new non-hormonal agents for the treatment of endometriosis are ongoing. Because oxidative stress has been considered a potential factor in the pathophysiology of endometriosis, new medical treatment options should be developed to reduce oxidative stress with minimal side effects [12]. In this study, we investigated the effects of hesperidin and nerolidol in a rat model of surgically induced endometriosis. Both hesperidin and nerolidol treatment were associated with decreased endometriotic lesion volume and histological improvements. Yildirim et al. conducted a prospective randomized controlled

Table 2. The levels of oxidative stress markers in endometriotic foci.

Group	MDA (nmol/g tissue)	GSH (nmol/mL)	SOD (U/mg protein)	CAT (kU/mg protein)	GPx (U/mg protein)
Endometriosis	8.25 ± 1.28[a]	39.76 ± 5.52[a]	37.35 ± 6.19[a]	0.0067 ± 0.0005[a]	225.9 ± 64.0[a]
E + nerolidol	6.22 ± 1.04[b]	47.38 ± 4.22[b]	60.13 ± 8.92[b]	0.0072 ± 0.0013[a]	479.1 ± 51.5[a]
E + hesperidin	7.39 ± 1.17[a]	47.85 ± 9.12[b]	53.84 ± 8.70[c]	0.0060 ± 0.0016[a]	452.9 ± 61.8[a]

CAT: catalase; E: endometriosis; GSH: glutathione; GPx: glutathione peroxidase; MDA: malondialdehyde; SOD: superoxide dismutase.
Means with different superscripts within the same column were significantly different ($p < .01$).

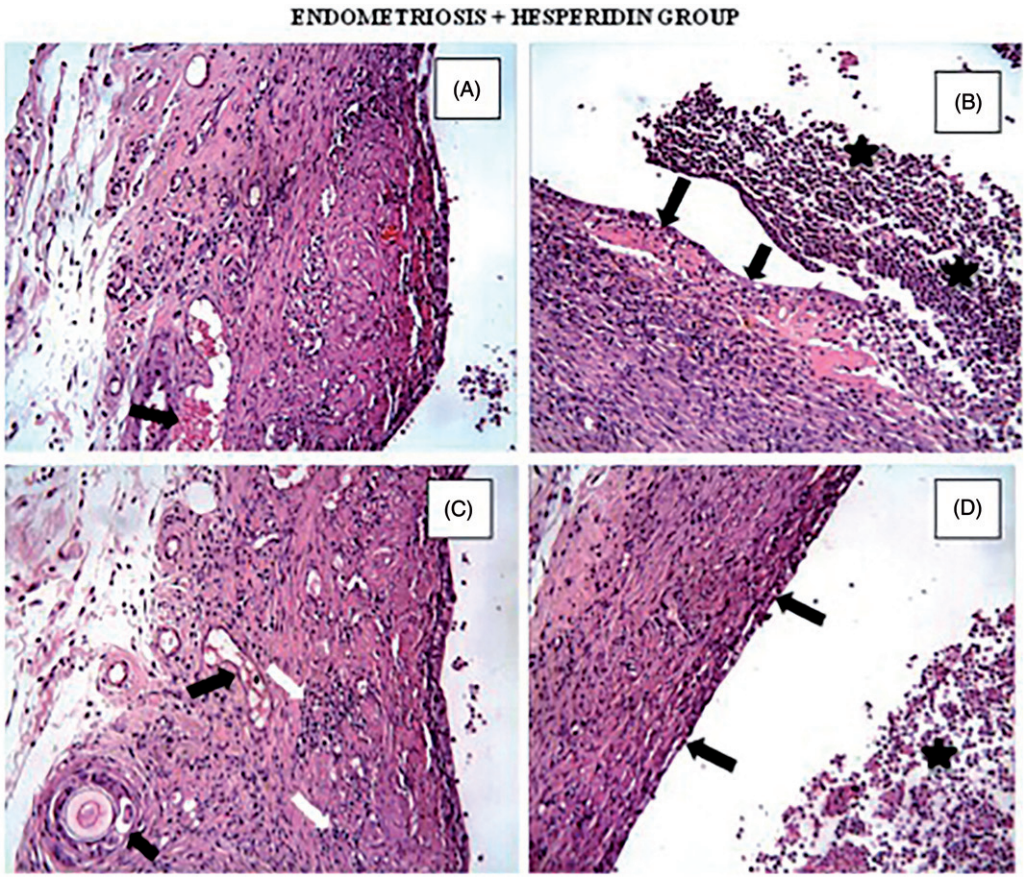

Figure 2. Histopathology of endometriotic lesions in the endometriosis + hesperidin group. Compared with the endometriosis group (Figure 1) there was decreased hemorrhage (A), vascular congestion (A, C, black arrows), necrosis (A, B, C), and inflammatory cell infiltration (C, white arrows) in the lesions. There were fewer blood cells in the lumen (black star), and the endometrial epithelial layer was preserved (B, D, black arrows). A–D: H&E, 20×.

experimental study that demonstrated the effects of melatonin (an antioxidant) in a rat endometriosis model. They showed that regression of endometriotic foci was more common with melatonin treatment than with letrozole treatment [13]. Ertan et al. similarly demonstrated the beneficial effects of vitamin C treatment on the prevention and regression of endometriotic implants in a rat model of endometriosis. They proposed that high dose ascorbic acid suppresses angiogenesis and exerts anti-inflammatory effects by inhibiting the pro-inflammatory nuclear factor kappa B (NF-κB). NF-κB is a major regulatory transcription factor that regulates the expression of pro-inflammatory cytokines. As with vitamin C, the anti-inflammatory effects of hesperidin were shown to be strongly related to the inhibition of NF-κB and the reduction of inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), interleukin (IL)-6, and IL-1β [14]. Additionally, nerolidol was demonstrated to decrease TNF-α and IL-1β levels, and the levels of polymorphonuclear cells [15]. Based on these anti-inflammatory effects, we believe that hesperidin and nerolidol may provide a novel strategy for the prevention and treatment of inflammatory diseases such as endometriosis. Consistently, the results of this study indicated that hesperidin and nerolidol have a remarkable effect on reducing the volume of endometriotic foci and improving the histological alterations caused by endometriosis.

Oxidative stress is characterized by an imbalance between free radicals (such as MDA) and the antioxidant defense system (SOD activity, CAT and GSH levels), which leads to lipid peroxidation and induces oxidative damage. Several studies have demonstrated the role of oxidative stress in the pathophysiology of endometriosis due to a general inflammatory response in the peritoneal cavity [16,17]. Donnez et al. reported that macrophages, red blood cells, apoptotic endometrial tissue, and cellular debris transplanted into the peritoneal cavity by retrograde menstruation are potential inducers of oxidative stress in the pelvic cavities of women with endometriosis. They proposed that the release of hemoglobin containing highly toxic heme and iron from erythrocytes is a primary factor in the development of an inflammatory peritoneal environment [18]. Additionally, the peritoneal levels of MDA and lipid hydroperoxides were significantly higher in women with endometriosis. After supplementation with vitamins C and E, a reduction in MDA was observed in both the serum and peritoneal fluid of affected women with endometriosis [19]. In this study, oxidative stress markers were significantly higher in the endometriosis group, and reduced lipid peroxidation and increased antioxidant activity were observed in the endometriotic foci of rats treated with hesperidin and nerolidol. Consistent with these results, in a recent systematic review, Scutiero et al. reported the role of oxidative stress in the development and progression of endometriosis. They proposed that therapeutic approaches targeting oxidative imbalance may play key roles in the prevention and treatment of endometriosis [20].

In the present study, we showed that hesperidin and nerolidol treatment improved histological parameters, such as hemorrhage, vascular congestion, necrosis, and inflammatory cell infiltration, in endometriotic foci in rats. In the hesperidin group, the histopathological regression of the endometriotic foci was greater than in the nerolidol group. However, the changes in the oxidant and antioxidant levels were not compatible with the histopathological

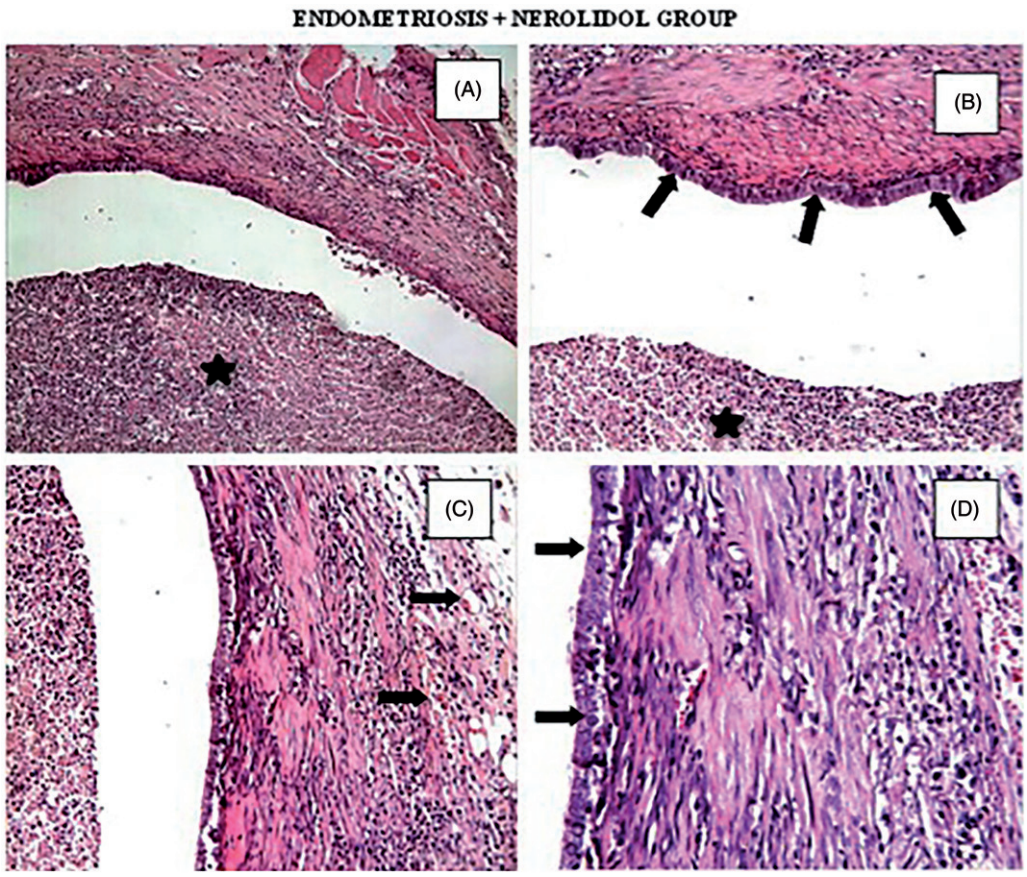

Figure 3. Histopathology of endometriotic lesions in the endometriosis + nerolidol group. Compared with the endometriosis group (Figure 1), there was decreased hemorrhage, vascular congestion (C, black arrows), necrosis (A, B, C) and inflammatory cell infiltration in the lesions, and fewer blood cells in the lumen (A, B, black stars). The endometrial epithelium layer was preserved (B, D, black arrows) and some of the epithelial cells displayed eosinophilic-stained cytoplasm and pyknotic nuclei (B, D). A: H&E, 10×; B, C: H&E, 20×; D: H&E, 40×.

Table 3. Histopathological scores of treatment groups.

Group	Histopathological score (mean ± SD)
Endometriosis	2.23 ± 0.80^{a}
Endometriosis + nerolidol	1.62 ± 0.81^{b}
Endometriosis + hesperidin	1.40 ± 0.67^{b}

Means with different superscripts within the same column were significantly different ($p < .01$).

changes. The pathogenic processes that underlie the development and maintenance of endometriosis are still unclear [21]. The induction and progression of the disease require a proinflammatory environment, increased angiogenesis, resistance to apoptosis, changes in structural and epigenetic elements, and oxidative stress [22]. Our study showed that hesperidin not only acts by decreasing oxidative stress, but probably also affects many of these steps. Uchiide et al. evaluated the detailed morphology of experimental rat endometriosis developed through the autotransplantation of uterine tissues to induce rat endometriosis. They found that secondary attachment of the endometrial epithelium to the peritoneum resulted in the proliferation and infiltration of cells related to allergic inflammation, such as mast cells, eosinophils, plasma cells, and macrophages in the peritoneal stromal tissue. These findings confirmed that the experimental rat endometriosis model is similar to human endometriosis tissue, especially during the first 14 days after autotransplantation [23]. Altinbas et al. evaluated the effectiveness of montelukast treatment in an experimentally induced endometriosis model in rats, and found a significant reduction in leukocyte and macrophage infiltration in the montelukast treatment group. They also demonstrated the protective effects of montelukast on endometrial epithelium due to inhibition of the proinflammatory activity of the leukotriene receptor [24].

Conclusions

Several studies have investigated novel non-hormonal medications for the treatment of endometriosis. Due to the potential role of oxidative stress in the pathophysiology of endometriosis, new therapeutic agents, as effective as hormonal treatments and with minimal side effects, are needed to target and reduce oxidative stress. Hesperidin and nerolidol are powerful antioxidants that show efficacy in this respect. This study demonstrated that the potent antioxidants nerolidol and hesperidin caused a significant regression in surgically induced endometriotic foci in rats. The beneficial effects of hesperidin and nerolidol indicate their potential utility for the treatment of endometriosis and warrant further investigation.

Disclosure statement

No potential conflict of interest was reported by the authors.

ORCID

Rauf Melekoglu http://orcid.org/0000-0001-7113-6691

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