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Ameliorative effects of apigenin on a rat model of endometriosis

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ABSTRACT

Objectives: Apigenin and parthenolide as natural products have potent antioxidant and anti-inflammatory outcomes that could make them a perfect option for endometriosis therapy. This study aimed to determine the effects of apigenin and parthenolide on created endometrial implants in a rat model of endometriosis.

Methods: Thirty-nine mature, female Sprague-Dawley rats were assigned randomly to six experimental groups four weeks after endometriosis induction. Group 1 (n = 5): Control (CTRL) that opened and closed the abdomen; Group 2 (n = 6): Peritoneal and ovarian endometriosis (POE) + drug-free; Group 3 (n = 7): POE+ Apigenin (APG) (50 mg/kg); Group 4 (n = 7): POE+ Parthenolide (PTL) (10 mg/kg); Group 5 (n = 7): POE+ Apigenin (APG) (50 mg/kg) + Parthenolide (PTL) (10 mg/kg); Group 6 (n = 7): POE+ DMSO. An endometriosis model was created, and histopathological analysis and biochemical evaluation were performed. Serum and peritoneal levels of pro-and-anti-inflammatory cytokine, and oxidative stress of implant tissue were measured.

Results: Serum IL-37 levels decreased significantly in the APG-treated group compared to the drug-free group ($p = 0.016$). The peritoneum and ovary endometriosis histopathologic scores were significantly lower in APG-treated ($p = 0.001$) and PTL-treated ($p = 0.001$) groups in comparison to the drug-free group. The oxidative stress index (OSI) values were increased statistically significantly in ovary endometriosis tissue in the drug-free group, ($p = 0.001$). However, compared to the drug-free group, OSI values decreased statistically significantly in the APG-treated group ($p = 0.003$)

Conclusions: The application of apigenin caused a decrease in oxidative stress and an improvement in histopathological grade. Apigenin may be a novel therapeutic agent for the treatment of endometriosis.

Keywords: Apigenin, parthenolide, oxidative stress, endometriosis, IL-6, IL-37

Endometriosis is an inherited condition that is affected by both genetic and environmental factors. It has been suggested that low progesterone levels and hormone imbalances in women with endometriosis

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may be genetic [1]. One of the most persuasive theories, the retrograde menstruation hypothesis indicates that endometrial fragments reach the pelvis via transtubal retrograde flow, implant onto the peritoneum and abdominal organs, and proliferate and cause endometriosis [2]. Due to the uncertain etiology of endometriosis, gonadotropin-releasing hormone (GnRH) agonists, progestin, and androgens are used for therapeutic purposes [3]. Therefore, new molecular studies that prevent or treat endometriosis are needed using therapeutic approaches for treatment.

Plant extracts that possess a broad range of biological activities have been investigated to prevent and treat various health issues, including gynecological disease, due to their anti-inflammatory and anti-oxidative properties [4]. Previous studies have also determined the effects of biological compounds on the restraint of endometriosis [4, 5]. For example, resveratrol and epigallocatechin-3-gallate have been shown to hinder a sharp increase in cell productivity and give rise to apoptosis in both in vitro and in vivo endometriosis models [5]. Rosaceae extracts have been determined to cause decreased cytokine levels and endometrial implants in surgically induced endometriosis [6].

Apigenin (4′, 5,7-trihydroxyflavone), found in many plants, belongs to the flavone class. Studies have shown that Apigenin has better pharmacokinetics and oral bioavailability than other flavonoids. Apigenin has recently attracted attention as a beneficial and health-promoting agent due to its striking effects, which are relatively non-toxic and non-mutagenic against normal cells compared to other flavonoids. It has been shown to have various biological properties such as antioxidant and anti-inflammatory [7]. While parthenolide is a natural product obtained from the shoots of Tanacetum parthenium, which has been shown to have immunomodulatory effects in several diseases [8]. In some studies, it has been suggested that parthenolide also shows anti-inflammatory and antioxidant activity [9].

There is an amount of evidence that inflammation and immune responses play a crucial role in the pathogenesis of endometriosis [10]. Especially, the cytokines – interleukin (IL)-1beta (IL-1β), 6, 10, and tumor necrosis factor-alpha (TNF-α), have been implicated in the pathogenesis of endometriosis [11]. Recent studies have proven that increased levels of IL-1β and TNF-α stimulate the production of IL-6 by peritoneal mesothelial cells, and as a result, it may cause local inflammation in endometriosis [12, 13]. In addition, it has been found that serum and peritoneal fluid IL-37 levels are significantly elevated in patients with endometriosis and are associated with the stage of endometriosis [14].

Furthermore, among the hypotheses put forward to explain the pathophysiology of endometriosis, oxidative stress is one of the leading theories. There is a body of evidence from animal and human studies that oxidative stress is one of the main processes in the pathogenesis of endometriosis [15].

Apigenin and Parthenolide are therapeutic agents with few side effects since they do not act directly hormonally. The effect of apigenin and parthenolide on pro-and anti-inflammatory cytokine secretion and oxidative stress in the pathogenesis of peritoneal and ovarian endometriosis has not been studied in-vivo yet.

In studies conducted to date, experimental peritoneal and ovarian endometriosis models have been created in rats, and many drugs' effects on endometriosis have been determined. The present study aimed to investigate the treatment effects of apigenin and parthenolide, which have not been used in endometriosis studies before and have proven potent anti-inflammatory and antioxidant activity, on endometriosis models in rats.

METHODS

The rats were obtained from the University of Health Sciences Animal Research Laboratory. In this study, 39 adult non-pregnant Wistar-Albino female rats weighing 145-208 g were used. Rats were housed under standard care and feeding conditions. All experimental procedures and protocols performed in this study were ethically approved by the Health Sciences University Hamidiye Animal Experiments Local Ethics Committee (Istanbul, Turkey) (No: 2020-01/01). All experimental animal procedures were performed following standard ethical guidelines.

Rats were randomly selected and divided into six groups (group 1 to group 6). All rats underwent laparotomy in the estrus phase. Only in the control group (n = 5) was the abdomen opened and closed. Experimen-

tal groups were listed below.

Group 1 (n = 5): Control (CTRL) group that opened and closed the abdomen. Group 2 (n = 6): Peritoneal and ovarian endometriosis (POE) + drug-free group. Group 3 (n = 7): POE+ Apigenin (APG) (50 mg/kg). Group 4 (n = 7): POE+ Parthenolide (PTL) (10 mg/kg). Group 5 (n = 7): POE+ Apigenin (APG) (50 mg/kg) + Parthenolide (PTL) (10 mg/kg). Group 6 (n = 7): POE+ Dimethyl sulfoxide (DMSO).

Establishment of the Endometriosis Model

The peritoneal area of the rats of the CTRL group was opened 3-4 cm in length after anesthesia and was sutured with 6/0 vicryl immediately afterward. In 34 rats other than the CTRL group, the right uterine horn was opened, turned inside out, and it was sutured in the right pelvic peritoneum of the rat uterus to create peritoneal endometriosis, and the left uterine horn was opened, turned inside out, and sutured to the left ovarian surface to form ovarian endometriosis.

In 34 rats (groups 2 to 6) with peritoneal and ovarian endometriosis, laparotomy was performed again 29 days later to detect the occurrence of endometriosis. Then, rats were rested for 3 days, and on the 3rd day (31st day of the experiment), APG (50 mg/kg) was given to POE + APG group, PTL (10 mg/kg) was given to POE + PTL group, and APG (50 mg/kg) + PTL (10 mg/kg) were given to POE + APG + PTL group. Intraperitoneal injection (IP) was applied five days a week for a month. POE + DMSO group was administered DMSO, IP in the concentration and volume in which we dissolved the apigenin and parthenolide herbal medicine five days a week for a month.
On 59 the day of the experiment, laparotomy was performed again on all rats. Rats were euthanized by cervical dislocation following intracardial blood collection (approximately 4 ml) under anesthesia. Peritoneal and ovarian endometriosis tissue was taken for clinical parameters and histopathological evaluation. In addition, the results of Hematoxylin and Eosin (H&E) staining were evaluated for gland-stroma structure in endometriosis foci and surface epithelial changes in the left ovary.

Tissue Procedures

Peritoneal and ovarian endometriosis tissue was divided into two, half of them for oxidative stress analysis, and another half of them for histopathological analysis. Tissues were weighed, homogenized with 1/5 (w/v) cold (1.15%) KCl solution in a homogenizer (Qiagen Tissue Layser LT) and centrifuged at 10,000 g for half an hour to separate the supernatants +4ºC. Protein measurements from tissues were analyzed colorimetrically by the Bradford method [16]. This method is based on the protein binding of Coomassie Brilliant Blue G250 dye (Sigma-Aldrich, Steinham, Germany). Bovine serum albumin (BSA) was used as a standard (Sigma-Aldrich, Steinham, Germany). 150 µL of Coomassie Brilliant Blue G250 were mixed with 10 µL of sample and then incubated for 10 minutes at room temperature with a shaker. The absorbance at 595 nm was measured (Synergy-HTX, Biotek, USA). The protein concentrations of the samples were calculated according to the standard protein curve.

Oxidative Stress Analyses

Peritoneal and ovarian endometriosis tissue samples were analyzed spectrophotometrically in the multi-plate reader (Synergy-HTX, Biotek, USA) for oxidative stress parameters. TAS and TOS were measured using commercially available kits. In agreement with the standard curve, TAS levels were presented as mmol ascorbic acid Eq/mL/mg protein and TOS levels were computed as µmol H2O2 Eq/mL mg protein. The oxidative stress index (OSI) was calculated as TOS/TAS.

Inflammation Biomarkers

During the last laparotomy surgery, 1 ml of PBS was injected into the abdominal cavity and allowed the PBS to spread all over the abdominal cavity. Then the PBS was withdrawn and used as the peritoneal fluid. IL-1β, IL-6, IL-10, TNF-α, and IL-37 cytokines, which are inflammation biomarkers were investigated in serum and peritoneal fluid. Cytokine levels were measured spectrophotometrically with ELISA kits (Abcam) in a multi-plate reader (Synergy-HTX, Biotek, USA) according to manufacturers' instructions.

Histopathological Investigation

Formalin-fixed, paraffin-embedded tissue samples were cut with 4 µm-thick and stained with H&E. A

semi-quantitative evaluation of all samples was performed in the ovary and peritoneum endometriosis tissue. Score 3: Well-protected endometrial glandular epithelium; Score 2: Moderately spilled endometrial glandular epithelium; Score 1: Highly spilled endometrial glandular epithelium; Score 0: No endometrial glandular epithelium left [17]. The histopathological analysis was conducted by a pathologist, who do not know each test group.

Statistical Analysis

IBM SPSS 21 statistical software was applied to analyze the data. The distribution of variables was evaluated using the Homogeneity of variance test. The difference between the groups was analyzed with the Kruskal-Wallis H test or the one-way ANOVA test. If there was a difference, compared furtherly in pairs using the Games-Howell test, or Tukey test, one of the post-hoc methods.

RESULTS

Oxidative stress-related biochemical parameters in peritoneal tissue and ovary

Oxidative stress biochemical parameters such as TAS, TOS, and OSI were measured. The Mean ± SD values achieved from the peritoneal and ovary endometriosis tissue are shown in Table 1.

Parameters of TAS, TOS, and OSI in peritoneum endometriosis tissue between groups

OSI values were increased in peritoneal endometriosis tissue in the drug-free group compared to the control group and were decreased compared to the other treatment groups. However, there were no significant differences identified between the groups (Table 1, Fig. 1).

Parameters of TAS, TOS, and OSI in ovary endometriosis tissue between groups

When compared with the control group, OSI values were increased statistically significantly in ovary endometriosis tissue in the drug-free group, (p = 0.001). Meanwhile, compared to the drug-free group, OSI values decreased statistically significantly in the APG-treated group (p = 0.003) (Table 2) (Fig. 1).

IL-1β, IL-6, IL-10, TNF-α, and IL-37 cytokines production in apigenin and parthenolide-treated endometriosis rat model

ELISA evaluated all serum samples to ascertain IL-1β, IL-6, IL-10, TNF-α, and IL-37 cytokines. IL-37 was quantified in all the analyzed samples and its serum concentration was significantly decreased in the POE+ APG group when compared to POE + drug-free (p = 0.016) group. However, no significant differences were identified in the production of IL-1β, IL-6, IL-10, and TNF-α between groups.

Peritoneal fluid samples for detecting IL-1β, IL-6, IL-10, TNF-α, and IL-37 cytokines were also evaluated. However, there were no significant differences

Table 1. TAS, TOS, and OSI biochemical parameters obtained from peritoneal endometriosis tissue

Groups	TAS (mmol Ascorbic acid Eq/L/mg protein)	TOS (μmol H_2O_2/L/mg protein)	OSI (AU)
CTRL	0.25 ± 0.04	0.38 ± 0.16	1.24 ± 0.60
POE + drug-free	0.15 ± 0.12	1.49 ± 1.11	6.49 ± 1.08
POE + APG	0.24 ± 0.05	0.98 ± 0.42	3.34 ± 1.89
POE + PTL	0.43 ± 0.18	0.73 ± 0.44	1.76 ± 1.48
POE + APG+ PTL	0.27 ± 0.02	1.69 ± 0.94	5.47 ± 3.35
POE + DMSO	0.26 ± 0.08	1.36 ± 0.36	4.40 ± 1.33

All values are expressed as mean ± SD. APG = apigenin, CTRL = control, DMSO = dimethyl sulfoxide, OSI = oxidative stress index, POE = peritoneal and ovarian endometriosis, PTL = parthenolide, TAS = total antioxidant status, TOS = total oxidant status

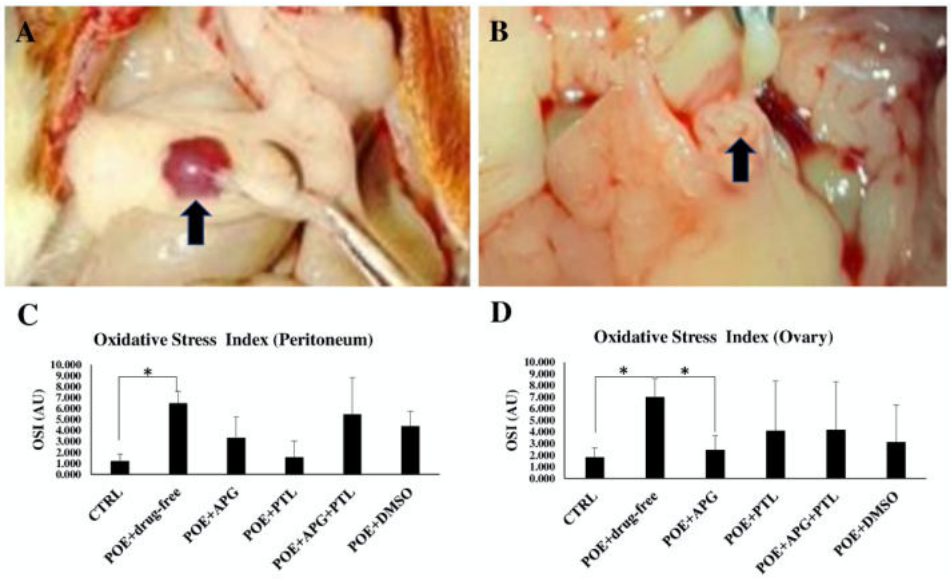

Fig. 1. The gross appearance of endometriotic lesion: pre-treatment enlarged cystic lesion (A), and the atrophic endometriotic lesion after treatment with APG (B). The oxidative stress index was evaluated by TAS, and TOS and analyzed spectrophotometrically. (OSI) for peritoneal endometriotic implants for all experimental groups (C), and ovarian endometriotic implants for all experimental groups (D).
Statistical comparisons were made using a Kruskal-Wallis H test with further Mann-Whitney U tests. A two-sided p-value of less than 0.05 was considered statistically significant. *$p < 0.05$. APG = apigenin, CTRL = control, DMSO = dimethyl sulfoxide, OSI = oxidative stress index, POE = peritoneal and ovarian endometriosis, PTL = parthenolide, TAS = total antioxidant status, TOS = total oxidant status

in the production of IL-1β, IL-6, IL-10, TNF-α, and IL-37 between groups.

Histopathological changes

We evaluated endometriosis foci in ovarian and peritoneal tissues with H&E staining. Histopathologically, grading evaluating the endometriosis that occurs in the peritoneum and ovary is given in Table 3. There was a significant difference between the groups in terms of preserved epithelium and inflammation in

Table 2. TAS, TOS, and OSI biochemical parameters obtained from the ovary endometriosis tissue

Groups	TAS (mmol Ascorbic acid Eq/L/mg protein)	TOS (μmol H_2O_2/L/mg protein)	OSI (AU)
CTRL	0.20 ± 0.03	0.41 ± 0.19	1.83 ± 0.81
POE+ drug-free	0.14 ± 0.03	1.22 ± 0.15	7.01 ± 1.58‡
POE+ APG	0.29 ± 0.07	0.96 ± 0.51	2.45 ± 1.25*
POE+ PTL	0.24 ± 0.05	1.33 ± 1.53	4.11 ± 4.30
POE+ APG+ PTL	0.20 ± 0.05	0.97 ± 0.38	4.17 ± 4.16
POE+ DMSO	0.21 ± 0.07	0.84 ± 0.19	3.13 ± 3.20

All values are expressed as mean ± SD. APG = apigenin, CTRL = control, DMSO = dimethyl sulfoxide, OSI = oxidative stress index, POE = peritoneal and ovarian endometriosis, PTL = parthenolide, TAS = total antioxidant status, TOS = total oxidant status

*$p = 0.003$ POE+APG versus POE+drug-free; ‡$p = 0.001$ POE+drug-free versus CTRL.

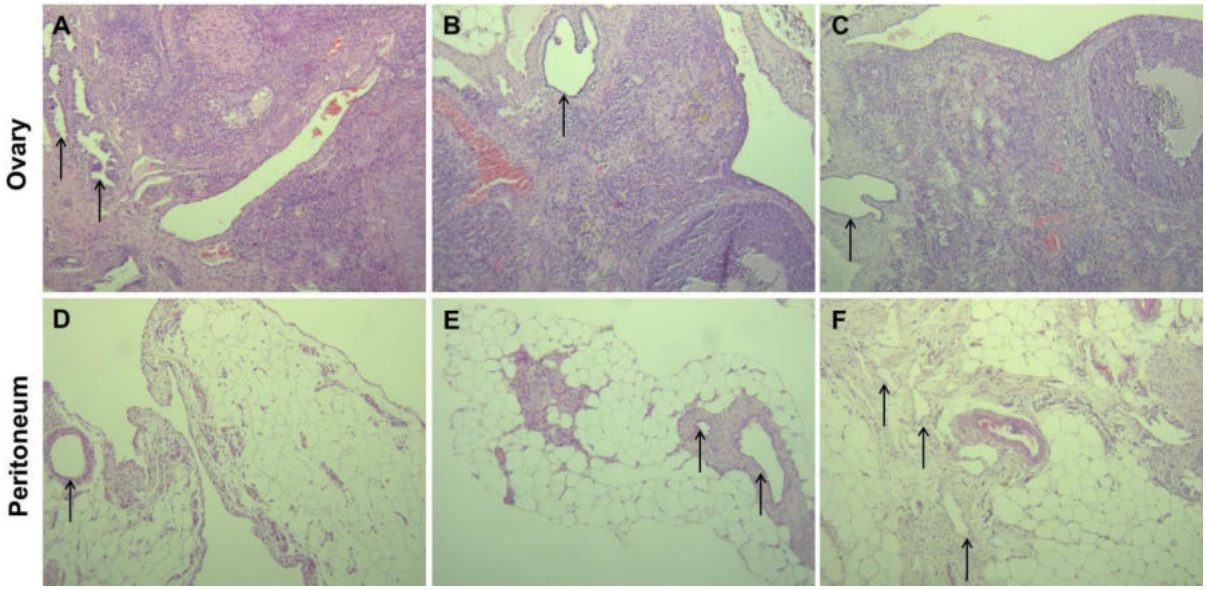

Fig. 2. Effect of APG or PTL treatment on the deterioration of the epithelial layer in endometriotic implants. The hematoxylin and eosin (H&E) images show representative histological sections of induced endometriotic implants in the ovary in the rat POE+drug-free group (A) and APG-treated rat POE+APG group (B), and PTL-treated rat POE+PTL group (C). In the peritoneum in rat POE+drug-free group (D) and APG-treated rat POE+APG group (E), and PTL-treated rat POE+PTL group (F). Arrows indicate endometrial glands. Magnification: 200×.

the peritoneum and ovary ($p < 0.001$) (Figs. 2 and 3).

DISCUSSION

Although many studies have been performed to better understand and explain the exact cause of endometriosis, the pathogenesis of endometriosis is still controversial and thus treatment options are not successful. In addition to modern treatment methods, some therapies have focused on oxidative stress in endometrial implants, and it has been proven that antioxidants can hamper endometriosis. In our rat models, apigenin and parthenolide, as strong antioxidant and anti-inflammatory herbal agents, were used to treat endometriosis.

Table 3. Endometriosis grading obtained from the peritoneum and ovary

Groups	Peritoneum	Ovary
POE+ drug-free	3.00 ± 0.00	3.00 ± 0.00
POE+ APG	0.33 ± 0.52*	0.50 ± 0.55*
POE+ PTL	0.33 ± 0.52‡	0.67 ± 0.52‡
POE+ APG+ PTL	2.20 ± 0.45	2.75 ± 0.50
POE+ DMSO	3.00 ± 0.00	3.00 ± 0.00

All values are expressed as mean ± SD. APG = apigenin, DMSO = dimethyl sulfoxide, POE = peritoneal and ovarian endometriosis, PTL = parthenolide

*p = 0.001 POE+APG versus POE+drug-free; ‡p = 0.001 POE+PTL versus POE+drug-free.

Iwabuchi *et al*. [18] has revealed that the inauguration of oxidative stress in endometriosis might be associated with the presence of degenerated endometrial tissue, and cell debris may have been transported to the abdominal cavity by menstrual reflux. In the present study, ovary endometrial implants showed significantly higher oxidative stress levels. These findings are in line with the results of studies showing that oxidative stress and inflammation have a pivotal function in the pathogenesis of endometriosis [17]. There may be a cause-result relationship between boosted oxidative stress and the pathogenesis of endometriosis [19]. It is well known that the disproportion between reactive oxygen species and antioxidants is associated with endometriosis [20]. We hypothesized in the current study that targeting oxidative stress probably results in a reduction in the histopathological grade of endometrial implants. In our study, the application of apigenin leads to the alleviation of lesional endometrial tissue in the rat ovary. This could be the mecha-

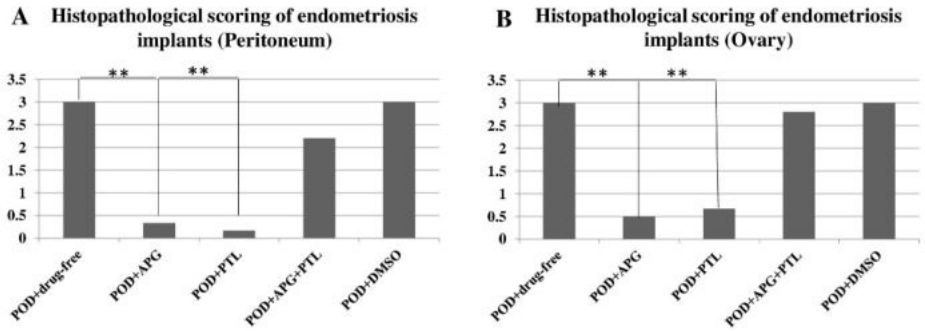

Fig. 3. Comparison of histopathological evaluations between groups. Histopathological scores were applied (stain: hematoxylin and eosin, H&E) Score 3: Well-protected endometrial glandular epithelium; Score 2: Moderately spilled endometrial glandular epithelium with inflammation; Score 1: Highly spilled endometrial glandular epithelium; Score 0: No endometrial glandular epithelium.
Statistical analysis was performed using a Kruskal-Wallis H test with further post-hoc Games-Howell tests. **$p = 0.001$. APG = apigenin, DMSO = dimethyl sulfoxide, POE = peritoneal and ovarian endometriosis, PTL = parthenolide

nism here that apigenin generates ROS-dependent apoptosis and endoplasmic reticulum stretching in endometriosis cells [21]. While Parthenolide inhibits cell proliferation in human endometriotic stromal cells, diminishes prostaglandin estradiol synthesis, and hinders the development of endometriosis in the murine model [22]. However, in the present study, histopathological grade and oxidative stress index could not match after the application of parthenolide treatment. Moreover, in the current study, there was no significant increase in serum levels of IL-1β, TNF-α, and IL-6 in the drug-free model group when compared to the CTRL group. Meanwhile, there was no remarkable decrease in cytokine IL-1β, TNF-α, and IL-6 in the APG and PTL-treated group after administration of apigenin and parthenolide. These findings are inconsistent with other studies which have reported that apigenin and parthenolide inhibit IL-1β, TNF-α, and IL-6 expression [23, 24]. This is probably due to the different degrees of inflammation in endometriosis foci, different doses of apigenin and/or parthenolide used, and the different types of experimental animals used in the studies. In our study, we found less inflammation in either ovary endometrial implants or peritoneal endometrial implants in contrast to the control. This is probably the reason why there are no significant differences identified between groups regarding pro-inflammatory cytokine production in our endometriosis model.

Differential expressions of anti-inflammatory cytokines and their roles in the pathogenesis of endometriosis are gradually emerging. Considering its immunological background, studies have shown that endometriosis is not only an inflammatory disorder but also a disease in which pro-and anti-inflammatory mechanisms work [25]. The current study analyzed the level of IL-10 and IL-37 in serum and peritoneal fluid with a rat model of endometriosis. We found that serum IL-37 levels were significantly lower than those measured in the drug-free group after treatment with APG; this suggests that APG may have inhibited immune responses. Based on present results, APG may play an anti-inflammatory role in endometriosis by regulating immune inflammation.

CONCLUSION

Apigenin application leads to a decreased histopathologic score of the induced endometrial implants and a depletion of oxidative stress in the rat model. Oxidative stress and inflammation are closely linked and have been suggested to play a role in the etiology of endometriosis. Apigenin can be a good target in the treatment of endometriosis.

Authors' Contribution

Study Conception: GH, SA, AD, KS, MT, NS, EMG, FM, HTC, HHP, TS, EK; Study Design: GH, SA, AD, KS, MT, NS, EMG, FM, HTC, HHP, TS, EK; Supervision: GH, SA, AD, KS, MT, NS, EMG, FM, HTC, HHP, TS, EK; Funding: GH, HTC, EK; Materials: GH, HTC, EK; Data Collection and/or Processing: GH, SA, AD, KS, MT, NS, EMG, FM, HTC, HHP,

TS; Statistical Analysis and/or Data Interpretation: GH, SA, AD, KS, MT, EK, NS, EMG, FM, HTC, HHP, TS; Literature Review: GH; Manuscript Preparation: GH and Critical Review: GH.

Conflict of interest

The authors disclosed no conflict of interest during the preparation or publication of this manuscript.

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