Archives of Medical Research 56 (2025) 103258

ORIGINAL ARTICLE

# Efficacy of Cyclosporin A and Tacrolimus in the Treatment of Endometriosis of Rats

Cagla Bahar Bulbul,[a] Gulay Turan,[b] Ceyda Sancakli Usta,[a] Ozgur Bulmus,[c] and Akin Usta[d]

[a] *Department of Obstetrics and Gynecology, Balikesir Ataturk City and Research Hospital, Balikesir, Turkey*
[b] *Department of Pathology, Balikesir University, School of Medicine, Balikesir, Turkey*
[c] *Department of Physiology, Balikesir University, School of Medicine, Balikesir, Turkey*
[d] *Department of Obstetrics and Gynecology, Balikesir University, School of Medicine, Balikesir, Turkey*

Received for publication September 16, 2024; accepted June 11, 2025 (ARCMED-D-24-00822).

*Background and Aims.* The molecular and cellular mechanisms underlying endometriosis are still under investigation. Cyclophilin A (CypA) is an inflammatory marker secreted by various types of cells in an inflammatory condition. During inflammation, CypA exacerbates the inflammatory response by activating calcineurin signaling, which increases cytokine secretion and tissue degradation in the inflammatory region. This study investigated the effect of inhibiting calcineurin signaling in treating endometriosis in rats.

*Methods.* Thirty-two albino Wistar rats were used in this study. All rats were divided into three groups: cyclosporin A ($n = 10$), tacrolimus ($n = 10$) and a control group ($n = 12$). The cyclosporin A (CsA) group received two intraperitoneal doses two weeks apart, and the tacrolimus group received the same two doses intravenously, also two weeks apart. All studies lasted eight weeks. The processed endometrial tissues were cut in half and embedded in paraffin. Histological sections (5 µm) were stained with Ki-67, Bcl-2, caspase-3 and VEGF.

*Results.* The endometriotic focus size was 204.7 ± 153.4 mm$^3$, 71.9 ± 85.4 mm$^3$, and 30.6 ± 36.7 mm$^3$ in the control, CsA, and tacrolimus groups, respectively. Compared to the control group, the endometriotic focus size was smaller in the CsA and tacrolimus groups ($p = 0.002$). Microscopically, Ki-67 ($p = 0.010$) and VEGF ($p = 0.007$) immunoreactivity were lower in the CsA and tacrolimus groups than in controls.

*Conclusions.* The inhibition of calcineurin signaling with CsA or tacrolimus treatment causes regression of the endometriotic focus by decreasing endometriotic cell proliferation and angiogenesis in ectopic endometriotic tissue. © 2025 Instituto Mexicano del Seguro Social (IMSS). Published by Elsevier Inc. All rights are reserved, including those for text and data mining, AI training, and similar technologies.

*Key Words:* Endometriosis, Cyclophilin a, Cyclosporin a, Tacrolimus, Rat, Calcineurin.

## Introduction

The endometrium is the inner lining of the uterus, composed of two layers: the functional layer and the basal layer. The endometrium undergoes cyclic changes during each menstrual cycle. The epithelial layer and the stroma cells that make up the endometrium form a layered structure, with a basal layer between them (1). Thus, cytokines remain in contact with the stromal cells and can regulate apoptosis and regulation. Disruption of this physiology can lead to endometrial pathologies such as adenomyosis or endometriosis.

Endometriosis is defined as the presence and growth of endometrial-like tissue outside of the uterine cavity (2). While some patients may be asymptomatic, others experience symptoms such as dyspareunia, dysmenorrhea,

Corresponding author: Cagla Bahar Bulbul, Balikesir Ataturk City and Research Hospital, Dept. of Obstetrics and Gynecology, Gaziosmanpasa 206, 10100, Balikesir, Turkey; Phone: (+90) 5063240528; E-mail: drcaglahanedar@gmail.com

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https://doi.org/10.1016/j.arcmed.2025.103258

bladder/bowel symptoms, chronic pelvic pain, and infertility.(3) Due to the difficulty of diagnosing endometriosis, diagnosis is often delayed, prolonging the time it takes to start treatment. Its prevalence is approximately 5–10% among women of reproductive age worldwide.

Several theories have been suggested in the literature to explain the development of endometriosis (4,5), although the molecular and cellular mechanisms underlying the condition are not yet fully understood. As a consequence, there is currently no consensus on the most appropriate medical and/or surgical approaches for the treatment of endometriosis (2,6).

Cyclophilin A (CypA) is a member of the immunophilin family of proteins that are secreted by various cells in response to inflammatory stimuli, such as hypoxia, oxidative stress, and infection. Previous studies have demonstrated increased tissue expression and/or higher blood levels of CypA in patients with inflammatory diseases, including asthma, rheumatoid arthritis, cardiovascular diseases, and various cancers (7–13). In inflammatory conditions, CypA plays a role in inflammatory cell migration and cytokine production. It also causes tissue destruction and cell invasion by increasing the secretion of matrix metalloproteinases (MMPs) in the inflammatory areas (14). The effects of CypA are mediated by the activation of calcineurin signaling (14).

Calcineurin, which is a $Ca^{+2}$-dependent serine/threonine protein kinase that plays an important role in the inflammatory response and implantation (15). It also plays a central role in the adaptive immune response by activating the nuclear factor of activated T cells (NFAT) and the nuclear factor kappa beta (NF-$\kappa$B) transcription factors in several cell types (16). The known mechanism of the calcineurin signaling pathway, which causes the production and releasing of proinflammatory cytokine and chemokine including TNF-$\alpha$, and IL-6 by activating NF-$\kappa$B. Cyclosporine A (CsA) and tacrolimus (FK506) are calcineurin inhibitors that act by a similar mechanism. The pharmacological effects of both drugs are based on the inhibition of calcineurin by binding to CypA, especially in immune effector cells (15).

In previous studies, we demonstrated increased CypA expression in eutopic and ectopic endometrial cells of patients with endometriosis (17,18). Moreover, we found CypA expression to be correlated with vascularity and endometrioma recurrence. Previous studies on cancer cell lines and culture media have shown that CypA deficiency or CsA (or tacrolimus) treatment decrease tumor growth and increase the efficacy of chemotherapeutic agents in relation to the treatment of glioblastoma multiforme and endometrial cancer (19,20).

Given the anti-inflammatory and immunomodulatory properties of CsA and tacrolimus, we investigated their added benefit in the treatment of endometriosis using an experimental rat model.

## Materials and Methods

### *Animals*

A total of 36 female nonpregnant, 10–12 week-old Wistar albino rats, weighing 250–300 g were used for the induction of the experimental model of endometriosis. All rats were housed at a room temperature of 20 ± 2°C during the adaptation and experimental procedure. The room had a 12 h light/ dark cycle and 50 ± 10% humidity, and the rats had access to food and water ad libitum. The animals were randomly classified into three groups: a control group, a cyclosporine A group, and a tacrolimus group. Before surgical induction of endometriosis, the rats underwent daily vaginal lavages to detect the phase of the estrous cycle. Vaginal secretions were examined under a light microscope to identify the estrous cycle by the dominance of the anucleate cornified cells. The estrous stage was monitored daily by vaginal lavage 2 h after the lights were turned on, beginning at least 2 weeks before surgery and continuing until the day of death. Only rats with regular 4 d cycles both before and after surgery were used.

### *Surgical Procedure*

Experimental endometriosis was induced by removing the right uterine horn, and trimming a piece of the tissue with microscissors. The removed endometrial tissue fragment was sutured into the abdominal wall of the same rat. One month after the initial surgery, a second procedure was performed under anesthesia to confirm the presence of endometrial foci. During the procedure, the endometriosis foci was measured and recorded. After the procedure, all rats were divided into three groups: CsA ($n$ = 10), tacrolimus ($n$ = 10) and control group ($n$ = 12). The CsA group was administered 5 mg/kg intraperitoneally, and the tacrolimus group was administered 0,3 mg/kg intravenously. Each group received two doses at 2 weeks intervals. The control group received a saline infusion at 50 mg/kg by intraperitoneal injection at the same intervals. All studies lasted for eight weeks. At the end of the treatment period, all rats were sacrificed, and the eutopic and ectopic endometrial tissues were removed.

### *Tissue Evaluation*

After 28 d of medication, the rats in the experimental group were euthanized with 10% ketamine and 2% xylazine, and a laparotomy was performed. The ectopic endometrial tissue was isolated and its three dimensions (length, width, and height) were measured using a digital meter. The macroscopic volume of the tissue was calculated with the prolate ellipsoid formula, described elsewhere (21). For histopathological and immunohistochemical evaluations, all endometriotic tissue samples were fixed in 10% formalin followed by an overnight

soak in phosphate-buffered saline. The samples were then stored in 70% EtOH until processing. The processed endometrial tissues were cut in the middle and embedded into paraffin. Histological sections (5 µm) were stained with Ki-67 (anti-human Ki-67 antibody, Cat. No. A0047 [1:300], Dako, California, USA), Bcl-2 (Bcl-2 antibody [C-2], Cat. No: sc-7382 [1:250], mouse polyclonal, Santa Cruz Biotechnology, California, USA), Caspase-3 (Anti-caspase-3 antibody, Cat. No. 200270-T08 [1:500], rabbit polyclonal, Sino Biological, Houston, TX, USA) and VEGF (Anti-VEGF183/VEGFA antibody, Cat. No. 10009-RP02 [1:500] Sino Biological, Paoli, PA, USA).

### *IHC Examination*

A 5 µm thick endometriotic tissue section was obtained and stained with hematoxylin and eosin. All samples were visualized under a light microscope (Olympus BX48, Tokyo, Japan) and evaluated according to the semi-quantitative method described previously (Nis Elements Advantages Research Microscope Imaging Software; Nikon Instruments Europe BV, Amsterdam, Netherlands). All histopathological and immunohistochemical evaluations were performed by the same histopathologist who was blinded during the evaluation.

### *Assessment of H&E Immunoreactivity*

Morphological analyses using light microscopy showed that the ectopic endometriotic tissue mainly consists of endometrial glandular cells, stroma and vascular cells. The endometrium was structurally intact in the control group when stained with H&E, and blood vessels and glands were clearly visible. In contrast, cystic dilatation of the endometriotic gland, hemosiderin deposition in the stroma, and localization of inflammatory cells in the endometriotic gland were visible when the ectopic endometriotic focus was stained with H&E.

### *Assessment of Ki-67, Bcl-2, Caspase-3 and VEGF Immunoreactivity*

The expression of Ki-67, Bcl-2, caspase-3 and VEGF was compared in all tissue samples according to IHC staining intensity. Ki-67 and caspase-3 immunoreactivity were predominantly localized in the nuclei of endometriotic cells. In contrast, the Bcl-2 immunoreactivity was predominantly localized on the intracytoplasmic and cell membrane surfaces of the same cells.

The degree of positive staining for Ki-67 and caspase-3 was calculated as the ratio of positively stained endometriotic cells to the total number of endometriotic cells at 400x magnification in the selected field. Any nuclear staining was considered positive.

A semiquantitative scoring system was used to evaluate the degree of Bcl-2 and VEGF immunoreactivity. Brown staining in the cell membrane surface of the endometriotic cells revealed the presence of Bcl-2 expression. Brown staining in the cytoplasm of the cells revealed the presence of VEGF expression. The degree of positive staining for Bcl-2 and VEGF was scored as follows: 0, negative; 1, weak positive; 2, moderate positive; and 3, strong positive.

## Ethical Approval

The study protocol was approved by the Balikesir University Animal Research Local Ethics Committee (Date: 25.06.2020, No. 2020/4–14) prior to the start of the study. This study was conducted at the Balikesir University Experimental Animals Laboratory. All of the experimental procedures and investigations were performed in accordance with international guidelines on the ethical use of animals.

## Statistical Analysis

All the statistical analyses were performed using the MedCalc statistical package for medical sciences (MedCalc Inc., Belgium) version v20.218. Pre- and post-treatment endometriotic volume evaluation was calculated using a paired samples *t*-test. All data were presented as mean ± SD or as a proportion, as appropriate. The parametric data were compared between study groups using one-way analysis of variance (ANOVA), followed by post hoc tests for subgroup analyses. A $p$ of less than 0.05 was considered statistically significant.

## Results

A total of 36 rats were randomly divided into three study groups; four of them were excluded from the study due to anesthesia complications. Finally, 32 rats were included in the study and all of them were divided as controls ($n$ = 12), cyclosporine A ($n$ = 12) and tacrolimus ($n$ = 10). No significant differences were observed in baseline characteristics among the groups (Table 1). After treatment, the size of the endometriotic focus was 204.7 ± 153.4 $mm^3$ in the control group, 71.9 ± 85.4 $mm^3$ in the cyclosporine A group, and 30.6 ± 36.7 $mm^3$ in the tacrolimus group.

Compared to the control group, the macroscopic sizes of the endometriotic foci were smaller in both the CsA and tacrolimus group ($p$ = 0.002) (Table 2).

However, subgroup analyses showed no significant difference between the CsA and tacrolimus groups ($p$ >0.05). There was no difference in endometriotic implant volume between pre-treatment and post-treatment in the control group ($p$ = 0.200). However, statistically signifi-

**Table 1.** Results of endometriotic implant volume and immunohistochemical evaluations by group

| | Control group ($n$ = 12) | Tacrolimus group ($n$ = 10) | Cyclosporin A group ($n$ = 10) | $p$[b] |
|---|---|---|---|---|
| Pre-treatment endometriotic implant volume | 165.3 ± 129.1 | 213.6 ± 166.8 | 214.8 ± 193.9 | 0.716 |
| Post-treatment endometriotic implant volume | 204.7 ± 153.4[a] | 30.6 ± 36.7 | 71.9 ± 85.4 | 0.002 |
| Ki-67 mean ± SD | 1.8 ± 0.7[a] | 1.1 ± 0.5 | 1.0 ± 0.6 | 0.010 |
| Bcl-2 mean ± SD | 1.8 ± 0.7 | 1.2 ± 0.8 | 1.1 ± 0.7 | 0.056 |
| Caspase-3 mean ± SD | 1.6 ± 0.7 | 2.1 ± 0.9 | 2.2 ± 0.9 | 0.178 |
| VEGF mean ± SD | 1.9 ± 0.7[a] | 1.1 ± 0.6 | 1.0 ± 0.8 | 0.007 |

In terms of all evaluated parameters (pre-treatment endometriotic implant volume, post-treatment endometriotic implant volume, Ki-67, Bcl-2, caspase-3 and VEGF immunoreactivity) there was no difference between the tacrolimus and cyclosporin A groups ($p$ >0.05).
[a]Control group is statistically different from the tacrolimus and cyclosporin A groups ($p$ <0.05).
[b]ANOVA. Bcl-2: B-cell lymphoma 2; VEGF: Vascular endothelial growth factor.

**Table 2.** Pre-treatment and post-treatment endometriotic implant volume of each group

| | Pre-treatment endometriotic implant volume | Post-treatment endometriotic implant volume | $p$[a] |
|---|---|---|---|
| Control group ($n$ = 12) | 165.3 ± 129.1 | 204.7 ± 153.4[a] | 0.200 |
| Tacrolimus group ($n$ = 10) | 213.6 ± 166.8 | 30.6 ± 36.7 | 0.002 |
| Cyclosporin A group ($n$ = 10) | 214.8 ± 193.9 | 71.9 ± 85.4 | 0.003 |

[a]Paired samples $t$-test.

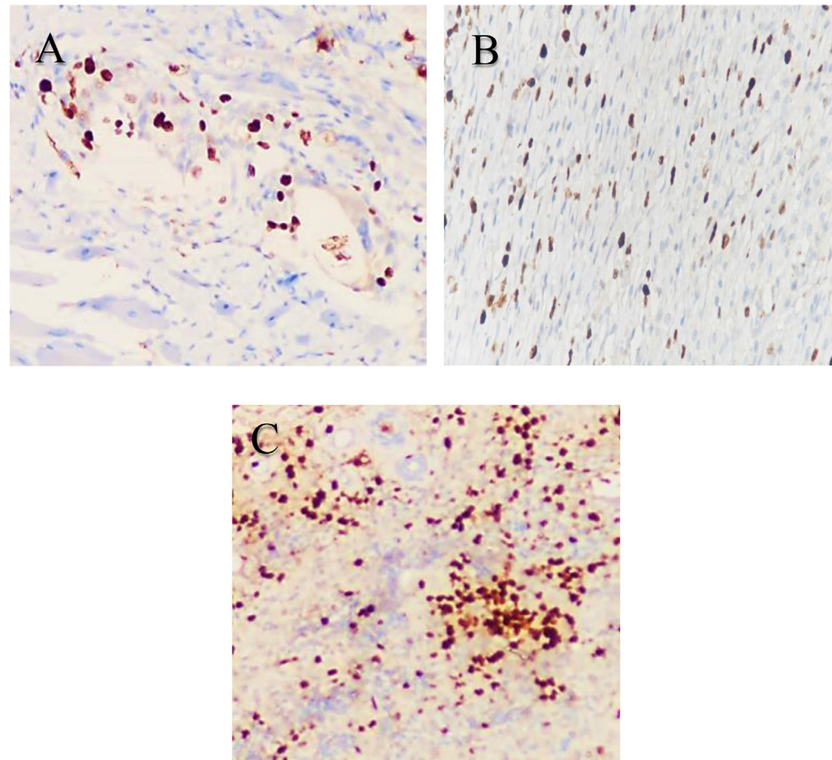


**Figure 1.** Immunoreactivity grades of Ki-67 (× 40). A. Weak immunoreactivity of Ki-67 in the cyclosporine A group. B. Weak immunoreactivity of Ki-67 in the tacrolimus group. C. Strong immunoreactivity of Ki-67 in the control group.

cant differences were observed between pre- and post-treatment endometriotic implant volumes in both the CsA and tacrolimus groups ($p$ = 0.003 and $p$ = 0.002, respectively).

Microscopically Ki-67 and VEGF immunoreactivity were lower in the CsA and tacrolimus groups than in the control group ($p$ = 0.010 and $p$ = 0.007, respectively) (Figures 1 and 2). Moreover, Bcl-2 immunoreactivity was lower in both the CsA and tacrolimus groups, though these differences were not statistically significant ($p$ = 0.056) (Figure 3). There were no significant differences in caspase-3 immunoreactivity between the groups ($p$ = 0.178) (Figure 4). Our subgroup analysis demonstrated that there were no significant differences in Ki-67,

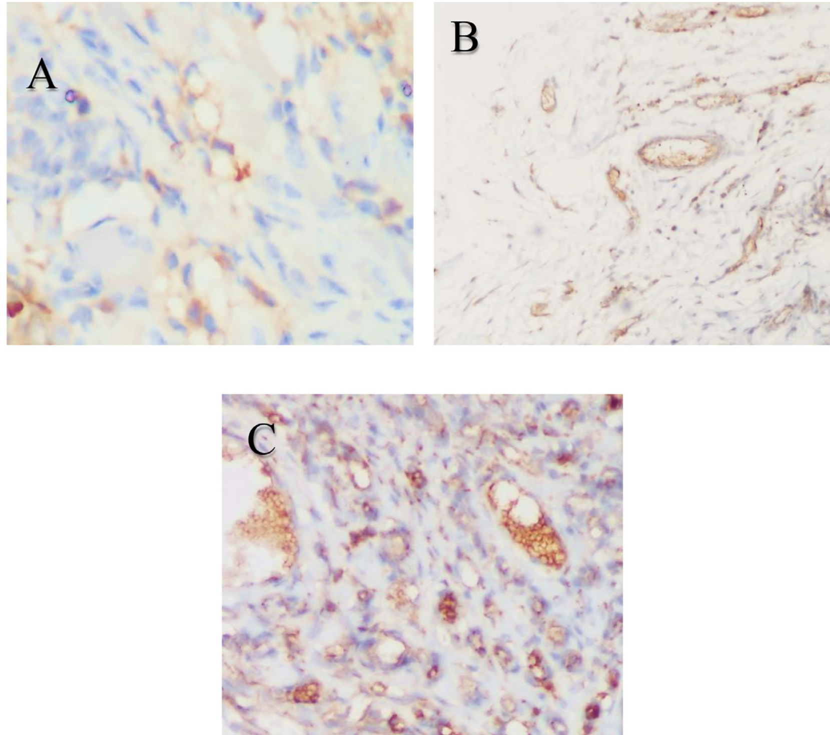


**Figure 2.** Immunoreactivity grades of VEGF (× 40). A. Weak VEGF immunoreactivity in the cyclosporine A group. B. Weak VEGF immunoreactivity in the tacrolimus group. C. Strong VEGF immunoreactivity in the control group.

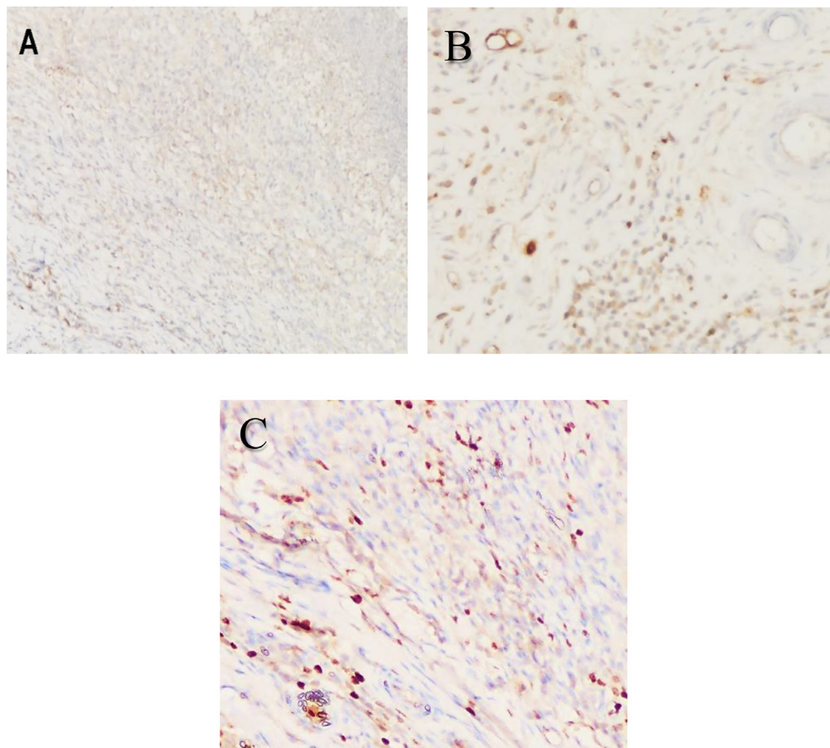


**Figure 3.** Immunoreactivity grades of Bcl-2 (× 40). A. Weak immunoreactivity of Bcl-2 in the cyclosporine A group. B. Weak immunoreactivity of Bcl-2 in the tacrolimus group. C. Strong immunoreactivity of Bcl-2 in the control group.

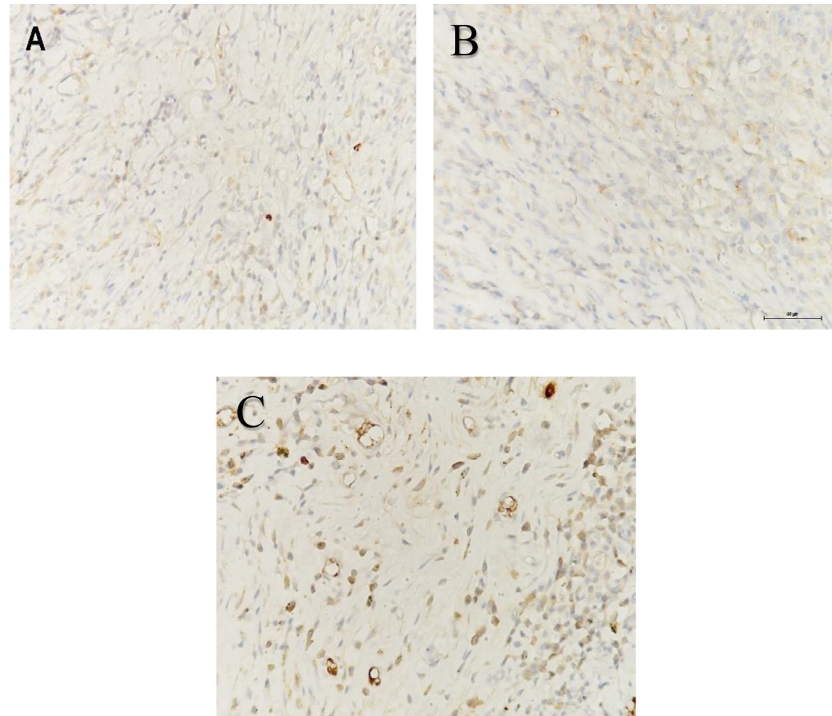


**Figure 4.** Immunoreactivity grades of caspase-3 (× 40). A. Weak immunoreactivity of caspase-3 in the cyclosporine A group. B. Weak immunoreactivity of caspase-3 in the tacrolimus group. C. Strong immunoreactivity of caspase-3 in the control group.

Bcl-2, caspase-3 or VEGF immunoreactivity between the CsA and tacrolimus groups.

## Discussion

In this experimental study, we observed that CsA and tacrolimus treatments were equally able to significantly reduce endometriosis size. We also found that they decreased the histological grade, as demonstrated by the Ki-67 and VEGF expression, in endometriotic implants in rats compared to the control group. Since the groups had comparable baseline characteristics, the observed effects may be attributed to the pharmacological blockade of the CypA-calcineurin axis rather than to intergroup variability. These data extend earlier work in the same model by confirming that two mechanistically similar, clinically approved calcineurin inhibitors (CNIs) yield overlapping therapeutic effects when administered at clinically relevant doses. To the best of our knowledge, this is the first study to evaluate the effects of CsA- and tacrolimus- mediated calcineurin signaling inhibition in the treatment of endometriosis.

CsA and tacrolimus are two well-known immunosuppressive drugs with similar therapeutic mechanisms. Both drugs work by binding to CypA and inhibiting calcineurin signaling. Importantly, calcineurin signaling plays a central role in the adaptive immune response and calcineurin activation depends on CypA binding to the calcineurin receptor. CypA mediates the action of these immunosuppressant drugs via peptidyl-prolyl cis-trans isomerases (PPIases) (22,23). Once CsA and tacrolimus bind to their respective proteins, PPIase activity is abolished (22). The effect of CsA and tacrolimus become active by forming complexes with the PPIases in the body. These complexes bind to calcineurin and inhibit its phosphatase activity. These drugs inhibit calcineurin by competitively binding to and inhibiting $Ca^{+2}$ and calmodulin-dependent phosphatase calcineurin (24). According to the findings of the present study, inhibiting calcineurin signaling with CsA or tacrolimus treatment causes endometriotic tissue regression, leading to decreased cell proliferation and angiogenesis.

Our effect sizes are comparable to those achieved with immunomodulators such as leflunomide, which induced ∼60% lesion regression in a similar rat model, as well as with mTOR blockade. Rapamycin reduced lesion volume and microvessel density by inhibiting VEGF in mice (25,26). These results exceeded the 30–40% regression typically obtained with hormonal agents in rodents and underscore the potency of targeting non–hormonal, inflammation–angiogenesis axes. Emerging reviews emphasize the need for such hormone–independent drug classes in the treatment of endometriosis.

In inflammatory conditions, CypA secretion exacerbates the inflammatory response in various benign and malignant diseases, such as atherosclerosis, asthma, rheumatoid arthritis, diabetes mellitus, and lung and endometrial

cancer (7–13). In a previous study, we demonstrated that women with endometriosis had increased CypA expression in both their eutopic and ectopic endometrial tissues when compared to control subjects. Moreover, CypA expression correlates with the vascularity of endometriotic tissue (17). In the present study, we showed that the inhibition of CypA-mediated calcineurin signaling caused the regression of the experimental endometriotic tissues in rats.

Endometriosis is characterized by the presence and growth of the functional endometrial glands and/or stromal tissue in abnormal regions outside the uterine cavity. The condition has a multifactorial etiology and previous studies have demonstrated that various inflammatory and immunological biomarkers play a significant role in its pathogenesis.

Natural killer (NK) cells, one of the most important cytokines involved in normal physiology, are found in the uterus and are called uterine NK (uNK). The amount of uNK cells in the circulation increases during the menstrual cycle (27). Endometriosis-associated cytokines have been examined in numerous studies to date (28–30). The expression of proinflammatory cytokines and chemokines increases in response to the presence of ectopic endometriotic tissue. Previous studies have shown increased expression of IL-1$\beta$, TNF-$\alpha$, IL-6, IL-8 and VEGF in the blood samples, pelvic peritoneum, and endometriotic tissue samples of patients with endometriosis (31,32). Similarly, prior studies have demonstrated that calcineurin signaling activation causes the upregulation of proinflammatory cytokines and chemokines, including TNF-$\alpha$ and IL-6, via NF-$\kappa$B activation (33). In addition, blocking calcineurin signaling with calcineurin inhibitors decreases TNF-$\alpha$ and IL-6 (33). According to these results, the CsA- and tacrolimus-mediated inhibition of calcineurin signaling may decrease proinflammatory cytokine and chemokine production. In this study, we focused especially on the macroscopic and microscopic effects of CsA and tacrolimus on endometriotic tissue and observed that the use of CsA and tacrolimus decreases endometriotic volume by inhibiting cell proliferation and angiogenesis in endometriotic tissue.

Previous studies have clearly shown that calcineurin acts by activating the NF-$\kappa$B and NFAT transcription factors. More specifically, the activation of the calcineurin/NFAT signaling pathway by angiotensin II increases the endometrial stromal cell proliferation and blastocyst implantation in rats by inducing cyclooxygenase-2 gene expression (34). On the other hand, NF-$\kappa$B activation in macrophages releases proinflammatory cytokines in endometriotic cells, which promotes cytokine production in ectopic endometriotic cells. This process increases the synthesis of anti-apoptotic factors, preventing apoptosis in endometrial cells, and contributing to endometriotic cell survival (35). In a recent study that sought to investigate the pathway leading to NF-$\kappa$B activation, the authors found that MAPKs, ERK1/2, JNK, and p38 were all activated by CypA treatment. However, only the ERK inhibitor PD98059 decreased NF-$\kappa$B activation and attenuated MMP-9 activity induced by CypA (36). In addition, previous studies using animal models have shown that CsA inhibits endometrial stromal cell proliferation by abolishing calcineurin/NFAT dephosphorylation and translocation to the nucleus (34). Recent studies on cancer cell lines and culture media have shown that CypA overexpression is associated with cancer cell proliferation, cell cycle progression, cell invasion, cell migration and apoptosis inhibition (9,37,38). Interestingly, the presence of CypA causes resistance to chemotherapeutic drugs. Recent studies have demonstrated that medical treatments with anti-cancer agents such as 5-aza-2-deoxycytidine, celecoxib, 5-fluorouracil, and paclitaxel are related to decreased CypA expression in cancer cells (20,39–41). However, the knockdown of CypA or CsA has been shown to increase the chemotherapeutic effects of cisplatin and paclitaxel in the treatment of glioblastoma multiforme and endometrial cancer (19,20).

Conversely, CsA and tacrolimus are known to have immunosuppressive effects, which can lead to adverse reactions such as nephrotoxicity, hepatotoxicity, and electrolyte imbalances. We suggest that non-immunosuppressive calcineurin signal inhibitors may be more effective for the treatment of endometriosis.

The main limitation of this study is that it was conducted in an experimental rat model, so we could not evaluate cytokine and chemokine levels in the blood, peritoneal fluid, or endometriotic tissue. Due to the absence of cytokine profiling, fertility readouts and long- term relapse data, immunosuppressive inhibitor doses may exceed acceptable clinical exposure. This hypothesis should be investigated in future studies. These should integrate multiple cytokine and transcriptomic analyses to graphically represent NFAT/NF-$\kappa$B signatures, monitor fertility and recurrence over an extended follow-up period, compare CNIs with non-immunosuppressive cyclophilin antagonists or mTOR/NF-$\kappa$B inhibitors, and develop intralesional depots or nanoparticle formulations that confine drug to pelvic lesions.

## Conclusions

For many years, medical researchers have investigated the treatment of endometriosis. Nowadays, while symptomatic treatment is mostly applied, permanent solutions to the underlying cause are being sought. The inhibition of calcineurin signaling with CsA and tacrolimus treatment could cause regression of endometriotic foci by decreasing endometriotic cell proliferation and angiogenesis in ectopic endometriotic tissue. Further studies are needed to support these results.

## Acknowledgments

The study was supported by the Scientific Research Projects Coordination Unit of the Balikesir University (Project number: BAP.2018/145). The authors declare no conflicts of interest.

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