

The Inhibitory Effects of *Pinus pinaster* Extract and Resveratrol on Aromatase Expression in the Eutopic Endometrium of Endometriosis Patients using Oral Contraceptives

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Abstract

Objective: To demonstrate whether a combination of resveratrol with *pinus pinaster* extract increases the inhibitory effect of oral contraceptives on aromatase expression in the eutopic endometrium of patients with endometriosis.

Methods: The study population consisted of 175 patients referred to this center for diagnostic hysteroscopy with endometrial biopsy. The patients were divided into five groups according to the treatment they were receiving at the time of hospital admission: Group A: 48 patients with a history of pelvic pain and/or infertility and no hormonal treatment; Group B: 36 patients using oral contraceptives in extended regimens; Group C: 30 patients using oral contraceptives with resveratrol; Group D: 15 patients using only the *pinus pinaster* extract with resveratrol; and Group E: 46 endometriosis patients who had been using oral contraceptives together with 100 mg of a *pinus pinaster* extract and 30 mg of resveratrol daily. Aromatase expression was determined in the endometrium by immunohistochemistry.

Results: The mean number of cases in which aromatase expression was positive in the endometrium decreased significantly in oral contraceptive users (Group B). In Group E, the combination of oral contraceptives with *pinus pinaster* extract and resveratrol further decreased the number of cases in which there was positivity for aromatase expression. In Group D, the mean percentage of positive aromatase expression was significantly lower compared to Group A (controls) ($p=0.01$) but not significantly different from Group B. In patients using oral contraceptives with resveratrol (Group C), the percentage of patients in whom aromatase expression was positive was still significantly higher than Group E but lower than that of Group B, although this difference failed to reach statistical significance ($p=0.07$).

Conclusion: The association of oral contraceptives with *pinus pinaster* extract and resveratrol was the most effective in inhibiting aromatase expression in the eutopic endometrium of endometriosis patients.

Keywords: *Pinus pinaster*; Resveratrol; Natural aromatase inhibitors; Endometriosis; Aromatase expression; Oral contraceptives

Introduction

Local estrogen production in endometrial cells of patients with endometriosis plays a pivotal role in the progression of the disease through the impediment of their phagocytosis by activated macrophages carried to the pelvis by retrograde menstruation [1,2]. This might explain the positive correlation between the severity of endometriosis and aromatase expression in the endometrium as determined using immunohistochemistry to measure either the mRNA transcripts for this enzyme or protein levels [3,4]. Progestins, used alone or in combined oral contraceptive pills, are capable of inhibiting aromatase expression in the eutopic endometrium of

endometriosis patients [5,6]. The suppression of aromatase activity correlates with the disappearance of symptoms, since this enzyme remains present in the endometrium of patients who remain symptomatic during oral contraceptive use [7,8]. Furthermore, the association of progestins with aromatase inhibitors was found to increase the efficacy of endometriosis treatment [9] not only for the management of pain but also to reduce endometriotic lesions [10,11]. Resveratrol, a natural aromatase inhibitor, was likewise found to be effective in ameliorating pain in endometriosis patients undergoing treatment with contraceptive pills containing drospirenone in continuous regimens through this mechanism [7].

The presence of a constitutively activated NF-Kappa.b transcription factor in endometriosis leads to the persistent activation of the cyclooxygenase type II enzyme and the resulting production of

proinflammatory prostaglandins [2]. Since aromatase expression in endometriosis is induced by prostaglandins, the failure to suppress NF-Kappa.b activity completely during treatment maintains this enzyme active, leading to enhanced inflammation and increased local estrogen production in both the endometrium and in the endometriosis lesions [2]. One logical approach for circumventing the failure of hormone therapy is to use natural NF- Kappa.b inhibitors concomitantly to further reduce inflammation and therefore potentiate the suppression of aromatase expression. One such inhibitor consists of a complex mixture of flavonoids extracted from the bark of a pine tree (*pinus pinaster*), which blocks NF-Kappa.B activity after its binding to DNA responsive elements [12]. Theoretically, the effects of *pinus pinaster* extract could be further potentiated with the concomitant use of resveratrol to inhibit aromatase activity in endometriosis patients using oral contraceptives, since these two products act in different steps. Resveratrol is able to inhibit aromatase at both enzyme and mRNA levels, while the flavonoids present in the *pinus pinaster* extract act indirectly by suppressing inflammation through the reduction of NF-Kappa.b translation of inflammatory genes after its binding to DNA [12,13]. Furthermore, it has recently been reported that resveratrol is likewise able to diminish inflammation by inhibiting nuclear translocation of NF-kappa B p65 by preventing its translocation to cell nuclei [14]. This suggests that combining resveratrol with *pinus pinaster* extract could result in a more effective inhibition of NF-Kappa.b activity by acting not only prior to its binding to DNA but also after this event. This would result in lesser inflammation and aromatase expression in the endometrium.

In the present study, the effect of the combination of resveratrol with *pinus pinaster* extract on aromatase expression in the endometrium was investigated in endometriosis patients undergoing treatment with oral contraceptives in continuous regimens.

Patients and Methods

The objective of this observational, case-control study was to determine the frequency of positive aromatase expression in endometrial tissue samples obtained from patients of reproductive age (19 to 45 years of age) diagnosed with endometriosis and infertility/pelvic pain and who were already using oral contraceptives, associated or not with *pinus pinaster* extract and resveratrol, prior to hospital admission. Eighty percent of the patients in this study were nulliparous. The study was carried out during a two year period between January 2013 and December 2014.

The study population consisted of 175 patients referred to this center for a diagnostic hysteroscopy with endometrial biopsy. In 48 patients, laparoscopy was performed concomitantly with hysteroscopy for the treatment of endometriosis. In the remaining cases, laparoscopy had been performed in the past to confirm this pathology. The main indication for hysteroscopy when performed alone was to evaluate the uterine cavity as part of the diagnostic work-up prior to an IVF procedure and to obtain endometrial samples for the determination of aromatase expression by immunohistochemistry, since endometrial positivity for that enzyme is associated with poor pregnancy outcome in this population [15]. The patients were divided into five groups according to the kind of treatment they were receiving at the time of hospital admission. Group A, an untreated control group, consisted of 48 patients with a history of pelvic pain and/or infertility who had been referred to this hospital for laparoscopy and hysteroscopy to confirm the presence of endometriosis. Only patients

with a positive diagnosis and no history of hormone therapy in the six months preceding hospital admission were included. During hysteroscopy, an endometrial biopsy was made to determine the presence of aromatase expression in this tissue by immunohistochemistry. Group B was composed of 36 patients who had been using oral contraceptives in extended regimens for at least two months prior to hysteroscopy. Group C consisted of 30 patients who had been using oral contraceptives with resveratrol for the same period of time. Group D consisted of 15 patients using the *pinus pinaster* extract (Fagron, the Netherlands) with resveratrol (Fagron, the Netherlands) and no oral contraceptives for 2-3 months at the time of hospital admission. Group E consisted of 46 endometriosis patients who had been using oral contraceptives with resveratrol and *pinus pinaster* extract for the same period of time. Patients in groups B, C and D and E had previously been diagnosed with endometriosis, as described above. All the hysteroscopies performed in patients in Groups B, C, D and E were performed in a day hospital setting (Itaigara Memorial Day Hospital). All the procedures were carried out by the same two surgeons (HM and CH) with the use of a paracervical block and light intravenous sedation with propofol. When evaluation of the uterine cavity was complete, the hysteroscope was removed and a 4 mm Karman curette attached to a 10 ml disposable plastic syringe was introduced and the endometrium was aspirated. The samples were immediately fixed in 4% formalin and sent to pathology. Routine histology using hematoxylin & eosin (HE) staining was performed on all samples. Both the routine pathology and immunohistochemical evaluation of the endometrium were performed by the same pathologist (NP). The *pinus pinaster* extract (100 mg) and resveratrol (30 mg) were prepared and placed in the same capsule at a local compounding pharmacy supervised by the same pharmacists (CH and WSDS) and prescribed to the patients by their attending physicians prior to hospital admission. All the resveratrol and *pinus pinaster* used in this study was obtained from the same supplier (Fagron, the Netherlands), with a quality control analysis conducted at their plant in Anapolis, Brazil. Both medications are approved by the Brazilian regulatory authorities (ANVISA) to be dispensed by compounding pharmacies.

At the time of hysteroscopy, 112 endometriosis patients in this study were in use of oral contraceptives in continuous regimens (Groups B, C and E). Information on the type of contraceptive used, the duration of treatment and whether breakthrough bleeding and pain were present was obtained at interviews with the patients at the time of admission to hospital. If breakthrough bleeding and/or pain were reported at that time, the patient was classified as symptomatic. The medications used by these patients were prescribed by the patients' attending physician either to treat dysmenorrhea or to increase the success rate of an in vitro fertilization (IVF) procedure, since pretreatment with oral contraceptives is known to increase pregnancy rates in endometriosis patients by decreasing aromatase expression [16]. The patients in groups B, C and E were using oral contraceptive formulations containing either 30 mcg of ethinylestradiol with 75 mcg of gestodene (Gestinol 28, Libbs Farmacêutica, Brazil) or 30 mcg of ethinylestradiol with 3 mg of drospirenone (Elani 28, Libbs Farmacêutica, Brazil). The oral contraceptives were used in extended regimens, since both formulations are already approved by the Brazilian regulatory authorities (ANVISA) for continuous use to suppress menstruation. Both contraceptive regimens were equally effective in reducing aromatase in the endometrium, as previously shown [8].

The study was approved by the internal review board of the Instituto da Mulher and the patients gave their written informed consent allowing access to their medical records and to the results of the immunohistochemistry studies. All hysteroscopy procedures were part of the standard medical care in our institution for patients undergoing IVF procedures. The medication used by the patients was dispensed prior to hospital admission and is approved by ANVISA for use in Brazil.

The presence of aromatase p450 expression in the endometrium was determined by immunohistochemistry following antigen retrieval. Aromatase expression was investigated using a commercially available monoclonal antibody, MCA2077, clone H4 (Serotech, Raleigh, NC, USA). Antigen retrieval was performed using the Tris-EDTA buffer at pH 8.0. The reaction was revealed using the DAKO EnVision Flex detection system + Linker followed by DAB+substrate chromogen mix (DAKO). The presence of aromatase expression was rated either as positive if there was any detectable staining reaction in the endometrium or negative when no reaction was observed. Placental

tissue and a sample of the atrophic endometrium were used as positive and negative controls, respectively, in all immunostaining reactions for aromatase p450.

Statistical analysis was performed using the chi-square test to detect differences in percentages. Significance was established at $p < 0.05$.

Results

Aromatase activity was detected in the stroma of patients in the untreated control group (Group A) with endometriosis (Figure 1). The distribution of positive staining was heterogeneous, varying from a focal to a more diffuse pattern of expression. When the proliferative and secretory phases of the menstrual cycle were compared, no statistically significant difference was found in the incidence of aromatase-positive endometria (80% versus 78%, respectively); therefore, both phases of the cycles were evaluated together. However the intensity of the staining reaction was not taken into consideration.

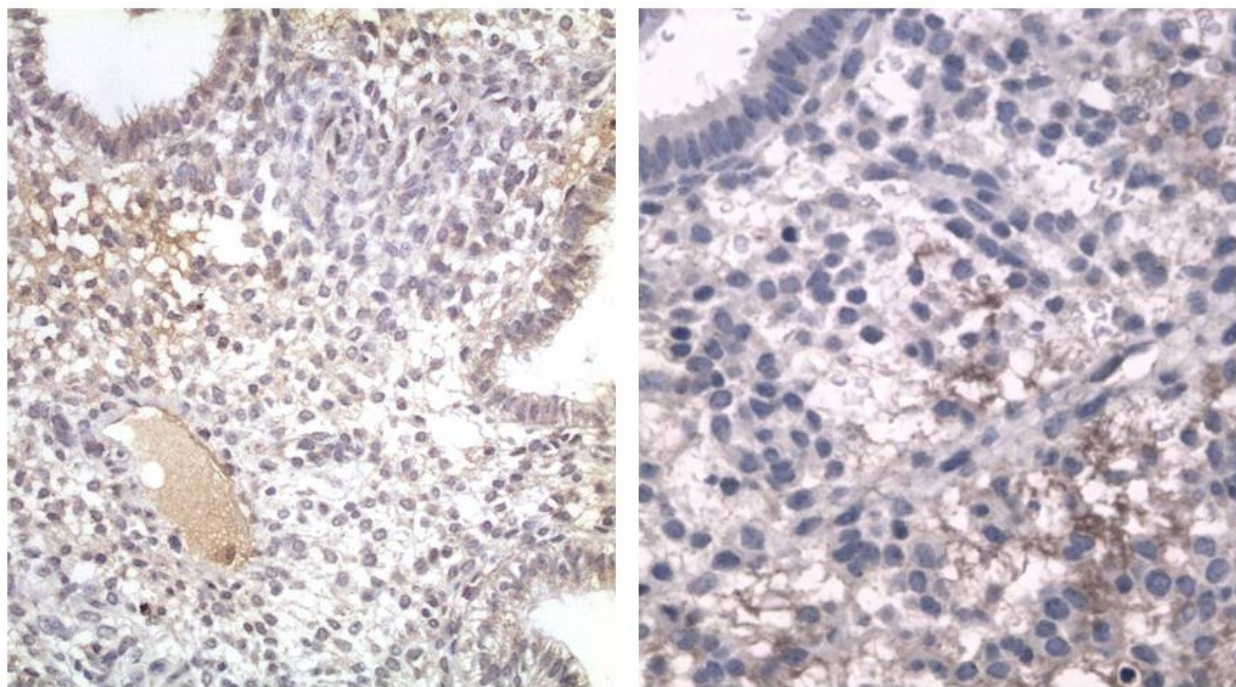


Figure 1: Positive aromatase expression in the stroma of the eutopic endometrium of a patient with endometriosis

The use of oral contraceptives in extended regimens for 2-3 months prior to hospital admission was associated with a significant reduction in the percentage of endometrial stroma showing positive aromatase expression. Since there was no statistically significant difference between the patients using the oral contraceptives containing drospirenone and those using the pills containing gestodene insofar as aromatase inhibition was concerned, they were grouped together for the purpose of analysis as a group of oral contraceptive users (Group B). In this group, the mean number of aromatase-positive cases decreased significantly to 50% ($p=0.005$) after a mean duration of pill use of 2-3 months.

Patients in Group E were using oral contraceptives in extended regimens associated with *pinus pinaster* extract + resveratrol. This

combination caused a further significant reduction in the percentage of endometria that were positive for aromatase expression when compared to the group of patients using oral contraceptives alone (Group B). In this group, the mean percentage of positive staining in the stroma was only 6%, which was significantly lower than that of Groups A and B ($p < 0.0001$). In Group D, which consisted of patients using *pinus pinaster* extract + resveratrol but who did not use oral contraceptives, the mean percentage of positive aromatase expression was significantly lower than in Group A (the control group) ($p=0.01$) but not different from those using oral contraceptives alone (Group B). However, it was significantly higher than in Group E ($p=0.0002$). In patients using oral contraceptives with resveratrol (Group C), the percentage of positive aromatase expression was still significantly

higher than in Group E but lower than in Group B, although this difference failed to reach statistical significance ($p=0.07$). These results are summarized in Table 1.

Group A	Untreated patients	34/48	79%
Group B	Users of oral contraceptives alone	18/36	50%
Group C	Oral contraceptives + resveratrol	10/30	33%
Group D	<i>Pinus pinaster</i> + resveratrol	7/15	47%
Group E	Oral contraceptives + <i>pinus pinaster</i> + resveratrol	3/46	6%
Group A x Group B- $p=0.005$; Group A x Group C- $p<0.0001$; Group A x Group D- $p=0.01$; Group B x Group C- $p<0.000001$; Group B x Group D-Not significant; Group E x Group D- $p<0.000001$			

Table 1: Percentage of cases in which the endometrium of patients with endometriosis was found to be positive for aromatase expression

The concomitant use of *pinus pinaster* extract + resveratrol with oral contraceptives (Group E) was associated with a decrease in the percentage of patients reporting break through bleeding and pain. These symptoms were reported by 15/36 patients in Group B (41%) and by only 7/46 in Group E (15%) ($p=0.04$). This decrease in the incidence of symptoms in Group E was associated with the more effective inhibition of aromatase expression in the eutopic endometrium. When patients in Groups B and E were analyzed together, the percentage of positive aromatase expression was found to be 68% in the endometria of symptomatic patients compared to only 4% in the asymptomatic women. This difference was statistically significant ($p=0.00006$).

Discussion

The present results showed that the combination of *pinus pinaster* extract with resveratrol not only significantly inhibited aromatase expression in the endometrium of endometriosis patients but also potentiated the inhibitory effects of oral contraceptives on this enzyme. It is noteworthy that this association proved more effective than when resveratrol was given alone to oral contraceptive users.

The aromatase gene is activated in the endometrium by proinflammatory prostaglandin E, which is generated in tissues as a result of Cox-2 activity. Since this is an NF-Kappa.b inducible gene, aromatase expression may depend on the inflammatory status of the tissues [17]. Endometriosis is an example of an estrogen-dependant inflammatory pathology in which NF-Kappa.b is constitutively expressed in the lesions and also in the eutopic endometrium [18]. The constant activation of NF-Kappa.b in patients with endometriosis and its binding to the DNA responsive elements enhances transcription of the cyclooxygenase gene, thus boosting aromatase gene expression in response to the increased exposure to inflammatory prostaglandins [2,19]. Progestins such as gestodene or dienogest can effectively block NF-Kappa.b DNA binding activity by preventing its translocation to cell nucleus. This halts the initiation of the inflammatory cascade that will ultimately provoke transcription of the aromatase gene [2,20]. In patients who experience breakthrough bleeding during continuous use of oral contraceptives containing gestodene/ethinylestradiol, NF-Kappa.b remains actively bound to cell nuclei where it will increase the transcription of several genes directly or indirectly related to inflammation, thus stimulating translation of the aromatase gene [2,21]. This will maintain estrogen production in both the endometrium and in lesions, thus preserving a vicious cycle of

increased inflammation and estrogen production that is pivotal in the progression of endometriosis. As shown in the present paper, the complete suppression of aromatase expression following hormone treatment is associated with higher rates of amenorrhea and pain-free intervals. Treatments that combine oral contraceptives with this association of natural NF-Kappa.b and aromatase inhibitors may prove more beneficial than their use alone, since they may increase the efficacy of treatment without increasing side effects [22]. The combination of resveratrol and oral contraceptives effectively suppressed aromatase expression in the endometrium of endometriosis patients; however, adding *pinus pinaster* extract to this combination further potentiated the inhibitory effects of this treatment on the expression of this enzyme in the endometrium. One plausible explanation is the blockade of NF-Kappa.b activity by the *pinus pinaster* extract, which will further decrease inflammation, thus leading to a greater reduction in aromatase activity in the endometrium. This will exert an additional inhibitory effect on aromatase expression, potentiating the direct inhibition of the aromatase gene transcription by resveratrol [10,23]. Since aromatase expression in the endometrium may serve as a marker of the severity of endometriosis, the findings reported here that the combination of resveratrol with *pinus pinaster* extract greatly potentiates the inhibitory effect of oral contraceptives suggest that combining hormone therapy with natural NF-kappa.b and aromatase inhibitors may be more beneficial for the treatment of endometriosis than the use of hormone therapy alone. However, well designed double-blind clinical trials are necessary to prove or refute this hypothesis, since this a case control clinical study.

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