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HUGO DA SILVA MAIA FILHO

EXPRESSÃO DE AROMATASE NO ENDOMÉTRIO E SEU PAPEL NO DESENVOLVIMENTO DE PATOLOGIAS UTERINAS

TESE DE DOUTORADO

Salvador
2013

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Tese apresentada ao Programa de Pós-graduação em Medicina e Saúde, da Faculdade de Medicina da Bahia, Universidade Federal da Bahia, como requisito para a obtenção do grau de Doutor em Medicina e Saúde.

Orientador: Profº. Drº Thomaz Rodrigues Porto Cruz

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Dissertação apresentada como requisito para obtenção do grau de Doutor em Medicina, Escola de Medicina da Universidade Federal da Bahia.

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A
Mª Conceição Maia, minha mãe amada, por me ensinar o que é a vida.
Hugo da Silva Maia, querido pai, que me fez aprender a ensinar como gerar a vida.

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Os sonhos devem ser ditos para começar a se realizarem. E como todo projeto, precisam de uma estratégia para serem alcançados. O adiamento destes sonhos desaparecerá com o primeiro movimento.

Paulo Coelho

SUMÁRIO

RESUMO

A expressão de aromatase no endométrio eutópico é desencadeada pela constante exposição a mediadores inflamatórios, que são produzidos durante o período menstrual e proliferativo do ciclo menstrual. A presença de aromatase nas células endometriais é um dos fatores desencadeantes de endometriose na cavidade peritonial, miomas submucosos e intra-murais, pólipos endometriais e adenomiose. Diante disso, esta tese tem como objetivo investigar os efeitos da expressão de aromatase no endométrio, compreendendo a ação desta e como se evitar o desenvolvimento das patologias endometriais.

Para isso, foram analisados resultados de biopsias de pacientes submetidas à histerectomia e laparoscopia, no período de janeiro de 2007 a março de 2009 de dois centros de tratamento da cidade de Salvador- Bahia, as quais apresentavam algumas das patologias citadas, seguindo os critérios da American Sciety of Reproductive Medicine.

Por fim concluiu-se que a diminuição da expressão de aromatase induzida por progestínicos foi acompanhada por uma redução na expressão de enzimas como ciclooxigenase-2 (Cox-2) ou de fatores angiogênicos como VEGF no endométrio. A inflamação no endométrio também foi reduzida pela progesterona ou por progestínicos e este mecanismo envolveu a inibição da ativação do NF-kappa B. Estes achados sustentam a hipótese do papel que teriam os progestínicos como agentes anti-aromatase e anti-inflamatórios no manejo atual da endometriose e de outras patologias ginecológicas. E que o uso contínuo de contraceptivos orais combinados contendo gestodeno ou o uso de sistemas intra-uterinos liberadores de levonorgetsrel são efetivos na prevenção tanto da recorrência de endometriose, quanto da menorragia associada a miomas.

Palavras-chaves: Aromatase, Endometriose, Endométrio, Mioma, Cox-2.

ABSTRACT

Aromatase expression in the eutopic endometrium is triggered by constant exposure to inflammatory mediators such as prostaglandins, which are produced during menstruation and in the proliferative phase of the menstrual cycle. The presence of aromatase in the endometrial cells is a prerequisite for the development of endometriosis in the peritoneal cavity, submucous and intramural myomas, endometrial polyps and adenomyosis. With this in mind, the objective of this thesis was to investigate the effects of aromatase expression in the endometrium, understand its mechanism of action and how to prevent the development of endometrial pathology.

To do so, the results of biopsies of patients undergoing laparoscopy or hysterectomy between January 2007 and March 2009 in two treatment centers in Salvador, Bahia, were analyzed. Patients found to have at least one of the aforementioned diseases, in accordance with the criteria established by the American Society of Reproductive Medicine, were enrolled to a study.

Results showed that the progestin-induced reduction in aromatase expression was accompanied by a decrease in the expression of enzymes such as cyclooxygenase-2 (Cox-2) or angiogenic factors such as VEGF in the endometrium. Inflammation in the endometrium was also reduced by progesterone or progestins and this mechanism involved the inhibition of NF-kappa.B activation. These findings support the role of progestins as anti-aromatase and antiinflammatory agents in the current management of endometriosis and other gynecological pathologies. In fact, the continuous use of combined oral contraceptives containing gestodene or the use of levonorgestrel-releasing intrauterine systems was found to be effective in preventing not only the recurrence of endometriosis but also of myoma-related menorrhagia, thereby supporting the hypothesis of a causal relationship between aberrant aromatase expression in the endometrium, inflammation and the development of pathology.

Keywords: Aromatase, Endometrium, Endometriosis, Myoma, Cox-2.

1 INTRODUÇÃO

O papel da aromatase e da produção local de estrogênio sempre foi um tópico que despertou um grande interesse. O conceito moderno da intracrinologia, no qual os hormônios podem ser produzidos localmente em tecidos, que não são necessariamente as tradicionais glândulas endócrinas, veio explicar muitas das doenças estrogênios dependentes. Nos últimos 6 anos, publicamos na literatura médica internacional 15 trabalhos de pesquisa sobre os vários aspectos da produção local de estrogênios nos tecidos e o seu papel tanto na fisiologia da menopausa tardia como no desenvolvimento de patologias ginecológicas. Neste estudo mostramos que pacientes que desenvolvem patologias como endometriose, por exemplo, têm um endométrio funcionalmente diferente daquelas pacientes que são normais.

Esta diferença não estava na histologia, mas sim no padrão enzimático do tecido. O atual estudo permitiu que verificássemos que estes endométrios expressavam a enzima aromatase p450, responsável pela síntese de estradiol, e aumento da Cox-2 e do NF-Kappa.b, ocasionavam um maior grau de inflamação. Esta inflamação endometrial induzia como sugerimos numa publicação contendo os trabalhos apresentados no World Congresso of Gynecological Endocrinology em Florença em março de 2012, a ativação do gene da aromatase no endométrio.

Nesse mesmo estudo analisamos o efeito dos progestagênios sobre a expressão da aromatase no endométrio de pacientes com adenomiose, mioma e endometriose. Em que mostramos pela primeira vez que esta expressão enzimática aberrante no endométrio era inibida pelo gestodeno, progestagênio presente na composição de vários anticoncepcionais orais. Em uma publicação em 2004, publicada em um suplemento do European Journal of Contraception and Reproductive Health relatamos que pílulas contendo gestodeno quando usados de maneira contínua eram eficazes para tratar endometriose e impedir a recorrência desta patologia em pacientes submetidas a um tratamento cirúrgico por laparoscopia. Escrevemos entre 2007-12 vários artigos para a classe médica brasileira e que foram publicados pela indústria farmacêutica responsável pela fabricação deste anticoncepcional de uso contínuo, onde o conceito de utilização da contracepção hormonal continua para o tratamento de patologias foi divulgado. Alguns destes artigos estão no anexo desta tese.

Fizemos também em 2008- 11 pesquisas mostrando que progestagênios como o gestodeno e a drospirenona, quando usados de maneira contínua inibiam a inflamação

endometrial através do bloqueia da enzima Cox-2 e do NF-Kappa.b. A parada do seu uso por sete dias como nas pílulas anticoncepcionais usadas em regime 21/7 era associada com a ativação da inflamação no endométrio e o aparecimento dos sintomas menstruais. Estes achados serviram de base para o lançamento de contraceptivos orais a base de drospirenona, que quando dados de maneira contínua ou estendida eram eficazes para o tratamento de sintomas menstruais. Estes estudos clínicos do qual participei foram utilizados para a aprovação pela ANVISA do Elani28 e foram publicados na revista Contraception.

Mostramos também pela primeira vez que DIUs medicados com levo-norgestrel (Mirena) e utilizados no tratamento de patologias endometriais inibiam a enzima aromatase no endométrio, explicando assim sua eficácia terapêutica. Estes achados foram inicialmente apresentados e publicados nos anais do World Congress on Menopause em Roma em 2011 e aceito pára publicação recentemente no International Journal of Women's Health.

Também faz parte desta tese o papel da aromatase no desenvolvimento das patologias estrogênios dependentes em pacientes que fazem reposição hormonal na menopausa usando tibolona. Ao contrario do modelo aceito para a ação deste esteróide, nós observamos que o principal efeito da tibolona era aumentar os níveis de testosterona livre através da redução da síntese hepática do SHBG (sex hormone binding globulin). A testosterona livre, por sua vez ao passar para os tecidos, agiria através da ativação do receptor androgênico ou como estrogênio após sua conversão a nível tecidual pela ação da aromatase. Assim os efeitos da tibolona nos tecidos seriam dependentes da atividade da aromatase e com isto pudemos explicar seus efeitos endometriais e na mama. Estes estudos foram divulgados no Brasil através de artigos patrocinados por dois das maiores indústrias farmacêuticas responsáveis pela fabricação da tibolona no Brasil.

Para finalizar gostaria de afirmar que esta tese representa um resumo dos estudos que fizemos sobre o papel da aromatase na produção tecidual de estrogênios e o seu papel no desenvolvimento de patologias como endometriose, adenomiose, pólipos endometriais e miomas. A maior parte destas observações sobre o papel da aromatase no desenvolvimento destas doenças já foi confirmado por outros investigadores. Nossos achados pioneiros de que os progestagênios inibem a Cox-2 e a aromatase no endométrio já foram também confirmados em muitos estudos posteriores feitos na Europa e Japão nos últimos 2 anos e servem de base para explicar a ação terapêutica dos progestagênios do tratamento da endometriose.

ARTIGOS

Artigo nº 1

Effect of a hormone-releasing intrauterine system (Mirena®) on aromatase and Cox-2 expression in patients with adenomyosis submitted or not, to endometrial resection.

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ORIGINAL RESEARCH

Effect of a hormone-releasing intrauterine system (Mirena®) on aromatase and Cox-2 expression in patients with adenomyosis submitted or not, to endometrial resection

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Objective: To investigate the effect of a levonorgestrel-releasing intrauterine system (Mirena®) on aromatase and cyclooxygenase-2 (Cox-2) expression in the endometrium of patients with adenomyosis who were submitted to endometrial resection at the time of insertion, compared to a group not submitted to endometrial resection and a group of controls with adenomyosis not submitted to any previous hormonal treatment.

Patients and methods: Patients with adenomyosis (n = 89) were included in this study. Twenty-two patients had been using Mirena® for 5 years but had not been submitted to endometrial resection prior to insertion of the device. Twenty-four patients were submitted to endometrial resection at the time of Mirena® insertion. The remaining 43 patients with adenomyosis had undergone no previous hormonal treatment and served as a control group. Cox-2 and aromatase expression were determined in the endometrium by immunohistochemistry.

Results: Use of Mirena® for 5 years reduced aromatase expression in the endometrium; however, this reduction was significantly greater in the uteri previously submitted to endometrial resection. The reduction in Cox-2 expression was significant only in the uteri submitted to endometrial resection followed by the insertion of Mirena®.

Conclusion: Endometrial resection followed by the insertion of Mirena® was associated with greater rates of amenorrhea in patients with adenomyosis, which in turn were associated with a more effective inhibition of aromatase and Cox-2 expression in the endometrium.

Keywords: aromatase, Mirena®, adenomyosis, Cox-2, endometrium, levonorgestrel

Introduction

Adenomyosis is a frequent cause of abnormal uterine bleeding and pain in women of reproductive age. Although hysterectomy has always been advocated as an effective treatment, less invasive procedures such as endometrial resection have been proposed as an alternative; however, these procedures may be associated with lower success rates.

Nevertheless, the poor rates of amenorrhea may be due more to the intrinsic capacity of the endometrium to regenerate rather than to any fault in the actual technique.[1] Several recent studies have shown the presence of aromatase expression in the endometrium of patients with adenomyosis, myomas, and endometrial polyps.[2,3] Locally produced estrogens upregulate several angiogenic and inflammatory factors in the endometrium, thus enabling this tissue to regenerate more effectively following

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hysteroscopic surgery. This would explain the resumption of bleeding and the low rates of amenorrhea achieved with endometrial ablation. It is therefore important that aromatase expression be suppressed in order to achieve better rates of amenorrhea following endometrial resection. Several different progestins have been shown to inhibit aromatase, vascular endothelial growth factor, and cyclooxygenase-2 (Cox-2) expression in the endometrium.[2,4–6] This effect may help improve the surgical efficacy of endometrial resection for the treatment of menorrhagia by creating a long-lasting suppressive effect on the activity of these enzymes and growth factors in the endometrium. This can be achieved by inserting a levonorgestrel-releasing intrauterine system (Mirena®) immediately following endometrial resection. The insertion of Mirena® would also provide effective contraception for patients undergoing endometrial resection.

In the present study, the effect of Mirena® on aromatase and Cox-2 expression in the endometrium was evaluated in the fifth year of use of this device by patients previously submitted to endometrial resection at the time of insertion. These patients were compared with another group of women with adenomyosis in the fifth year of use of Mirena® who had not been submitted to endometrial ablation and to a control group of women who had undergone endometrial resection for adenomyosis but who had not been submitted to any hormonal treatment in the 3 months preceding the study.

Patients and methods

Patients (n = 89) aged 30–48 years with a history of menorrhagia and adenomyosis were included in this observational, case-control study. Enrolled patients had already completed their families and were seeking an alternative to hysterectomy. For the purpose of analysis, the patients included in the study were separated into three groups.

Group A consisted of 22 patients who had completed 5 years of Mirena® use. The device was originally inserted in the women in this group as an outpatient procedure to treat excessive menstrual bleeding due to adenomyosis. In this group, hysteroscopy including an endomyometrial biopsy was carried out to establish the diagnosis of adenomyosis at the time of Mirena® insertion. The myometrial biopsy was carried out using a 5 mm forceps used in laparoscopy for ovarian biopsy, which was inserted through the cervix immediately prior to Mirena® insertion. The use of this forceps permits a sufficiently large fragment of the myometrium to be obtained to enable a histological diagnosis of adenomyosis to be made. Eighteen of the patients in this group were submitted to an endometrial resection at the time of insertion of the replacement device. The procedure was carried out using the bipolar resectoscope (Versapoint™ Gynecare, Ethicon, Inc, Somerville, NJ) under paracervical block, associated with intravenous propofol as previously described.[6] In brief, the surgical technique consisted of dilating the cervix to 9 mm to allow the introduction of a bipolar resectoscope. A 0.9% solution of isotonic saline was used as the distention medium for the uterine cavity. The endometrium was removed during surgery together with approximately 5 mm of the underlying myometrium. This procedure was carried out in a private hospital setting and the patients were discharged 6 hours after its completion. In the remaining four patients in this group, a replacement Mirena® was inserted but endometrial ablation was not performed; however, an endomyometrial biopsy was taken at the time of the procedure to establish whether or not adenomyosis was present.

Group B consisted of 24 patients with histologically proven adenomyosis in whom Mirena® was inserted at the time of endometrial resection for the treatment of menorrhagia. All the women in this group had been using the device for at least 5 years when they returned to this center to have it replaced. Outpatient hysteroscopy was performed using a Bettocchi hysteroscope (Karl Storz, Tuttlingen, Germany) under paracervical block established with 10 mL of lidocaine 1% prior to reinsertion of a replacement Mirena®. The previous device was easily removed and an endomyometrial biopsy was performed by inserting a 5-mm forceps into the uterus as described previously. A replacement Mirena® was then inserted without any difficulty.

Group C consisted of 43 patients submitted to endometrial resection between March 2009 and September 2011 in whom pathology revealed adenomyosis. Since they had not been submitted to any hormonal treatment for menorrhagia in the 3 months preceding hysteroscopic surgery, these patients were included as untreated controls to determine the prevalence of aromatase and positive Cox-2 expression in the endometrium. This group was necessary because when Mirena® was inserted in groups A and B the pathology laboratory was not equipped to perform immunohistochemistry.

All surgical procedures were carried out by the same group of surgeons. Pathology and immunohistochemical analysis of the endometrium and myometrium were performed by the same pathologist.

Data on the menstrual-related symptoms such as menorrhagia, dysmenorrhea, and the premenstrual syndrome reported by these patients prior to treatment were obtained from their medical records.

During hysteroscopy performed using the Versapoint™ resectoscope, the endometrium and approximately 0.5 cm of the myometrium were removed as described on previous page, fixed in 4% formalin, and sent to pathology for histological and immunohistochemical evaluation. Immunohistochemistry was performed following antigen retrieval to detect the presence of aromatase p450 and Cox-2 expression. Aromatase expression was investigated using a commercially available monoclonal antibody, MCA2077, clone H4 (Serotech, Raleigh, NC), while Cox-2 expression was assessed using CX-294. Antigen retrieval was performed using the Tris-EDTA buffer at pH 8.0. The reaction was revealed using the streptavidin-biotin method. The presence of aromatase expression was rated either as positive if there was any detectable staining reaction in the glandular epithelium or negative when no reaction was observed. Cox-2 expression was graded as 0 when no expression was detected, +1 when there was a light staining in more than 10% of the glands, +2 when staining was moderate, and +3 when there was strong expression in the endometrium. In all cases, slides were evaluated by a pathologist. 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 and Student's *t*-test for comparison of means. Significance was established at $P < 0.05$. All patients gave their written informed consent authorizing the immunohistochemical studies to be performed on the endometrial tissue, as determined by the hospital's internal review board. The insertion of Mirena® following endometrial resection has been the standard treatment for menorrhagia in this day hospital for a decade and all patients were adequately counseled prior to the procedure. A previous study conducted by our group had shown that endometrial resection without the concomitant use of Mirena® was associated with much lower amenorrhea rates than when this device was inserted at the time of surgery.[6] For this reason, patients submitted to an endometrial resection for the treatment of adenomyosis-related menorrhagia were always adequately counseled to have a Mirena® device inserted right after completion of the procedure.

Results

Histology and hysteroscopy findings

In the group of untreated patients with a histological diagnosis of adenomyosis, the endometrium was in the proliferative phase in 28 cases and in the secretory phase in the remaining 15. On the other hand, in patients using Mirena®, the most common histological pattern reported consisted of an eutopic endometrium with atrophic glands in the presence of stroma with a decidual reaction or atrophic changes (Figure 1 and 2). These results are summarized in Table 1.

In eight patients of group B who had adenomyosis at the time of endometrial resection and Mirena® insertion, examination of the myometrium failed to detect the presence of ectopic endometrial tissue when the device was replaced 5 years later.

The uterine cavity appeared normal at hysteroscopy in 80% of the patients in the untreated control group; however, in 20% of cases an endometrial polyp was also detected.

In patients using Mirena® who had not previously been submitted to endometrial resection, the endometrium was thin; however, the presence of dilated blood vessels was detected, a finding that was not present in the group of women who had an endometrial resection at the time of insertion (Figure 3).

Menstrual symptoms in Mirena® users

The incidence of amenorrhea at the fifth year of use was 84% in the group of patients who had an endometrial resection at the time of Mirena® insertion and only 19% in the group not submitted to this procedure ($P < 0.0001$). Complete resolution of dysmenorrhea was reported by 91% of the patients who had undergone endometrial resection followed by Mirena® insertion, while in the group not submitted to resection, only 20% of patients reported complete disappearance of this symptom at the fifth year of use ($P = 0.0005$). The occurrence of dysmenorrhea was consistently associated with the presence of uterine bleeding.

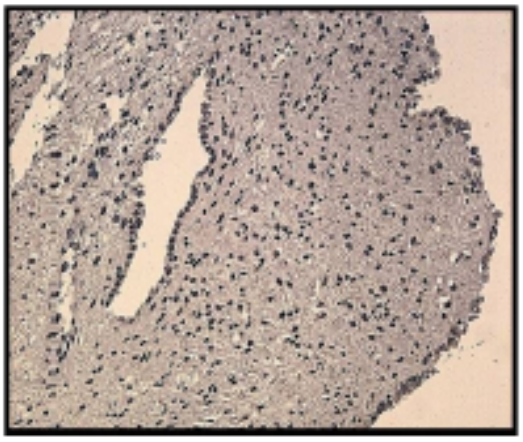

Figure 1 Atrophic endometrium in a patient who had been using Mirena® for 5 years.

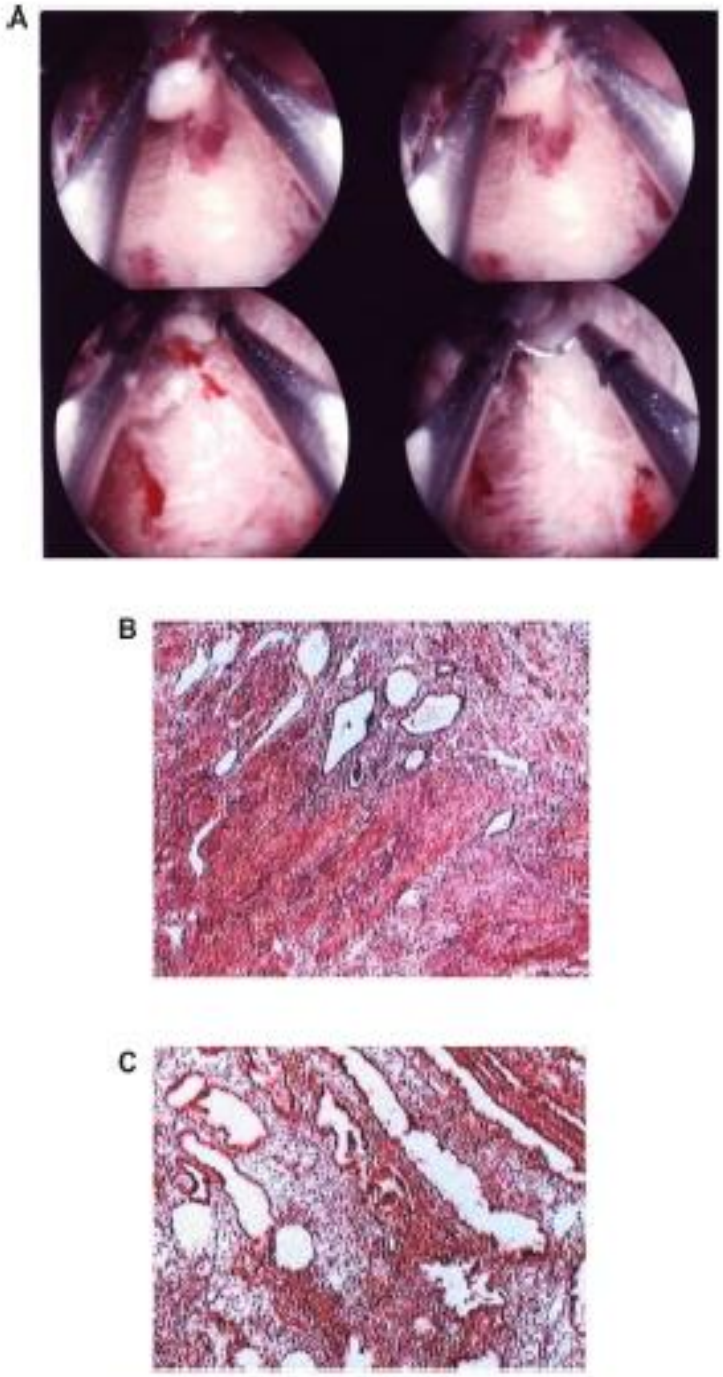

Figure 2 Secretory endometrium in a patient with adenomyosis not previously treated with hormones. (A) Hysteroscopy; (B) Adenomyosis; (C) Secretory eutopic endometrium.

Aromatase expression

The staining reaction for aromatase was detected using immunohistochemistry not only in the glandular epithelium of the eutopic endometrium but also in the ectopic glands embedded in the myometrium in the untreated controls with adenomyosis (Figure 4). In all cases there was agreement between the positive staining in both the eutopic and ectopic endometrium. In the untreated group, aromatase expression was present in 23/28 cases during the proliferative phase and in 9/15 cases in the luteal phase of the menstrual cycle, a difference that was not statistically significant ($P = 0.2$). For this reason, the results were

Table 1 Endometrial histology in patients with adenomyosis according to group

	Group A (n)	Group B (n)	Group C (n)
Proliferative endometrium	0	0	28
Secretory endometrium	0	0	15
Atrophic glands + decidual stroma	16	15	0
Atrophic glands + atrophic stroma	6	9	0

Abbreviation: n, number of patients.

pooled together and analyzed as a single group of untreated control patients (group C).

In contrast, in patients with adenomyosis using Mirena®, there were significantly fewer cases in which a positive staining reaction for aromatase could still be detected in both the eutopic endometrium and in the ectopic glands embedded in the myometrium at the end of the fifth year of use when compared to the untreated patients with adenomyosis (group C) (Figure 5). Although this decrease in the number of cases was statistically significant in both group A and group B, it was significantly greater in the group previously submitted to an endometrial resection when compared to the group of women who had not undergone resection, being detected in only 1/24 of cases in the former. The difference between groups A and B was statistically significant ($P < 0.01$). These results are summarized in Table 2.

Cox-2 expression

The mean intensity of Cox-2 expression did not vary significantly between the proliferative and luteal phases of the menstrual cycle in the untreated patients with adenomyosis, and for this reason the results were analyzed together (group C) (Figures 6 and 7). However, in the patients who had been using Mirena® for 5 years, a significant reduction occurred in the mean intensity of Cox-2 expression only in the group of patients who had been submitted to endometrial resection at the time of Mirena® insertion (group B) (Figure 8). Conversely, in patients not submitted to this procedure, the mean intensity scores for Cox-2 in the glandular epithelium were not significantly different from those found in the untreated controls and significantly higher than those found in group B. These results are summarized in Table 3.

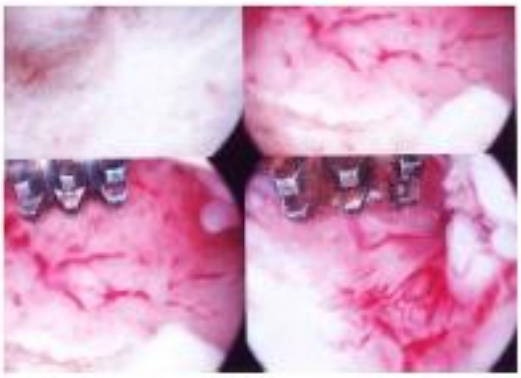

Figure 3 Presence of dilated vessels in the endometrium of a patient with adenomyosis who had been using a Mirena® device for 5 years.

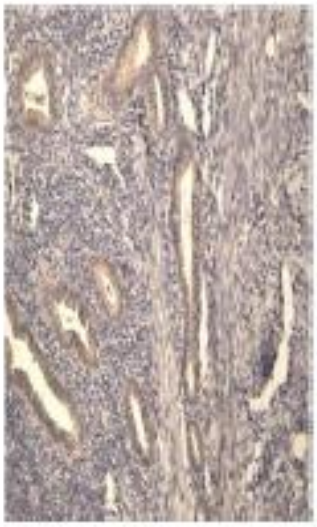

Figure 4 Aromatase expression in the glandular epithelium of a patient with adenomyosis during the proliferative phase.

Discussion

The findings of the present study show that the use of Mirena® following endometrial resection in uteri with adenomyosis is associated with higher rates of amenorrhea and lower rates of dysmenorrhea compared to a group of women who had Mirena® inserted but who were not submitted to endometrial resection. Although this was not a prospective clinical trial designed specifically to address the question of whether endometrial resection improves rates of amenorrhea in Mirena® users, the amenorrhea rates reported here are similar to those cited in the literature for patients with adenomyosis who had not been submitted to prior endometrial ablation.[7] The presence of Mirena® inhibited the expression of aromatase and p450, thus exerting a local anti-estrogenic effect on the endometrium, which is beneficial in the treatment of adenomyosis. The suppression of aromatase in this tissue may explain the efficacy of Mirena® in halting the progression of endometriosis, since the seeding of aromatase-positive cells through retrograde menstruation may play an important role in the development of this disease.[8]

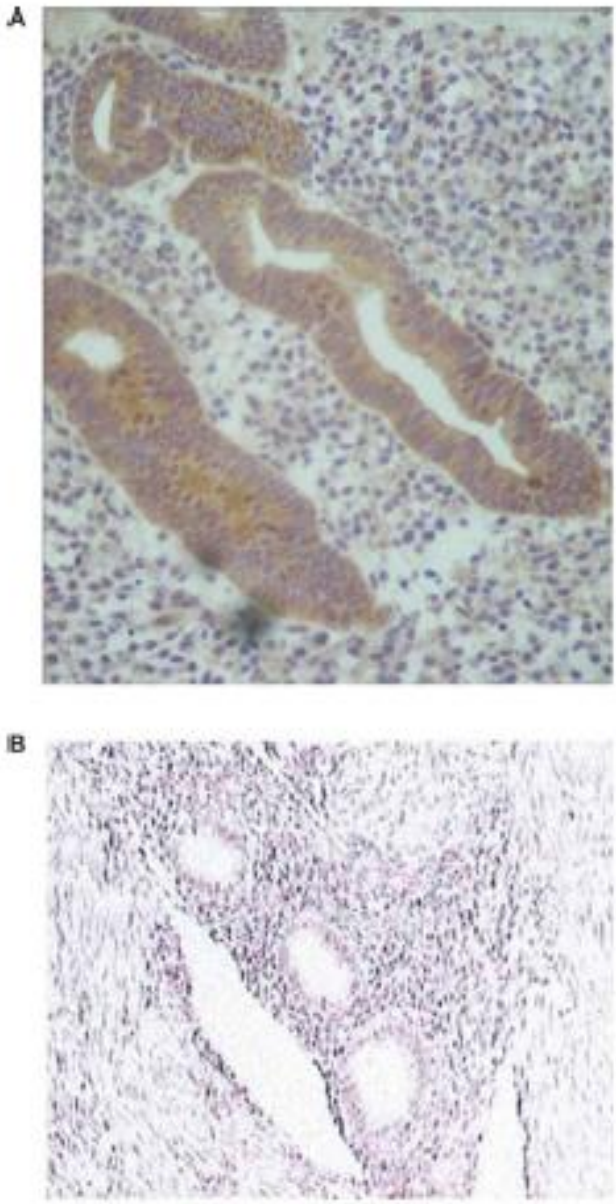

Figure 5 Positive aromatase expression in the eutopic endometrium of a patient with adenomyosis (A) and negative expression in a patient after using a Mirena® device for 5 years (B).

Aromatase expression in the endometrium is activated by exposure to prostaglandin E2, thus establishing a link between inflammation and the development of this pathology.[9] The greater Cox-2 expression in the endometrium of women using Mirena® who had not been submitted previously to ablation correlates positively with the occurrence of uterine bleeding, which is consistent with the role of prostaglandins and the ensuing inflammation in the pathogenesis of both menorrhagia and breakthrough bleeding during the use of oral contraceptives and the progestin-only pill.[10–12]

The rate of amenorrhea reported here during the fifth year of Mirena® use also compares favorably with rates reported previously by our group 1 year after insertion of this device.[5] These rates are also far superior to those achieved with the insertion of Mirena® alone in patients with adenomyosis, myomas, or menorrhagia.[13–15]

The presence of adenomyosis specimens removed at hysterectomy from patients with persistent bleeding following the insertion of Mirena® or microwave endometrial ablation[16–18] is noteworthy and suggests that the presence of

Table 2 The effect of Mirena® and endometrial resection on aromatase expression in the glandular epithelium of adenomyosis lesions

Group	Positive endometrium
Group A: Mirena® alone	8/22 36%
Group B: Mirena® after endometrial resection	1/24 4%
Group C: untreated adenomyosis	32/43 74%

Notes: Chi-square test: difference between groups A and B: $P < 0.01$; difference between groups A and C: $P = 0.02$; difference between groups B and C: $P < 0.0001$.

adenomyosis increases the risk of failure of both forms of treatment due to the resumption of menorrhagia, probably caused by persistent Cox-2 activation and the less effective inhibition of aromatase in patients not submitted to endometrial resection. Another factor which may contribute to the high incidence of amenorrhea in patients using Mirena® after endometrial resection would be the direct exposure of the ectopic glands in the myometrium to levels of levonorgestrel higher than those achieved when the endometrium is intact. This would lead to a more effective inhibition of both aromatase and Cox-2 in the ectopic glands deeply embedded in the myometrium, resulting in better rates of amenorrhea. This is in agreement with the findings of a previous study showing that the levonorgestrel levels in the endometrium of patients using Mirena® are many times higher than levels in the myometrium, which are similar to those found in extrauterine tissue.[20] This suggests that the intact endometrium may act as a barrier, preventing the diffusion of levonorgestrel to the underlying myometrium, which receives levonorgestrel only through the systemic circulation. This may explain why the rates of amenorrhea in the present study were far higher in the group of women with adenomyosis submitted to endometrial resection compared to those using Mirena® alone. In the latter, because the endometrium remains intact, the levonorgestrel levels in the myometrium may not be high enough to effectively suppress both aromatase and Cox-2 expression in the deeply embedded ectopic glands. This would explain the increased efficacy of Mirena® in inhibiting these enzymes in uteri with adenomyosis when the endometrium is removed prior to insertion of the device. Although this was not a subject of the present study, it is likely that endometrial resection may improve the diffusion of levonorgestrel into the myometrium; however, for the present moment this hypothesis has to be considered speculative. This may also explain the reduction in breakthrough or irregular bleeding with this combined method compared to rates reported in the literature when Mirena® is used without prior endometrial resection in patients with adenomyosis or myomas.[21] The decrease in aromatase expression in adenomyosis uteri bearing a Mirena® device seems to occur even when Cox-2 expression is not suppressed by the treatment. Since prostaglandins are an important inductor for the activation of the aromatase gene in the endometrium, a direct effect of the progestin inhibiting gene transcription would be the likely mechanism.[8,9,11]

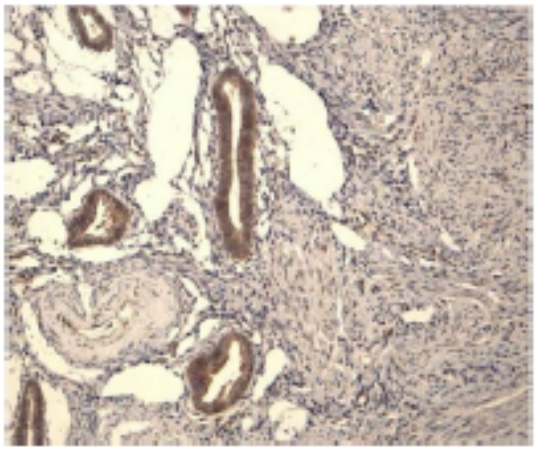

Figure 6 Cox-2 expression in a patient with adenomyosis during the proliferative phase.

The combination of Mirena® with endometrial resection represents a viable and effective alternative therapy to hysterectomy for the management of menorrhagia and dysmenorrhea in patients with adenomyosis and menorrhagia. In patients undergoing endometrial resection alone or Mirena® insertion alone for the treatment of menorrhagia, it is important to remember that success rates are lower than when both methods are used together. This has been shown in a previous study conducted by our group to compare the amenorrhea and success rates of the combination of Mirena® and endometrial resection versus endometrial resection alone for the treatment of adenomyosis-related menorrhagia.[6] Similar results were also reported more recently by another group.[22]

In conclusion, Mirena® is an effective option for the suppression of aromatase and Cox-2 expression in the endometrium of patients with adenomyosis; however, inhibition appears to be greater when this device is inserted immediately following endometrial resection. Nevertheless, it should be noted that endometrial resection is an alternative to hysterectomy and is not an option for women who still want to preserve their fertility. In this case, particularly when used to treat adenomyosis-related menorrhagia, the insertion of Mirena® alone is a viable option, since fertility returns promptly after removal of the device.[23]

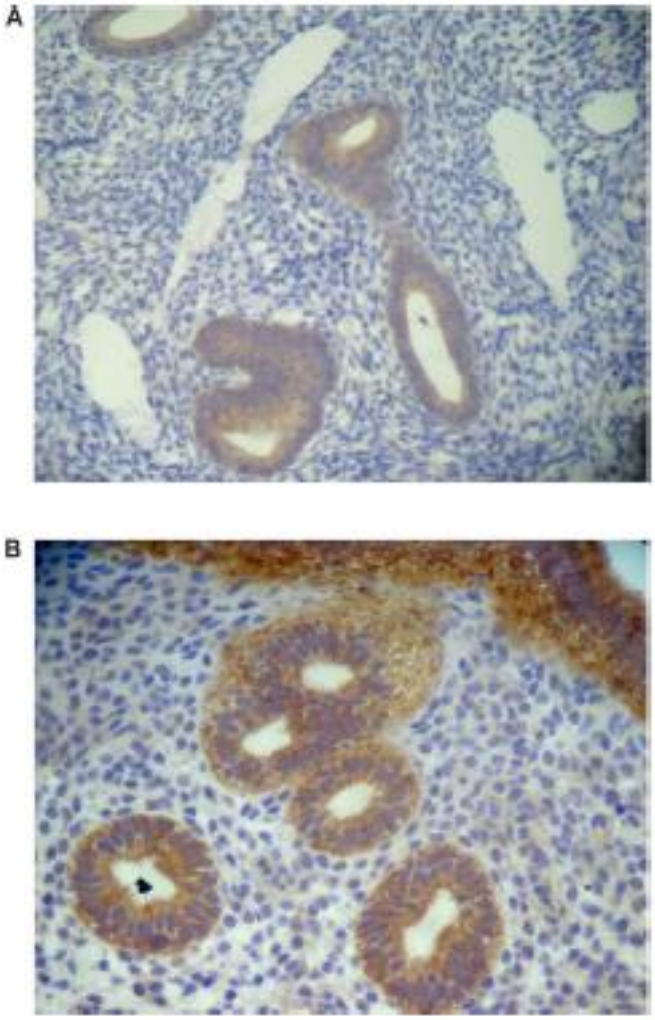

Figure 7 Immunohistochemical grading of Cox-2 expression in the eutopic endometrium of patients with adenomyosis. (**A**) 1+ expression and (**B**) 2+ expression.

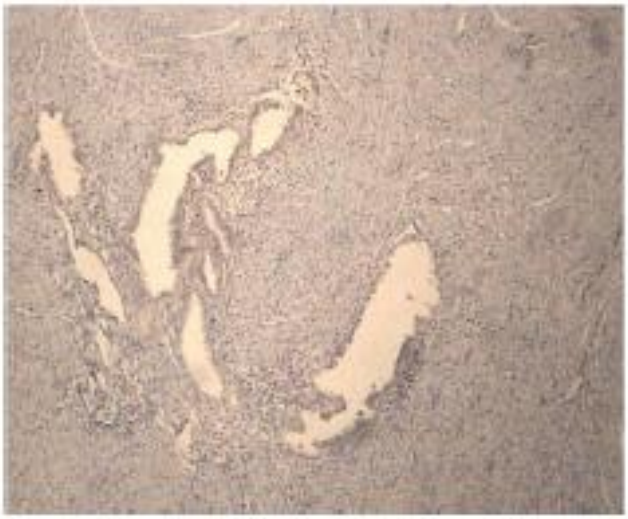

Figure 8 Cox-2 negative expression in a patient using Mirena® who had been previously submitted to an endometrial resection.

Table 3 Mean Cox-2 expression in the endometrium of patients with adenomyosis in use of Mirena®

Group	Mean Cox-2 expression
Group A: Mirena® alone	1.6 ± 0.9 ($n = 22$)
Group B: Mirena® after endometrial resection	0.7 ± 1 ($n = 24$)
Group C: untreated adenomyosis	1.7 ± 0.7 ($n = 43$)

Notes: Student's t-test: difference between groups A and B: $P < 0.0001$; difference between groups A and C: $P = 0.02$; difference between groups B and C: $P < 0.0001$.

Because of the negative impact on quality of life caused by the pain and excessive bleeding associated with adenomyosis, hysterectomy remains the standard treatment for this pathology.[24] Development of alternative methods of treatment capable of offering effective relief of these symptoms without constituting a major surgical procedure would be desirable. In this aspect, the combination of Mirena® with endometrial resection fulfills these requisites, since it is a less invasive procedure compared to hysterectomy and its success rate is high. However, further prospective studies must be conducted before changes in standard medical practice can be contemplated.

Disclosure

All authors report no conflicts of interest in this work.

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Artigo nº 2

Correlation between aromatase expression in the eutopic endometrium of symptomatic patients and the presence of endometriosis.

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ORIGINAL RESEARCH

Correlation between aromatase expression in the eutopic endometrium of symptomatic patients and the presence of endometriosis

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Objective: To investigate whether aromatase expression in the eutopic endometrium correlates with the presence and severity of endometriosis in patients with infertility and/or dysmenorrhea undergoing laparoscopy and hysteroscopy.

Patients: The study involved 106 patients of reproductive age with symptoms of dysmenorrhea and infertility. Sixteen endometriosis-free asymptomatic patients were used as a control group.

Methods: Concomitant laparoscopy and hysteroscopy was carried out in all cases. An endometrial biopsy was taken to determine aromatase p450 expression by immunohistochemistry. Endometriosis was staged according to the American Society of Reproductive Medicine classification.

Results: Endometriosis was diagnosed by laparoscopy in 92/106 symptomatic patients. In this group, aromatase expression was detected in the eutopic endometrium of 66/92 patients with endometriosis (72%) and in 13/14 (95%) patients in the symptomatic, endometriosis-free group ($P = 0.09$). Aromatase expression was not detected in any patients from the control group. In the endometriosis group, aromatase expression was detected in the eutopic endometrium of 28/45 patients (62%) with American Society of Reproductive Medicine classification stage I of the disease, in 11/14 patients (78%) with stage II, 14/20 patients (70%) with stage III, and in 12/13 patients (92%) with stage IV; however, the difference was only statistically significant between stages I and IV ($P = 0.04$).

Conclusion: Aromatase expression in the endometrium was associated with the presence of dysmenorrhea and infertility irrespective of the presence of endometriosis. When endometriosis was present, however, there was a tendency for aromatase expression to be positively correlated with dysmenorrhea severity.

Keywords: aromatase, endometrium, endometriosis, Cox-2, dysmenorrhea

Introduction

The onset of clinical symptoms associated with endometriosis may predate clinical diagnosis by many years.[1] Although this may simply reflect a lack of sensitive, non-invasive methods capable of diagnosing this pathology at an early stage, it is also possible that the enzymatic and inflammatory changes that signal the development of endometriosis may be initiated in the endometrium before the disease is established in the pelvis. These functional changes may involve the upregulation of enzymes related to estrogen synthesis that are overexpressed in the eutopic endometrium of patients with endometriosis, where they may play a pivotal role not only in the implantation failure associated with this pathology but also in the onset of menstrual symptoms.[2-4]

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It is known that both endometriotic lesions and the eutopic endometrium are able to express aromatase, a key enzyme in the transformation of androgens into estrogens. The release of these enzymes is stimulated by inflammatory mediators such as the prostaglandins produced by the action of cyclooxygenase-2 (Cox-2).[2,3] The aggressiveness of endometriosis appears to correlate with the intensity of the expression of this enzyme in lesions, with the most aggressive occurring in red peritoneal lesions.[2] A correlation was also found between the severity of endometriosis and the intensity of Cox-2 expression in both lesions and the eutopic endometrium, thus suggesting a role of locally produced estrogens in the upregulation of this enzyme.[3-5]

Nevertheless, the role played by aromatase and Cox-2 expression in the eutopic endometrium in relation to the development of endometriosis and in the intensity of menstrual-related symptoms is not totally understood. It was recently suggested that endometriosis may commence as an endometrial disease provoked by the aberrant expression of aromatase in this tissue, as this appears to play a pivotal role in its survival in ectopic locations.[4] Gene array studies have shown that the expression profiles of endometriosis lesions and the eutopic endometrium are similar, which supports the hypothesis that endometriosis is an endometrial disease.[6] It is biologically plausible that the presence of aromatase activity in the endometrium is pivotal for the development of endometriosis by blocking phagocytosis by activated macrophages and may also be responsible for the exacerbation of menstrual symptoms whose onset predates the diagnosis of this pathology.[1,4,7] In this hypothesis, endometriosis would be a late consequence of enzymatic changes that occur in the endometrium prior to its establishment outside the uterus, which could explain the lag that is often reported between the onset of symptoms and diagnosis of the disease.

This hypothesis could be tested by examining patients with severe dysmenorrhea or infertility to determine whether laparoscopy reveals endometriosis despite aromatase expression already being positive in the endometrium. In the present study, aromatase expression was determined in the eutopic endometrium of patients with severe menstrual symptoms and/or infertility who underwent laparoscopy and hysteroscopy as a part of their diagnostic work-up.

Methods

A total of 106 patients of reproductive age (range = 18–47 years) received a laparoscopy and hysteroscopy with endometrial biopsy that was carried out using a 4 mm Karman curette attached to a 10 mL syringe to produce a vacuum, which is the standard procedure for the evaluation of menorrhagia, severe dysmenorrhea, or infertility in the day hospital where the current study was carried out. All patients included in this group had reported at least two of these three symptoms for periods ranging from 2 to 8 years. The control group consisted of sixteen patients who received a laparoscopy for reasons unrelated to infertility or severe dysmenorrhea and who were found to be endometriosis-free during the examination. The indications for laparoscopy in this group were either benign non-endometriotic ovarian cysts (seven patients) or tubal ligation (nine patients). In these patients, an endometrial biopsy was performed at the time of the laparoscopy using a 4 mm Karman curette.

The patients enrolled in the study gave written informed consent for immunohistochemical studies to be carried out on the endometrial biopsy specimens obtained as part of their standard medical care. This study was approved by the institution's internal review board.

Laparoscopies were performed either during the proliferative phase (n = 72) or in the secretory or menstrual phase (n = 34), since the timing of the procedure depended on the availability of the surgical theater and the specifics of the institution's waiting list. The laparoscopy reports and color images were retained in the patients' files for further evaluation if necessary. During the laparoscopy procedure, the surgical team ascertained the presence of endometriosis and graded the stage of the disease in accordance with the American Society of Reproductive Medicine (ASRM) classification.[8] Using laparoscopic classification criteria, the patients with endometriosis were divided into four groups according to the severity of their lesions, using the ASRM classification of minimal (stage I), mild (stage II), moderate (stage III), or severe (stage IV). The severity of menstrual symptoms such as dysmenorrhea, the presence or absence of infertility, and the reason for laparoscopy were recorded in the patients' files.

Endometrial samples were fixed in 10% formalin before being assessed. Immunohistochemistry was performed following antigen retrieval to detect the presence of aromatase p450. Aromatase expression was investigated using the commercially available monoclonal antibody MCA2077 clone H4 (Serotech, Raleigh, NC). Antigen retrieval was performed using Tris-EDTA buffer (pH 8.0; Sigma-Aldrich, São Paulo, Brazil). The reaction was revealed using the streptavidin-biotin method. The presence of aromatase expression was rated as positive if there was any detectable staining reaction in the glandular epithelium or negative when no reaction was observed. Placental tissue and an atrophic endometrial sample were used as positive

and negative controls, respectively, in all immunostaining reactions for aromatase p450. Statistical analysis was performed using the one-tailed chi-square test for differences in percentages, using an unnamed online interactive calculation tool for chi-square tests of goodness of fit and independence, which is hosted at http://quantpsy.org. Results were considered statistically significant at $P < 0.05$.

Results

In the group of patients with severe dysmenorrhea, endometriosis was diagnosed in 90/106 patients. In the remaining 16 cases, the pelvis was found to be normal during the laparoscopy.

Aromatase expression in the eutopic endometrium was detected by immunohistochemistry mainly in the glandular epithelium (Figure 1). The staining reaction was positive in 66/92 patients (72%) with endometriosis and symptoms of dysmenorrhea and/or infertility, and there were no significant differences between the phases of the patient's menstrual cycle. In symptomatic patients who were found to have normal pelvic results during the laparoscopy, aromatase expression was still positive in the endometrium in 13/14 cases (95%), though this difference was not statistically significant ($P = 0.09$). In the asymptomatic control group without endometriosis, however, aromatase expression was negative in the eutopic endometrium in all cases.

In the subset of symptomatic patients with a laparoscopic diagnosis of endometriosis, the percentage of endometrial samples positively expressing aromatase in the glandular epithelium showed a trend toward greater positivity as the severity of the disease increased in accordance with the ASRM criteria. Aromatase expression was detected in the eutopic endometrium of 28/45 patients with stage I endometriosis (62%), 11/14 patients with stage II (78%), 14/20 patients with stage III (70%) and 12/13 patients with stage IV endometriosis (92%). However, only the difference between stages I and IV of the disease was statistically significant ($P = 0.04$) (Table 1).

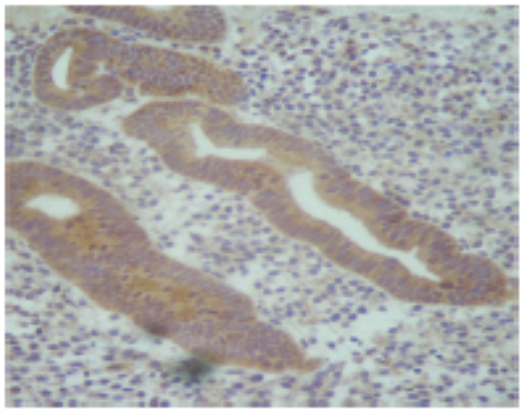

Figure 1 Aromatase expression in the eutopic endometrium detected by immunohistochemistry.

Discussion

The present study not only showed an association between the presence of aromatase expression in the eutopic endometrium and the severity of endometriosis lesions at laparoscopy but also suggests that this expression was already present in symptomatic patients in whom pelvic endometriosis was not found. These findings agree with the hypothesis that endometriosis may commence as an endometrial disease resulting from functional changes in the endometrium before the disease establishes outside the uterus.

The presence of similar epigenetic changes in the eutopic endometrium and the endometriotic lesions further corroborates the hypothesis that endometriosis is an endometrial disease.[6] The positive correlation between mRNA levels in the eutopic endometrium and the severity of endometriosis and adenomyosis also supports this hypothesis.[9] Whether endometriosis starts as an endometrial disease before establishing itself elsewhere in the pelvis[6,7] cannot be proven by the present study, but previous work shows that the onset of symptoms associated with this pathology may commence many years prior to its diagnosis, which support this hypothesis.[1] The occurrence of severe dysmenorrhea for many years prior to a diagnosis of endometriosis may reflect these initial endometrial changes favoring the upregulation of both estradiol and prostaglandin production in this tissue.[4,6,7] The occurrence of such symptoms in patients with a normal pelvis at laparoscopy, as shown in the present study, may suggest that these functional changes in the endometrium are not only associated with the onset of endometriosis-related symptoms but that they may occur prior to the diagnosis of any pelvic pathology by laparoscopy. Functional endometrial changes leading to an increase in aromatase expression

Table 1 Aromatase expression in the eutopic endometrium and the laparoscopic classification of endometriotic lesions according to American Society of Reproductive Medicine classification

Type of lesion	Aromatase positive
Stage I	28/45 (62%)
Stage II	11/14 (78%)
Stage III	14/20 (70%)
Stage IV	12/13 (92%)

Notes: Chi-square test: stages I and IV, $P < 0.05$. The other differences were not statistically significant.

may therefore predate the development of endometriosis, initially provoking dysmenorrhea and possibly infertility before triggering the development of the disease outside the uterus.[4] It still remains to be determined whether this group of symptomatic patients represents a high-risk population for the development of endometriosis. In patients with an established diagnosis of endometriosis, however, endometrial positivity for aromatase tends to increase with the severity of the disease. This may be due to the greater possibility of spontaneous regression of early stage endometriosis, which may occur because of reduced aromatase activity in the eutopic endometrium.

Although aromatase expression in the eutopic endometrium may be pivotal for the onset and progression of endometriosis, it has yet to be clarified how this enzyme affects the development of endometriosis. The role played by estrogens in inhibiting immune surveillance by activated macrophages has been suggested as one of the likely mechanisms by which aromatase affects the course of endometriosis.[4,7,10] This local estrogen production in the endometrial cells shed to the pelvis in retrograde menstruation may favor their survival in these ectopic locations by directly preventing their destruction by activated immune cells through the inhibition of the phagocytosis mechanism.[10–13]

The presence of aromatase in the eutopic endometrium may therefore represent the initial step in the development of endometriosis and could be necessary for the development of lesions outside the uterus.[7] The similar genetic alterations found between endometriotic lesions and the respective eutopic endometrium also indicate a common histological origin.[7]

The current study's finding that aromatase expression is detectable by immunohistochemistry in the endometrium of symptomatic, endometriosis-free patients agrees with the hypothesis that this enzyme initially exacerbates dysmenorrhea or causes infertility prior to facilitating the development of endometriosis elsewhere in the pelvis. Nevertheless, a temporal relationship cannot be established solely from these data. A previous study has shown that aromatase expression in the endometrium reduces implantation rates in patients undergoing in vitro fertilization, however, which may help to explain the lower fertility rate of women with endometriosis.[14]

The presence of aromatase and other enzymes related to estrogen metabolism in the eutopic endometrium will ultimately create a hyperestrogenic milieu, resulting in increased prostaglandin production, particularly because Cox-2 expression is upregulated by estradiol in this tissue.[6–8,12,13] This would explain why the pelvis of a patient with severe dysmenorrhea or infertility may be normal, as was reported in the current study – aromatase expression in the eutopic endometrium may be sufficient to provoke these symptoms by enhancing the production of proinflammatory prostaglandins in this tissue. However, the possibility that some of these endometriosis-free patients have adenomyosis cannot be completely excluded in this study, despite the fact that myometrial findings were normal at transvaginal sonography.

Estrogens produced locally in the endometriotic lesions and eutopic endometrium result in the mechanism of decreased immune surveillance and enhanced tolerance found in endometriosis,[14] which may explain the inverse relationship between estrogen levels and impaired NK-cell function found as a function of the severity of the disease.[15] Local estrogen production would also stimulate the production of VEGF, growth factors, and inflammatory cytokines by immune cells, thus favoring the progression of endometriosis.[12,15,16] The blockade of phagocytosis by the local production of estrogens and the concomitant stimulation of growth and inflammatory factors would explain the paradoxical effects of macrophages in endometriosis, which seem to interact with rather than destroy these ectopic endometrial cells, thus enhancing their growth and angiogenesis.[12,17]

In conclusion, a vicious cycle of enhanced inflammation and local estrogen production mediated by Cox-2 and aromatase expression in the eutopic endometrium of endometriosis patients may play a pivotal role not only in determining the clinical course of this disease but also in defining the intensity of its debilitating symptoms. In the pelvic cavity, the ensuing inflammatory reaction provoked by the arrival of endometrial cells will further exacerbate their preexisting aromatase activity, thus augmenting local estrogen production and halting phagocytosis by activated macrophages.[6,7]

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Disclosure

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Artigo nº 3

Activation of NF-kappaB and COX-2 expression is associated with breakthrough bleeding in patients using oral contraceptives in extended regimens.

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CONTRACEPTION

Activation of NF-κB and COX-2 expression is associated with breakthrough bleeding in patients using oral contraceptives in extended regimens

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Abstract

The objective of the present study was to determine whether there is an increase in endometrial inflammation associated with the occurrence of breakthrough bleeding in patients using an oral contraceptive in extended regimens. The presence of nuclear factor NF-κB and Cox-2 expression was determined by immunohistochemistry in endometrial samples removed by hysteroscopy from patients with breakthrough bleeding during continuous use of an oral contraceptive containing gestodene. All patients had a history of menorrhagia associated or not with the presence of uterine pathology. The percentage of endometria showing a positive staining reaction for NF-κB in cell nuclei was significantly higher in patients with breakthrough bleeding than in those with amenorrhea. Cox-2 expression in the endometrium was also significantly more frequent in patients with breakthrough bleeding. The occurrence of breakthrough bleeding in patients with uterine pathology using combined oral contraceptives is associated with the activation of endometrial inflammation through the NF-κB pathway.

Keywords: *NF-κB, breakthrough bleeding, inflammation, extended regimen, Cox-2*

Introduction

Inflammation appears to play an important role in the mechanism of breakthrough bleeding during the use of progestins. For instance, the levels of inflammatory mediators such as interleukin 13 and 15 are elevated in the endometrium of levonorgestrel implant users who develop breakthrough bleeding [1]. In fact, the enhanced endometrial inflammation observed in these patients with breakthrough bleeding seems to play a crucial role in the mechanisms responsible for generating abnormal angiogenesis and the ensuing uterine bleeding [2]. The triggering event for the onset of breakthrough bleeding at endometrial level may be the downregulation of progesterone receptors induced by the constant exposure to progestins [3]. The antagonism of progesterone action at receptor level is known to result in an upregulation of key local inflammatory mediators including NF-κB, interleukin-8 (IL-8), monocyte chemotactic peptide-1 (MCP-1) and cyclooxygenase-2 (COX-2) among others in the decidua [4]. Progesterone and progestational compounds, on the other hand, may attenuate the expression of inflammatory mediators such as IL-8 by reducing TNF-α-induced NF-κB activation in endometriotic stromal cells [5]. NF-κB is an important transcription factor for the activation of genes related to the regulation of the inflammatory cascade, as its translocation to cell nuclei from the cytoplasm reservoir and the binding to DNA triggers the transcription of these genes [6,7]. In the endometrium, the activation of NF-κB and its subsequent migration to cell nuclei is inhibited by progesterone, which behaves as an anti-inflammatory hormone exhibiting a mechanism of action that bears a great similarity to cortisone [5,8]. The activation of progesterone receptors in the endometrium is, therefore, important in curtailing the inflammatory reaction in

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the uterus, a mechanism that plays an important role in pregnancy [8]. The observation that progestins are able to diminish the expression of inflammatory mediators in the endometrium through mechanisms involving the suppression of NF-κB activation suggests that activation of this transcription factor may play a role in the mechanism of breakthrough bleeding in patients using oral contraceptives.

Methods

Collection of human endometrial tissue

This is an observational study carried out in paraffin-embedded tissue samples obtained from 53 patients submitted to endometrial resection or biopsy in our institute between January 2007 and March 2009. These patients had a history of menorrhagia and were using an oral contraceptive containing 30 mcg of ethinylestradiol and 75 mcg of gestodene (Gestinol, Libbs Farmacêutica, Brazil) in a continuous regimen, as prescribed by their attending physician to reduce bleeding and thin the endometrium prior to hysteroscopy. This specific formulation of oral contraceptive has been approved by the Brazilian regulatory agency (ANVISA) to be used continuously to suppress menstruation. All patients were premenopausal and were in the 34- to 52-year age-bracket. Endometrial resection was carried out in these patients using the bipolar resectoscope (Versapoint Gynecare, NJ, USA), as previously described, to control bleeding [9]. Using this technique, it is possible to remove not only submucous myomas but also, when indicated, the surrounding endometrium and approximately 5 mm of the myometrium, allowing the pathologist to make a diagnosis of adenomyosis. There were no statistically significant differences in the frequency of myomas, adenomyosis and endometrial polyps between the group of women with amenorrhea and the group with breakthrough bleeding, the percentage of women with these pathologies being, respectively, 17, 42 and 17% in the amenorrhea group and 22, 37 and 15% in the breakthrough bleeding group. The percentage of women with a normal uterus in the amenorrhea group was 25% compared to 27% in the group of women with breakthrough bleeding.

The medical records of these patients revealed that prior to the initiation of oral contraceptive use progesterone levels were compatible with ovulatory cycles and thyroid function was normal. The duration of use of Gestinol 28 varied from 2 to 6 months. At the time of hysteroscopy, 41 patients were in amenorrhea while the remaining 12 were experiencing breakthrough bleeding. All surgical procedures were carried out as day-hospital procedures under propofol sedation and paracervical block. The tissue samples were fixed in formalin 10% before being sent to pathology. The patients gave their informed consent for the immunohistochemical studies to be carried out in order to determine NF-κB and Cox-2 in the paraffin-embedded endometrial tissue samples removed during the hysteroscopic procedure.

Immunohistochemical staining

Immunohistochemistry was carried out following antigen retrieval to detect the presence of Cox-2 and NF-κB. Commercially available monoclonal antibodies manufactured by Novocastra (Newcastle, UK), (Clone 4H-12) were used for Cox-2 staining. Antigen retrieval was carried out using the Tris-EDTA buffer at pH 8.0. For NF-κB immunohistochemical staining, the primary antibody was obtained from Zymed (San Francisco, USA) (Clone P65) and was used at a dilution of 1:450 in a citrate buffer pH 6.0. The reaction was revealed using the streptavidin-biotin method. The presence of nuclear NF-κB expression was rated either as positive if there were cell nuclei showing any detectable staining reaction or negative when no reaction was observed. The presence of Cox-2 staining reaction in the glandular epithelium was rated as positive if more than 10% of the glands were positive.

Statistical analysis

Statistical analysis was carried out using the StatsDirect software program, version 2.3.8 (StatsDirect Ltd. Cheshire, UK, 2004). The χ^2 test was used to compare proportions of positive Cox-2 and nuclear NF-κB endometria between the group of women with amenorrhea and the group with breakthrough bleeding. Significance was established as $p < 0.05$.

Results

Histological analysis

Endometrial histology in patients using oral contraceptives containing 75 mcg of gestodene and 30 mcg of ethinylestradiol revealed a pattern characterized by the presence of atrophic glands with stroma showing either a decidual or quiescent pattern. In all the patients with breakthrough bleeding at the time of hysteroscopy, histology of the endometrium was characterized by the presence of a decidual reaction in the stroma while the glandular epithelium was atrophic. The same histological pattern was also found in 30/41 amenorrheic oral contraceptive users (73%) at the time of hysteroscopy. In the remaining patients (11/41, 27%), the endometrium was quiescent, showing atrophic glands and no histological evidence of decidual reaction in the stroma, which was cellular and compact.

NF-κB activation

In this sample of patients with gynecological pathologies and in continuous use of an oral contraceptive, there was an association between the presence of positive endometria for nuclear NF-κB and the occurrence of breakthrough bleeding. Nuclear NF-κB was detected simultaneously in the decidual stroma and atrophic glandular epithelium of a significantly higher percentage of patients with breakthrough bleeding compared to the group of patients with amenorrhea, irrespective of whether a decidual reaction was present or not in the stroma ($p < 0.0001$) (Figure 1). In the group with amenorrhea, the percentage of endometria positive for nuclear NF-κ was low and there were no statistically significant differences between patients with a decidual stroma and those with a quiescent stroma. These results are shown in Table I. NF-κB staining reaction in the cytoplasm, on the other hand, was always present in the stroma and glands of the endometrium and was unaffected by the presence of breakthrough bleeding.

Cox-2 expression

The presence of breakthrough bleeding was associated with a higher percentage of positive staining for Cox-2 in the glandular epithelium of endometria showing a decidual reaction when compared with the amenorrheic group with the same histology ($p = 0.004$) (Figure 2). In patients with an atrophic endometrium and amenorrhea, on the other hand, this difference did not reach statistical significance ($p = 0.09$) although the percentages of Cox-2-positive glands were lower in the amenorrhea group compared to the breakthrough bleeding group. These results are summarized in Table II.

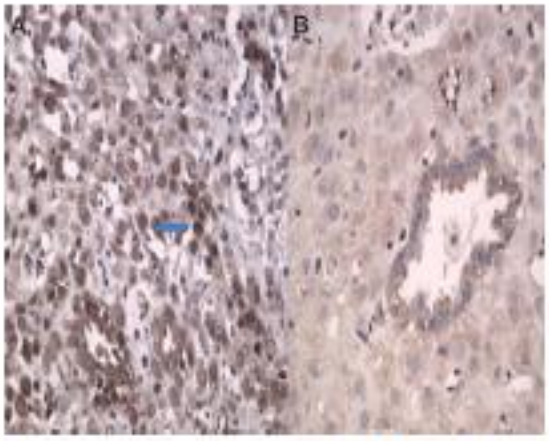

Figure 1. Presence of nuclear NF-κB staining in the decidual stroma of a patient with breakthrough bleeding during the use of an oral contraceptive containing gestodene in an extended regimen (A). Note the absence of nuclear staining reaction in the endometrium of a patient who developed amenorrhea following the use of this oral contraceptive regimen (B).

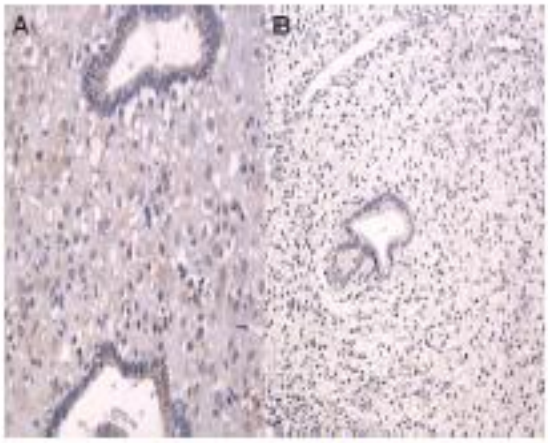

Figure 2. Presence of positive Cox-2 staining in the endometrial glands of a patient with breakthrough bleeding during the use of an oral contraceptive containing gestodene in an extended regimen (A). Note the absence of Cox-2 expression in the endometrium of a patient who developed amenorrhea while using the same contraceptive formulation (B).

Table I. Frequency of positive nuclear NF-κB in the endometria of patients with breakthrough bleeding during the use of an oral contraceptive in an extended regimen according to histological pattern.

	Amenorrhea		Bleeding	
	n	%	n	%
Decidual stroma/atrophic gland	2/30	7	9/12	75*
Non-decidual stroma/atrophic gland	1/11	10**	0/0	0

χ^2 test:
*$p < 0.0001$ when compared to decidual amenorrhea.
**$p < 0.01$ when compared to decidual bleeding.

Table II. Frequency of positive reaction for Cox-2 in the endometria of patients with breakthrough bleeding during the use of an oral contraceptive in an extended regimen according to the histological pattern.

	Amenorrhea		Bleeding	
	n	%	n	%
Decidual stroma	9/27	33*	10/12	83**
Non-decidual stroma	4/10	40***	0/0	

χ^2 test:
*Not different from non-decidual amenorrhea; $p = 0.99$.
**Significantly greater than decidual amenorrhea; $p = 0.004$.
***Not significantly different from decidual bleeding; $p = 0.09$.

Discussion

The present study shows that breakthrough bleeding during the continuous use of oral contraceptives containing gestodene is associated with a significant increase in positive nuclear staining of NF-κB in the endometrium. This suggests that a flare-up of inflammation at endometrial level is associated with the initiation of breakthrough bleeding in these patients, although not all the mechanisms involved in this process are as yet fully understood. Our findings showing the presence of NF-κB in the cytoplasm of endometrial cells during the use of oral contraceptives suggest that the main effect of a progestin is to keep the reservoir of this transcriptional factor dormant in the endometrium, only to be activated when this inhibitory action is somewhat diminished. This is what occurs when the effect of progestin wanes off either as a result of its removal from circulation or by the downregulation of its receptor in the endometrium [3,10,11]. Whenever this occurs, NF-κB is activated and the ensuing inflammation results in endometrial bleeding and stimulation of Cox-2 expression and other inflammatory cytokines [10,12]. This hypothesis is in agreement with previous reports showing that progesterone withdrawal triggers the translocation of NF-κB to cell nuclei where, following its binding to the response elements in the DNA, there is an increased transcription of inflammation-related genes such as Cox-2 [10].

Cox-2 will, in turn, stimulates the synthesis of prostaglandins which then affect the intensity of menstrual flow and pain [13]. Another prostanoid also produced by Cox-2 enzymatic action is prostacyclin (PGI2), which is a potent vasodilator and an inhibitor of platelet aggregation [14]. As a result of NF-κB activation in the endometrium, Cox-2 and other genes are turned on, thus resulting in enhanced inflammation, endometrial breakdown and bleeding. The enhanced expression of other proinflammatory mediators such as interleukin 13 and 15 observed in the endometria of patients experiencing episodes of breakthrough bleeding during the use of levonorgestrel implants is also in agreement with the hypothesis that an increased inflammatory reaction is the cause of irregular uterine bleeding [1].

The findings reported here are also in agreement with recent reviews showing that nonsteroidal anti-inflammatory drugs (NSAID) are more effective than placebos for the treatment of heavy menstrual flow [15]. The presence of irregular bleeding in depot-medroxyprogesterone users was also more effectively controlled with valdecoxib, a Cox-2 inhibitor, than with placebo [16]. Prostacyclin is a potent vasodilator mainly produced by Cox-2 and the blockade of its synthesis by selective Cox-2 inhibitors will cause vasoconstriction and a reduction in endometrial bleeding [14,16]. The presence of increased Cox-2 in women, who bleed while in use of oral contraceptives, as reported here, confirms previous observations [17] and also confers biological plausibility for the use of these inhibitors in the treatment of uterine breakthrough bleeding during progestin therapy [16]. The enhancement of the inflammatory reaction in the endometrium and the occurrence of breakthrough bleeding in oral contraceptive users are, therefore, two interconnected events triggered by the decrease in the antiinflammatory effect of the progestin on the uterus. Because inflammatory cytokines and prostaglandins constitute the underlying mechanism in triggering endometrial bleeding [13], the present findings that NF-κB was detected in the nuclei of endometrial cells in patients with breakthrough bleeding but not in the amenorrheic women suggest that inflammation is constitutively activated under these circumstances, causing bleeding and pain similar to symptoms found in patients with endometriosis [18].

During menstruation, endometrial bleeding is triggered by the fall in progesterone production associated with the regression of the corpus luteum. In fact, when endometrial stromal cells were incubated with MG132, a potent inhibitor of NF-κB activation, the increases in both PGF2 α production and COX-2 mRNA expression during estrogen–progesterone withdrawal failed to occur [10]. This supports the conclusion that estrogen–progesterone withdrawal stimulates COX-2 expression through NF-κB activation, thus suggesting that progesterone withdrawal induces the transformation of the decidualization-associated hemostasis of the endometrium in the luteal phase to the hemorrhagic milieu of menstruation through inflammatory mediators [10,19]. However, when progestins are used in a continuous fashion as in extended regimen oral contraception, the triggering factor for NF-κB translocation is not hormone withdrawal but probably the downregulation of progesterone receptors in the endometrium induced by the continuous exposure to progestin present in these formulations [3]. In fact, short-term exposure to mifepristone in new starters of DMPA was found to increase the expression of endometrial receptors for estrogens and progesterone and promote cell proliferation, thus suggesting a suppressive receptor mediated effect of a progestin on its own receptor [11]. Likewise, in patients using a monophasic oral contraceptive, stromal PR staining was significantly reduced when compared to the secretory phase [20]. In levonorgestrel implant users, the levels of progesterone receptor mRNA in the endometrium of patients with amenorrhea were significantly higher in subjects experiencing amenorrhea than in those with breakthrough bleeding [3]. This supports the idea that the depletion of progesterone receptors in endometrial cells induced by the constant exposure to

progestin leads to an enhancement of inflammation and bleeding through the activation of the NF-κB pathway. However, although immunohistochemistry is an adequate method for evaluating protein expression, validation by more precise techniques is required before a final conclusion can be reached.

Declaration of interest: Hugo Maia Jr. declares that he is a speaker on extended contraception for Libbs Farmacêutica Ltda. The other authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Artigo nº 4

Effect of oral contraceptives on vascular endothelial growth factor, Cox-2 and aromatase expression in the endometrium of uteri affected by myomas and associated pathologies.

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Original research article

Effect of oral contraceptives on vascular endothelial growth factor, Cox-2 and aromatase expression in the endometrium of uteri affected by myomas and associated pathologies

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Abstract

Background: The study was conducted to evaluate vascular endothelial growth factor (VEGF), Cox-2 and aromatase expression in the endometrium of uteri with myomas and other associated pathologies.
Study Design: Hysteroscopy was performed in 118 women of reproductive age with myomas and menorrhagia, 40 of whom were using a pill containing 75 mcg gestodene+30 mcg ethinylestradiol. Aromatase p450, VEGF and Cox-2 expression was detected using immunohistochemistry. Fisher's Exact Test and the Mann–Whitney test were used in the statistical analysis, with significance established at $p<.05$.
Results: In patients with myomas and menorrhagia, associated pathologies such as adenomyosis, endometrial polyps and endometriosis were found in 32%, 12% and 17% of cases, respectively. Aromatase, Cox-2 and VEGF expression was greater during the proliferative phase compared to the luteal phase of the cycle or following oral contraceptive use.
Conclusion: Endogenous progesterone or combined oral contraceptives are potent inhibitors of VEGF, aromatase and Cox-2 expression in the endometrium of patients with myomas and menorrhagia.

Keywords: Gestodene; VEGF; Aromatase; Cox-2; Myoma; Menorrhagia

1. Introduction

Oral contraceptives decrease uterine bleeding associated with myomas without stimulating their growth [1]. Their mechanism of action is complex and may involve not only the blockade of ovulation but also suppression of local estrogen production through a direct inhibitory effect of the progestin component on aromatase expression in the endometrium [2]. This effect was originally reported in studies carried out with gestodene; however, the suppressive effect in the endometrium has also been observed with other progestins such as medroxyprogesterone acetate and dydrogesterone [3]. Studies using human endometrium implanted in the peritoneum of nude mice have shown that the inhibitory effect of progestins on aromatase expression occurs at gene transcription level, decreasing the synthesis of mRNA for this enzyme [3]. When used in the form of a levonorgestrel-releasing intrauterine device, progestins can be efficacious in the treatment of myoma-associated menorrhagia, mainly through a local endometrial effect since this intrauterine system seldom blocks ovulation [4]. In this case, the decrease in the amount of menstrual bleeding was associated with a reduction in the expression of vascular endothelial growth factor (VEGF) in the endometrium of these patients [5]. However, it remains to be established

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whether a similar inhibitory effect on VEGF is also shared by other progestins such as gestodene that are effective when used in combined oral contraceptive pills to treat menorrhagia in patients with myomas [1,6]. The observation that VEGF mRNA levels in the endometrial stroma were higher during the proliferative phase than during the secretory phase suggests that endogenous progesterone may have an inhibitory effect similar to that of levonorgestrel on VEGF gene transcription [5,7]. In vitro studies carried out using cultures of endometrial stromal cells revealed that the addition of medroxyprogesterone acetate to the medium had an inhibitory effect on the release of VEGF from stromal cells [8]. Progesterone withdrawal, on the other hand, has been found to up-regulate VEGF mRNA in stroma cells in the premenstrual phase in the nonhuman primate [9]. Because oral contraceptives are effective in decreasing menorrhagia associated with myomas [6], it is important to determine their effects on VEGF expression in the endometrium, since increased levels of this angiogenic factor are detected in the endometrium of patients with menorrhagia, thus suggesting its pivotal role in the etiology of abnormal uterine bleeding [10]. However, other factors such as Cox-2 expression may also influence the intensity of uterine bleeding, since gene transcription for this enzyme correlates positively with the severity of menorrhagia [11]. In oral contraceptive users, Cox-2 expression in the endometrium is lower in comparison with the proliferative phase but not with the luteal phase of the menstrual cycle, thus suggesting an inhibitory role for progesterone and progestins in Cox-2 expression [12]. Since VEGF expression is also triggered by inflammatory mediators and since both estrogens and prostaglandins may exacerbate inflammation in the uterus while progesterone exerts a contrary effect [2,13–15], it is important to evaluate whether the use of combined oral contraceptives interferes with the expression of angiogenic factors such as VEGF and Cox-2. In this article, the effects of an oral contraceptive containing gestodene on aromatase, VEGF and Cox-2 expression were investigated in the endometrium of patients with menorrhagia caused by the presence of myomas alone or in association with other gynecological pathologies. The aim of the present study was to evaluate the changes in the expression of angiogenic factors in the endometrium of oral contraceptive users and their correlation with the noncontraceptive benefits in the treatment of menorrhagia caused by myomas [6].

2. Materials and methods

This is a retrospective observational study carried out in paraffin-embedded endometrial tissue samples obtained from 118 women of reproductive age (range, 35–49 years) with symptoms of menorrhagia and an ultrasonographic diagnosis of myomas or other associated pathologies. The present study does not involve any direct intervention in patients or any change to the standard treatment for menorrhagia, and it was approved by the Institutional Review Board. Endometrial resection was performed in these patients as an alternative to hysterectomy using the bipolar resectoscope (Versapoint, Gynecare, Somerville, NJ, USA), as previously described [14]. The use of the bipolar loop permits the resection not only of the endometrium but also of 5 to 10 mm of the underlying myometrium. Whenever submucous myomas or endometrial polyps were present, they were removed during the procedure. Since more than 5 mm of myometrium is usually present in the endometrial sections removed with the resectoscope, this allows the pathologist to make the diagnosis of adenomyosis. In 20 patients, laparoscopy was carried out simultaneously to excise endometriotic lesions after preoperative vaginal sonography revealed the presence of ovarian endometrioma. No attempts were made to either remove or coagulate deep intramural myomas during laparoscopy or endometrial resection; however, patients with subserous myomas but no abnormal uterine bleeding were not included in the present study since only patients with symptoms of menorrhagia are submitted to endometrial resection in this institution. The concomitant presence of other pathologies such as endometrial polyps, endometriosis or adenomyosis in these patients with myomatous uteri did not constitute exclusion criteria for this study. All patients included in the present study had a history of progesterone levels compatible with ovulatory cycles and normal thyroid function as inferred by their medical records. The patients gave their informed consent to carry out immunohistochemical studies for the measurement of VEGF, aromatase, p450 and Cox-2 expression in the paraffin-embedded endometrial tissue removed during the hysteroscopic procedure.

Endometrial resection using the bipolar resectoscope is standard procedure for the treatment of menorrhagia in our minimally invasive surgical unit, and the use of continuous oral contraceptives is an acceptable medical indication for the treatment of menorrhagia in Brazil. It was, therefore, possible to identify 40 patients from their medical records who, at the time of endometrial resection or myomectomy, were using an oral contraceptive pill containing 30 mcg of ethinylestradiol and 75 mcg of gestodene (Gestinol, Libbs Farmacêutica, Brazil) in a continuous regimen, as prescribed by their attending physician for the reduction of bleeding and treatment of dysmenorrhea. This specific formulation of oral contraceptive is the only one approved by the Brazilian regulatory agency (ANVISA) to be used continuously for the suppression of menstruation. The time of use of gestodene/ethinylestradiol prior to hysteroscopy varied from 1 to 24 months. The remaining patients had regular menstrual cycles of 25–32 days and no history of previous hormone use in the 3 months preceding surgery. The concomitant presence of other associated pathologies such as endometrial polyps, adenomyosis and endometriosis was confirmed histologically in all cases. Adenomyosis was defined as the presence of endometrial glands in the myometrium at least 2.5 mm from the basal layer, surrounded by hypertrophied

and hyperplastic smooth muscle cells. In patients with endometriosis, diagnosis made during laparoscopy was confirmed by biopsies taken at the time of the procedure. The endometrium was dated according to histological criteria. Early luteal phase was arbitrarily defined as the first four postovulatory days when mitotic figures could still be seen in the glandular epithelium, and the late luteal phase was characterized by any endometrium displaying full secretory changes in the glands and/or decidual changes in the stroma.

All tissue samples were fixed in formalin before they were sent to pathology. Immunohistochemistry for p450, VEGF and Cox-2 was carried out following antigen retrieval. Aromatase expression was investigated in 108 endometrial samples using a commercially available monoclonal antibody manufactured by Serotech (Burlington, Ontario, Canada), clone H4. The presence of Cox-2 and VEGF immunohistochemical staining in the endometrium was investigated in 118 cases using commercially available monoclonal antibodies purchased from Novocastra, Newcastle-upon-Tyne, UK (clone 4H12), and Invitrogen (Carlsbad, CA, USA), respectively. Antigen retrieval was carried out using Tris–EDTA buffer at pH 9.0 for aromatase and VEGF and citrate buffer at pH 6.0 for Cox-2. After washing in Tween 20–0.1% phosphate-buffered saline, tissues sections were incubated overnight with the primary antibodies at 4°C in a humidifier chamber. Blockade of the endogenous peroxidase was accomplished using 3% H_2O_2 for 10 min at room temperature. The staining reaction was obtained using 3,3-diaminobenzidine tetrahydrochloride (Dako, Carpinteria, CA, USA). Endometrial sections were counterstained with Harris hematoxylin for 2 min, dehydrated and mounted with Permount mounting medium (Fisher Scientific, Waltham, MA, USA). Negative control sections in which the primary antibody was either omitted or replaced by normal mouse serum were used in all staining reactions. The presence of aromatase expression was rated either as positive if there was any detectable staining reaction or as negative when no reaction was observed. Placental tissue and an atrophic endometrial sample were used as positive and negative controls, respectively, in all immunostaining reactions for aromatase p450. For Cox-2 reaction, the positive controls consisted of samples of luteal phase endometrium previously known to be Cox-2 positive in the surface epithelium and negative in the glands. For VEGF, tissue samples of a glioblastoma brain tumor were used as positive controls. VEGF expression in the endometrial stroma was rated as diffusely positive, focal or negative. Cox-2 and aromatase expression, on the other hand, was rated in the endometrial glands or stroma as either positive or negative. Statistical analysis was carried out using the StatsDirect software program, version 2.3.8 (StatsDirect Ltd., Cheshire, UK, 2004). The chi-square test and Fisher's Exact Test were used to compare proportions of positive expression between the various groups. Significance was established at $p<.05$.

3. Results

In patients with myomas and symptoms of menorrhagia associated with varying degrees of pelvic pain, other associated pathologies were diagnosed in a greater number of cases. In the present group of patients with symptomatic intramural or submucous myomas, endometriosis was present in 20/118 cases (17%), adenomyosis in 37/118 cases (32%) and endometrial polyps in 14/118 cases (12%).

In the endometrium of patients with myomas, aromatase expression was detected by immunohistochemistry in both the glandular epithelium and stroma, although it was present more frequently in the stroma (73%) than in the glands (27%) (Fig. 1). The percentage of endometrial samples in which aromatase expression was positive, however, varied during the menstrual cycle. During the proliferative phase, 70% of the endometrial samples were positive for aromatase expression; however, this number decreased to only 11% during the late luteal phase. The percentages of aromatase-positive endometria were significantly higher during the proliferative phase than during the late luteal phase ($p<.001$) but not during the early luteal phase ($p=.66$). In the group of patients who were using a combined pill containing gestodene, there was a significant reduction in the percentage of endometrial samples showing positive aromatase expression when compared to either the proliferative ($p<.001$) or early luteal phase ($p=.018$) of the cycle but not to the late luteal phase ($p>0.99$). These results are summarized in Table 1.

Cox-2 expression was detected by immunohistochemistry in endometrial glands and in the surface epithelium and was negative in the stroma during the menstrual cycle. The percentage of Cox-2-positive glands was significantly higher during the proliferative phase of the menstrual cycle than during either the early proliferative phase ($p=.024$) or the

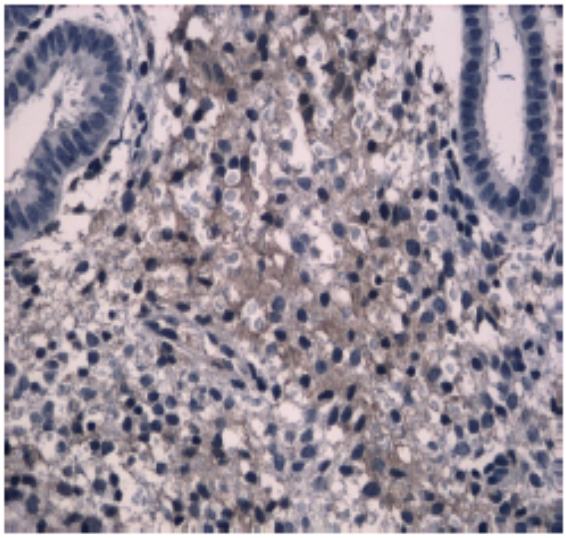

Fig. 1. Aromatase expression in the endometrial stroma of a patient with an intramural myoma and adenomyosis. Brown staining is indicative of positive immunoreactivity.

Table 1
Percentage of positive aromatase expression in the endometrium (stroma+gland) during the menstrual cycle and following oral contraceptive use in patients with myomatous uteri

Groups	Aromatase positive	
	n/*N*	%
Proliferative phase	24/34*	70
Early luteal phase	4/7	54
Late luteal phase	3/27	11
Oral contraceptive users**	5/40	12

n, number of cases; *N*, total number of patients.
* Significantly higher than late luteal and oral contraceptive users ($p<.001$) (Fisher's Exact Test).
** Significantly lower than proliferative phase ($p<.001$) and early luteal phase ($p<.05$) (Fisher's Exact Test).

luteal phase ($p<.001$). However, in the surface epithelium, Cox-2 expression remained positive throughout the menstrual cycle with no decrease in staining intensity during the luteal phase. In the group of patients who were using gestodene/ethinylestradiol and had no breakthrough bleeding ($n=31$), Cox-2 expression in the glandular epithelium was significantly lower compared to the proliferative phase ($p<.001$) but not to the early or late luteal phase ($p=.32$ and $p=.6$, respectively). However, in patients with breakthrough bleeding during oral contraceptive use, Cox-2 positivity was detected in either the glands or decidual stroma in all nine patients. The percentage of endometrial samples that were positive for Cox-2 expression in oral contraceptive users with irregular bleeding (100%) was significantly higher than that in the amenorrhea group ($p<.001$). When compared to the menstrual cycle, this percentage was significantly higher than that during the early ($p=.009$) and late luteal phase group ($p<.001$) but similar to that found in the proliferative phase ($p=.31$). These results are summarized in Table 2.

Table 2
Cox-2 expression in endometrial glands during the menstrual cycle and following oral contraceptive use in patients with myomatous uteri

Groups	Cox-2 positive		Comparisons	p
	n/*N*	%		
Proliferative phase (G1)	30/37	81	G1×G2	.024[a]
Early luteal phase (G2)	3/8	37	G1×G3	<.001[b]
Late luteal phase (G3)	7/32	22	G1×G4	<.001[b]
Oral contraceptive users in amenorrhea (G4)	5/31	16	G1×G5	.31[a]
Oral contraceptive users with breakthrough bleeding (G5)	9/9	100	G2×G3	.34[a]
			G2×G4	.32[a]
			G2×G5	.009[a]
			G3×G4	.6[b]
			G3×G5	<.001[a]
			G4×G5	<.001[a]

n, number of cases; *N*, total number of patients.
[a] Fisher's Exact Test was used.
[b] Chi-square test was used.

VEGF expression was detected by immunohistochemistry principally in the endometrial stroma, with three patterns of staining reaction being observed during the menstrual cycle. During the proliferative phase, VEGF staining was diffuse, being detected throughout the entire endometrial stroma in 92% of cases, while in the remaining 8%, the pattern of expression was either focal (5%) or entirely negative (3%) (Fig. 2). Following ovulation or the use of oral contraceptives, there was a progressive decrease in VEGF expression in the endometrium. When compared to the proliferative phase, the percentage of samples with a diffuse positive pattern of VEGF expression in stroma was significantly lower following ovulation, being detected in only 33% of the cases during the late luteal phase as defined by histological criteria. On the other hand, the percentage of endometria in which no VEGF expression was detected in the stroma increased to 42% in the late luteal phase. In patients using a combined contraceptive pill containing gestodene, there was a significant decrease in the percentage of endometria with a positive diffuse expression for VEGF when compared to nonusers during either the proliferative phase ($p<.001$) or the late luteal phase ($p=.046$) of the menstrual cycle, while the percentage of endometria showing no VEGF expression in the stroma increased to 58% (Fig. 3). These results are summarized in Table 3.

4. Discussion

The present findings suggest that hormonally dependent fluctuations in VEGF expression occur in the endometrial stroma during the menstrual cycle. Our results show that VEGF increases during the proliferative phase of the cycle when compared to the luteal phase, and this is probably a consequence of the unopposed effect of either locally or

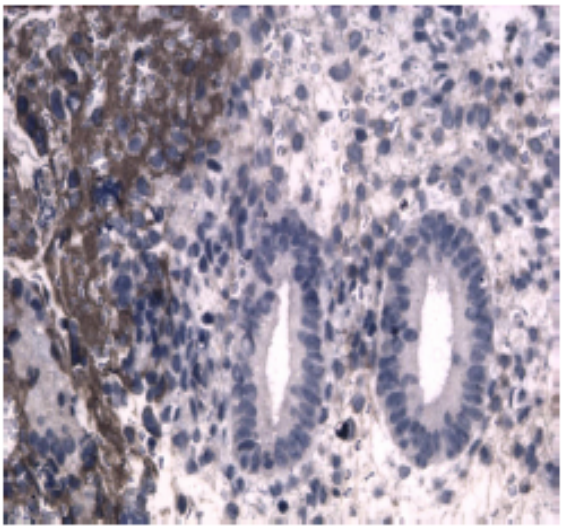

Fig. 2. Diffuse VEGF expression in the endometrial stroma of a patient with an intramural myoma. Tissue sample was obtained during the proliferative phase of the cycle. Brown staining is indicative of positive immunoreactivity.

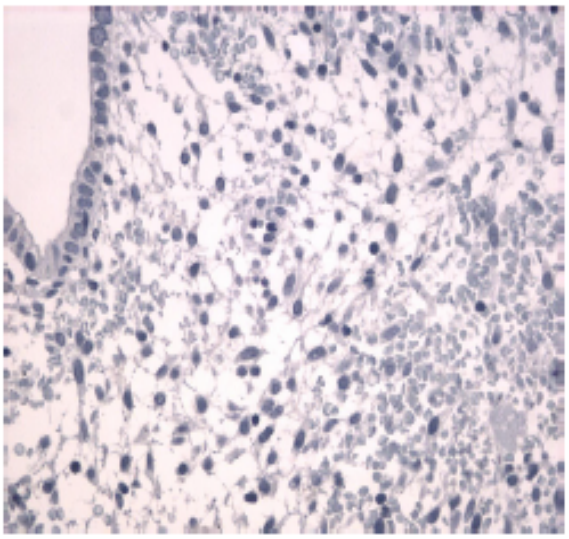

Fig. 3. Negative VEGF expression in the decidual endometrial stroma of a patient with a submucous myoma who had been using an oral contraceptive containing gestodene continuously for 2 months.

ovarian-produced estrogens since these hormones are known to up-regulate VEGF expression in the uterus [15]. It may be speculated that the increase in VEGF expression induced by estrogens may have a stimulating effect on angiogenesis in the endometrium, thus explaining the menorrhagia associated with the presence of myomas. In fact, the presence of aromatase expression in the endometrium shows a positive correlation with the occurrence of menorrhagia in patients with myomas. In uteri affected solely by subserous myomas, which are known to attain considerable sizes without provoking extensive blood loss during menstruation, aromatase expression is not found in the endometrium, whereas it has been detected in a significant number of patients with intramural/submucous myomas and symptoms of menorrhagia [2]. This is in agreement with the hypothesis that estrogen probably promotes angiogenesis in the endometrium by up-regulating VEGF expression in both glandular epithelial and stromal cells [16]. Although VEGF expression was assessed only semiquantitatively in the present study and no comparisons were made with respect to the levels of expression in pathology-free uteri during the menstrual cycle, previous studies have already shown that VEGF expression is greater in the endometrium of myoma-bearing uteri than in the normal uterus [17]. This is in agreement with the hypothesis that aberrant aromatase expression in the endometrium of myoma-bearing uteri will further stimulate angiogenesis in this tissue during the proliferative phase, since local estrogens may potentiate the effect of the systemically produced hormones, further stimulating VEGF expression in the endometrial stroma. However, this remains speculative and further studies are necessary to confirm this hypothesis. Animal studies have shown that the promoting effects of estrogens on endometrial angiogenesis are mediated by the increase in VEGF expression, thus suggesting that the blockade of VEGF action may curb the stimulatory effects of estrogens on angiogenesis [18]. Angiogenesis, increased uterine inflammation and abnormal aromatase expression in the endometrium are frequently associated conditions that support the pivotal role of estrogens in the development of various uterine pathologies associated with abnormal menstrual bleeding, pain and increased inflammation [13]. The clinical association between VEGF expression and abnormal uterine bleeding became evident in a recent study, the results of which showed that when there was a high prevalence of myomas in families, both the severity of menorrhagia and the intensity of VEGF expression detected in these tumors were greater than when these tumors were found in families in which their occurrence was sporadic [19]. Likewise, in patients with adenomyosis and menorrhagia, VEGF expression in the endometrium was stronger than that observed in patients with asymptomatic myomas [10]. Since aromatase expression is absent in the endometria of patients with asymptomatic subserous myomas [2], these findings support the concept that excessive exposure of the endometrium to estrogens probably plays a pivotal role in up-regulating VEGF production and angiogenesis during the proliferative phase, thus exacerbating uterine bleeding during menses. Previous reports have shown that aromatase expression is detected in the endometrium of patients with adenomyosis, endometriosis and endometrial polyps, thus supporting the causal relationship between local estrogen in the uterus and the development of these pathologies [2,20–24]. Local estrogen production will up-regulate both VEGF and Cox-2 expression in the endometrium, thereby exacerbating the symptoms of pain and bleeding during menses. Based on the results of the present study and previous reports [2,20,22,23], it is, therefore, plausible from the biological point of view to conclude that menorrhagia associated with the presence of myomas and other uterine pathologies may arise as a consequence of this local increase in estrogen synthesis caused by aberrant aromatase expression in the endometrium, although this remains to be confirmed. However, tissue levels of prostaglandin $F_{2\alpha}$ were found to be

Table 3
Diffuse VEGF-positive staining in endometrial stroma during the menstrual cycle and following the use of oral contraceptives in patients with myomatous uteri

Groups	Diffuse VEGF staining		Comparisons	p
	n/*N*	%		
Proliferative phase (G1)	34/37	92	G1×G2	.002[a]
Early luteal phase (G2)	3/8	38	G1×G3	<.001[b]
Late luteal phase (G3)	12/36	33	G1×G4	<.001[b]
Oral contraceptive users (G4)	6/40	15	G2×G3	>.99[a]
			G2×G4	.14[a]
			G3×G4	.046

n, number of cases; *N*, total number of patients.
[a] Fisher's Exact Test was used.
[b] Chi-square test was used.

significantly higher in the nodule and corresponding endometrium of women with submucous and intramural myomas compared to cases of subserous myomas or pathology-free uteri [25]. Since Cox-2 expression is up-regulated by estrogens, these results are in agreement with the findings previously reported by our group that aromatase expression is detected in the endometrium of uteri affected by intramural and submucous myomas but not asymptomatic subserous ones [2,25]. The association of myomas with other pathologies in which aromatase is also detected in the endometrium, as reported here, further supports the concept that these pathologies have common pathogenic mechanisms [2,20,22]. Aromatase expression in the endometrium will create a hyperestrogenic milieu in the uterus during the proliferative phase of the menstrual cycle, leading to further enhancement of the expression of angiogenic and inflammatory factors such as VEGF and Cox-2, which will trigger a constant state of inflammation that will predispose to the development of pathology [13,26]. The brief exposure to progesterone in nonfertile cycles may not suffice to abate all these changes caused by this local hyperestrogenism, although aromatase, VEGF and Cox-2 expression is lower during the luteal phase in response to the rising levels of progesterone, as reported in this article and in other previous papers [2,12,23]. However, a longer time of exposure to the anti-inflammatory effects of progesterone may be required in order to cause regression of these changes in uteri affected by pathologies, since progesterone withdrawal prior to the onset of menstruation will trigger the activation of these inflammatory mediators, including the up-regulation of mRNA levels for VEGF [9,27]. One practical way of achieving this is through the continuous use of oral contraceptives. Our observation that there was a significant reduction in VEGF, aromatase and Cox-2 expression in the endometrium of patients previously treated with combined oral contraceptives may explain some of their noncontraceptive health benefits in the treatment of menorrhagia associated with myomas and other uterine pathologies [6]. Studies using cultured leiomyoma cells have shown that the blockade of aromatase expression with letrozole is effective in inhibiting estrogen synthesis induced by prostaglandin E_2, thus augmenting myoma cell death in vitro [28]. This suggests not only that aromatase expression plays a pivotal role in the survival of myoma cells but also that there is a need for further investigation of the use of aromatase inhibitors as a therapeutic option for the treatment of leiomyomas. In this aspect, it should be emphasized that progestins present in the formulation of combined oral contraceptives are potent inhibitors of aromatase expression in the endometrium as shown in this article and in previous reports [2,3,23]. The inhibitory effect of progestins on aromatase expression may provide the basis for their use in the treatment of menorrhagia associated with myomas and other uterine pathologies [6].

The results reported in this article suggest that these noncontraceptive health benefits associated with the use of oral contraceptives may be a consequence of their suppressive effects on the expression of angiogenic and inflammatory factors in steroid target tissues such as the endometrium. This provides a rationale for the use of oral contraceptives in the management of menstrually related symptoms since many of the health benefits of oral contraceptives on menorrhagia and pelvic pain are a consequence of their curtailing effects on inflammation at the endometrial level, thus explaining their frequent use in the treatment of various gynecological conditions associated with increased inflammation [6].

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Artigo nº 5

Is aromatase expression in the endometrium the cause of endometriosis and related infertility?

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healthcare

ENDOMETRIOSIS

Is aromatase expression in the endometrium the cause of endometriosis and related infertility?

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Abstract

Aromatase expression in the endometrium seems to play a pivotal role in the development of endometriotic lesions. Because inflammatory mediators such as prostaglandin E2 appear to activate aromatase in the cells of the endometrial stroma, it was hypothesised that the ensuing inflammation caused by the arrival of aromatase-positive cells in the peritoneal cavity would stimulate local estrogen production, which would in turn facilitate the development of endometriotic lesions by suppressing macrophage phagocytosis. Aromatase expression in the eutopic endometrium will also hamper ovum nidation, thus causing infertility. Progestins, such as gestodene and danazol, are potent inhibitors of aromatase expression in the endometrium, and the use of vaginal rings with danazol in doses that do not block ovulation is associated with the occurrence of pregnancy in patients with severe endometriosis without the need for surgery. A local effect on the endometrium suppressing aromatase expression has been suggested as a possible mechanism of action for the danazol ring.

Keywords: *Aromatase, endometriosis, endometrium, infertility, danazol*

Introduction

Endometriosis is defined as the presence of endometrial glands and stroma outside the uterus, most commonly within the pelvic cavity [1,2]. The current clinical definition of the disease involves a wide spectrum of symptoms ranging from dysmenorrhea, menorrhagia and chronic pelvic pain to infertility, although a certain percentage of women may be asymptomatic [3]. However, from a medical point of view, diseases are only diagnosed when they provoke symptoms resulting in morbidity; therefore, incidence rather than prevalence should be used in the evaluation of endometriosis, because this will exclude women with an asymptomatic form of the disease in whom the condition was detected during unrelated surgery. A broad definition of endometriosis should thus be used, comprising not only the presence of ectopic endometrial tissue within the pelvic cavity but also evidence that the lesions are active and provoking symptoms [4]. The medical or surgical treatment of endometriosis and its related symptoms present several unresolved challenges, because, despite being efficacious in the short term, they are associated with high rates of recurrence following discontinuation of therapy [5–8]. There are several explanations for the long-term failure of endometriosis therapy, but one likely rationalisation is the resumption of menstruation. An intriguing aspect of this problem is that retrograde menstruation is a risk factor for endometriosis in a certain group of patients, but not in others [9]. Although retrograde menstruation seems to be a rather universal phenomenon that affects almost 90% of women during menstruation, endometriosis occurs in a much smaller proportion of cases [10,11]. This discrepancy may provide important clues to the etiology of endometriosis, which may affect management strategies. One fundamental issue related to the treatment of endometriosis is whether this disease consists of one single pathological event in the reproductive life of a woman that can be resolved with a specific form of treatment or whether it is a chronic, recurrent condition that may be controlled but never completely cured [12,13]. The answer to this fundamental question may lie in understanding the pathogenesis of endometriosis, and this brings us back to the important issue of retrograde menstruation [2,14,15].

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Retrograde menstruation and endometriosis

The answer to the question of why retrograde menstruation does not result in endometriosis in all patients may provide clues to explain not only the etiology of this disease but also the high recurrence rates following both medical and surgical modes of treatment. The explanation for this may lie in the endometrium itself. One important aspect of the pathogenesis of endometriosis is whether or not there are differences with respect to gene expression between the eutopic endometrium of these patients and the endometrium of disease-free women [16,17]. This question is fundamental not only in understanding the basic aspects of the disease process, but it may also be pivotal for the design of better therapeutic approaches. The main drawback in the treatment of endometriosis is that its long-term efficacy has not yet been established, because most of the meta-analyses carried out so far indicate high recurrences rates following the discontinuation of both medical and surgical treatment modalities [6,8,18,19]. Nevertheless, rather than lack of efficacy of the various surgical and medical approaches for the treatment of this disease, these high recurrence rates may reflect more subtle differences between the endometrium of women with endometriosis and that of disease-free patients, predisposing the former to recurrence [14,15]. Therefore, the Achilles heel of endometriosis treatment may lie in the endometrium itself and in the widespread occurrence of retrograde menstruation.

Aromatase expression in the eutopic endometrium

What, then, would be the differences between the eutopic endometrium of patients with endometriosis and that of disease-free patients that could predispose the former to treatment failure and the consequent return of the condition? The answer to this question may lie in the abnormal expression of the aromatase enzyme, which may be detected in the eutopic endometrium of patients with endometriosis but not in disease-free women [14,16,20], although this has not been reported in all studies [21]. These discrepancies, however, may be explained either by differences in the sensitivity of the techniques used or by the stage of the menstrual cycle in which the endometrial samples were obtained, because aromatase expression is detected less frequently during the luteal phase [22,23]. Besides endometriosis, aromatase expression in the endometrium is also detected in patients with adenomyosis, endometrial polyps and myomas [22–27].

The abnormal expression of aromatase in the endometrium is probably maintained by the constant exposure of this tissue to inflammatory mediators [28], because E-type prostaglandins may activate the gene for aromatase [27]. However, prostaglandin E2 only stimulates aromatase in the endometrium in which basal levels of this enzyme already exist, having no effect on the aromatase-negative endometrium of disease-free women [17,27]. It is not known which factors trigger activation of the aromatase gene in the endometrium; however, the occurrence of incessant menstruation may be a risk factor [3,16].

The role of aromatase in the development of endometriosis

During retrograde menstruation, endometrial cells may be carried to the pelvis, which upon their arrival there would trigger an intense inflammatory reaction, bringing an efflux of immunologically active cells including macrophages and natural killer (NK) cells to clear the menstrual debris [16,29–34]. However, this intense inflammatory reaction would generate great amounts of prostaglandins and other inflammatory mediators that could stimulate aromatase expression exponentially in endometrial cells that already have basal levels of enzyme expression, but not in negative cells [27]. The rise in aromatase activity will trigger a local production of estrogen, which will further enhance prostaglandin synthesis by activating the cyclooxygenase II enzyme. This will ultimately create a vicious cycle of augmented inflammation and aromatase expression, and the resulting excessive local estrogen production would facilitate the implantation of endometrial cells in the peritoneum [16,17,20] by sparing them from destruction by activated macrophage and NK cells. The observations that estrogens are known to inhibit the mechanisms of phagocytosis by macrophages and NK cells are in agreement with this hypothesis [28–30]. One common example of this effect of estrogens is the well-known decrease in bone resorption induced by estrogen, because osteoclasts are modified macrophages that respond to estrogens by decreasing phagocytosis in the extracellular matrix of the bone. Aromatase-negative endometrial cells, on the other hand, would be eliminated from the pelvic cavity in the days following menstruation by activated macrophages and NK cells, because phagocytosis would not be inhibited by the local production of estrogens [28,31,32]. The high recurrence rates of endometriosis even after successful medical or surgical treatment may also be explained by the implantation of newly arrived, aromatase-positive cells following the resumption of menstruation, because the underlying cause in the endometrium was not corrected [7,8,19].

This makes endometriosis a chronic condition that may be controlled but not cured unless aromatase expression in the eutopic endometrium is suppressed [33,16,17]. This suggests that the control of endometriosis may only be achieved by suppressing menstruation.

Medical treatment of endometriosis

Drugs that suppress aromatase expression or modulate immunological activity would be expected to play an important role in endometriosis therapy [34]. It was recently reported that aromatase inhibitors may be effective in the treatment of unusually aggressive forms of endometriosis associated with high levels of aromatase expression [35]. Aromatase is, therefore, crucial not only in defining predisposition to the development of endometriosis, but also in determining the aggressiveness of its clinical course [34–36]. However, the use of current aromatase inhibitors in premenopausal patients may lead to high FSH levels, which may revoke the inhibitory effect on ovarian steroidogenesis, causing a rebound increase in circulating estrogen levels. This may diminish some of the advantages of using this medication. One possible solution is the use of combined oral contraceptive pills, not only to suppress elevated FSH but also to prevent the onset of osteoporosis, which may impede the long-term use of this drug in young women [37]. In addition, the cost of these medications represents another drawback.

Recent observations have indicated that progesterone and progestins are able to suppress aromatase expression in the endometrium and in peripheral blood leucocytes [38] by decreasing gene transcription for this enzyme [22,39]. The inhibitory effect of combined oral contraceptives on aromatase expression may be one of the mechanisms responsible for their therapeutic effects on endometriosis. The efficacy and the low incidence of side effects with the use of oral contraceptives render them an ideal medication for the suppression of menstruation and aromatase expression in patients with endometriosis [22]. Short-term treatment with GnRH analogs will halt the disease process only temporarily, because their prolonged use is contraindicated not only because of the risks associated with the resulting hypoestrogenism but also in view of the high cost of treatment. Because GNRH-induced hypoestrogenism will also ultimately lead to suppression of aromatase expression in the endometrium, the blockade of local estrogen production is, therefore, one the mechanisms of action of this drug in addition to ovulation suppression [24]. Because oral contraceptives are able to accomplish the same feat at a much lower cost and with substantially fewer side effects, they should be the first line of therapy for the control of recurrences of endometriosis. In fact, there is no evidence that GnRH analogs are any more effective than oral contraceptives in the treatment of endometriosis-related symptoms [40]. Combined oral contraceptives containing gestodene and ethynylestradiol, when given continuously, are effective in preventing the recurrence of endometriosis following laparoscopic surgery when compared to no treatment [41]. The presence of aromatase-positive cells in the eutopic endometrium, rather than the failure to eradicate all lesions, may be the main culprit for the recurrence of endometriosis [42] following successful surgical treatment, because the resumption of menstruation will carry these cells back into the pelvic cavity in a retrograde fashion [16,20,24].

Treatment of endometriosis in infertile patients

In infertile patients, the ideal medical treatment would consist of the inhibition of aromatase expression in the endometrium without the concomitant suppression of ovulation; however, this is a requirement not fulfilled by most of the medical treatments using currently available progestins [43].

Danazol is very effective in controlling endometriosis symptoms such as pain; however, its androgenic side effects such as oily skin, hirsutism and weight gain may affect patient compliance with treatment [44–48]. The delicate balance between efficacy and side effects may be achieved by using other routes of administration. Although danazol is able to suppress ovulation, there is evidence that it may have a local effect on endometriosis and in the endometrium by suppressing the aromatase enzyme. In adenomyosis, the use of danazol was effective in inhibiting aromatase activity both at gene transcription and enzymatic levels [24].

The vaginal use of danazol

One of the benefits of the use of the vaginal route for the administration of danazol is avoidance of the first passage through the liver. Because the vaginal route is less effective in lowering SHBG levels compared to the oral route, free testosterone levels and the incidence of androgenic side effects will be lower. Another beneficial effect of the vaginal absorption of danazol is that the concentration of the drug reaching the uterus may be higher than that achieved by the oral route, although systemic levels will be lower. This theoretically would result in similar or even greater efficacy in the control of the symptoms of endometriosis with much fewer side effects. The vaginal use of danazol may also allow ovulation to occur, because lower circulating levels of the drug may be insufficiently high to provoke anovulation [49]. Initial trials using danazol at a dose of 1500 mg in a vaginal ring to be replaced every 2 months showed that it was indeed possible to treat endometriosis-related pain without blocking ovulation. In addition, significantly fewer side effects were reported compared with those observed when the oral route was used [45,49]. Most importantly, because ovulation was not blocked, over half the patients became pregnant while using the danazol-loaded vaginal ring with no untoward effects on the ongoing pregnancy [49]. Although the effects

of the vaginal ring containing danazol on aromatase expression in the endometrium were not investigated in this preliminary clinical study, it is plausible to assume that the beneficial effects on both fertility and on the symptoms of endometriosis may be the result of a direct inhibitory effect of danazol on aromatase gene transcription as shown previously *in vitro* [24]. However, danazol may also have other effects on endometriotic lesions in addition to inhibiting aromatase, because local danazol therapy may also reduce endometriosis by directly suppressing DNA synthesis or inducing apoptosis in endometriotic cells [46,47]. The inhibition of aromatase expression in the endometrium may also be one of the mechanisms of the pain-reducing effects of danazol-containing vaginal rings in patients with endometriosis of the ovary and vaginal septum [24,49]. The report of the occurrence of ongoing pregnancies in these patients is also in agreement with the hypothesis that danazol exerts a local effect on the endometrium consisting of both aromatase inhibition and the consequent improvement in ovum implantation rates. Recently, the presence of aromatase was reported to decrease endometrial receptivity to implantation in *in vitro* fertilisation cycles [50]. Because danazol suppresses aromatase expression at gene transcription level, it is possible that this may have occurred in those patients using vaginal rings loaded with this drug, thereby increasing endometrial receptivity to implantation of the fertilised ovum [24,50]. The paradigm of endometriosis and infertility may be explained by the presence of aromatase expression in the endometrium, which favours the survival of the shed endometrial cells in the peritoneum while simultaneously causing subfertility by decreasing the implantation rates of the fertilised ovum in the uterus [50]. Most importantly, in patients undergoing IVF treatment, the use of danazol before the initiation of ovarian stimulation almost doubled the rate of clinical pregnancies, and this effect may be attributed to better endometrial receptivity achieved with the prior use of danazol [51]. In this aspect, inhibition of aromatase expression in the endometrium would be a plausible biological explanation for the fertility enhancing effects of vaginal danazol in endometriosis patients.

Conclusion

Endometriosis may an endometrial disease, because the expression of the aromatase enzyme in the eutopic endometrium may be pivotal in establishing the disease process. Local estrogen production in the endometriotic foci may suppress their phagocytosis by peritoneal macrophages and NK cells, thereby facilitating their implantation and development in ectopic locations in the pelvis [52].

Danazol and progestins, such as gestodene, are able to suppress aromatase activity in the endometrium, thus explaining their efficacy in the treatment of endometriosis [22–26]; however, all these medications also suppress ovulation at the doses currently used, and are therefore unsuitable for the treatment of endometriosis-related infertility, although they are effective in treating pain. The initial results obtained with vaginally administered danazol suggest that it is possible to design formulations that exert local effects on the uterus and on the symptoms of endometriosis without blocking ovulation.

The possibility of treating endometriosis by suppressing aromatase expression at endometrial level without blocking ovulation may represent a true advancement in the management of endometriosis, particularly in the infertile patient, because this form of management would correct the primary defect in the endometrium without compromising fertility by blocking ovulation.

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Artigo nº 6

The effect of oral contraceptives on aromatase expression in the eutopic endometrium of patiens with endometriosis.

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ENDOMETRIOSIS

The effect of oral contraceptives on aromatase expression in the eutopic endometrium of patients with endometriosis

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Abstract

Objective. To determine the effect of oral contraceptives containing gestodene on aromatase expression in the endometrium of patients diagnosed with endometriosis.

Patients and methods. Endometrial biopsies were taken at the time of laparoscopy in 40 patients with endometriosis, 16 of whom were using an oral contraceptive containing gestodene at the time of laparoscopy. The remaining 24 patients were receiving no form of treatment for endometriosis. Endometrial biopsies taken from 23 patients with normal echographic signs and no symptoms were used as controls. Aromatase expression was evaluated in endometrial samples using immunohistochemistry.

Results. In the untreated, symptomatic endometriosis patients, aromatase expression was detected during the proliferative phase in 92% of cases, while in the symptom-free control patients aromatase was expressed in only 9% of cases. In patients with endometriosis who were using oral contraceptives, there were significantly fewer cases of positive endometria compared with the untreated patients with endometriosis (6%).

Conclusion. Oral contraceptives containing gestodene are effective in decreasing aromatase expression in the eutopic endometrium of patients with endometriosis.

Keywords: *Endometrium, aromatase, gestodene, endometriosis, menstrual cycle*

Introduction

The eutopic endometrium of patients with endometriosis is capable of aberrantly expressing the enzyme aromatase, which stimulates the transformation of androgen precursors into estrogens [1]. In the endometrium, the aromatase gene is activated by inflammatory mediators such as prostaglandin E_2, thus establishing a vicious circle between inflammation and local estrogen production. This mechanism is greatly enhanced during menstruation when endometrial cells capable of expressing aromatase are transported in a retrograde fashion into the peritoneal cavity, thereby triggering an intense inflammatory response that will ultimately augment aromatase activity exponentially [1,2]. The inducing effects of prostaglandin E_2 on the promoter of the aromatase gene is observed only in endometrial cells from patients with endometriosis, being absent in the endometrium of disease-free women [3]. This locally increased estrogen production is thought to play a pivotal role in the mechanism of implantation and in the development of endometriotic lesions, since a hyperestrogenic milieu is known to be a facilitating factor for the development of endometriosis [1]. However, the relationship between aromatase expression in eutopic endometrial cells and the capacity of these cells to implant in the peritoneal cavity, thus escaping the surveillance of the immunological system, is not yet fully understood [4]. The hormonal and inflammatory factors that regulate aromatase expression in the endometrium are therefore important for the survival of endometrial cells in the peritoneal cavity, their subsequent implantation and

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the formation of endometriotic lesions [5]. In patients with myomas, aromatase expression in the endometrium is lower during the luteal phase, thus suggesting an inhibitory effect of progesterone [6]. Similarly in peripheral macrophages, aromatase expression was also found to diminish significantly during the luteal phase compared with the other phases of the menstrual cycle [7]. Oral contraceptives (OCs) and danazol are able to mimic this inhibitory effect of endogenous progesterone on aromatase expression when used in patients with adenomyosis [8,9]. Aromatase inhibitors, either alone or in combination with OCs, are also effective for the treatment of endometriotic lesions including those with aggressive clinical behavior [10,11]. Moreover, when OCs were used in an extended regimen in patients with endometriosis who had been successfully treated by laparoscopic surgery, they were found to be more effective for the treatment of pain recurrence than when the same contraceptives were used cyclically [12,13]. These non-contraceptive health benefits of the pill in the treatment of endometriosis-related pain are probably a consequence of inhibition of cyclooxygenase-2 (COX-2) expression in the endometrium and the consequent reduction in prostaglandin production in this tissue, although other mechanisms may also work synergistically, including the suppression of menstruation itself [14,15].

In the present observational, case–control study, aromatase expression was investigated using immunohistochemical methods in endometrial biopsies obtained from patients with a laparoscopically proven diagnosis of endometriosis, who were either untreated or had been using OCs containing gestodene in an extended regimen prior to surgery.

Patient and methods

This was a retrospective, observational study carried out in paraffin-embedded endometrial tissue to detect aromatase expression in both untreated and OC-treated patients with endometriosis. Endometrial biopsies were performed using a 4 mm Karman catheter (IPS, USA) in 40 patients submitted to laparoscopy and hysteroscopy for the treatment of endometriosis in our institute between January 2005 and January 2007. All patients submitted to this procedure had a history of pelvic pain associated or not with abnormal ultrasonographic findings. In this group, 17 patients had a history of pelvic pain and a previous diagnosis of ovarian endometrioma made following transvaginal sonography (TVS) and confirmed by laparoscopy. In the remaining patients ($n = 23$) preoperative TVS findings were normal but peritoneal foci of endometriosis were detected in the Douglas pouch, bladder, peritoneum and broad ligament during laparoscopy. Patients with normal uteri at sonography but who were referred to our unit for hysteroscopic evaluation of the uterine cavity, including endometrial biopsy, as part of the diagnostic work-up prior to *in vitro* fertilization procedures because of male-factor infertility, served as symptom-free controls ($n = 23$). These patients had no history of pelvic pain or abnormal uterine bleeding, and the uterine cavity was normal at hysteroscopy. Laparoscopy was carried out in all 40 patients not only to confirm the diagnosis of endometriosis but also to coagulate peritoneal lesions or to drain and excise ovarian endometriotic cysts. Hysteroscopy and endometrial biopsies were carried out concomitantly. All patients were included in the present study retrospectively and had progesterone levels compatible with ovulatory cycles and normal thyroid function as inferred by their admission medical records. At the time of laparoscopy and endometrial biopsy, 16 patients with endometriosis were using an OC containing 30 μg of ethinyl estradiol and 75 μg of gestodene (Gestinol®; Libbs Farmacêutica, Sao Paulo, Brazil) in a continuous regimen as prescribed by their attending physician to ameliorate pelvic pain and uterine bleeding. The duration of OC treatment varied between 2 and 6 months in 15 patients, while one patient with an ovarian endometrioma had been using the pills for 24 months. In the untreated patients with endometriosis, laparoscopy was carried out in the proliferative phase of the menstrual cycle. All patients were premenopausal and were in the 22- to 40-year age bracket.

The endometrial samples were fixed in formalin 10% before being sent to pathology. Immunohistochemistry was carried out following antigen retrieval to detect the presence of aromatase p450. Aromatase expression was investigated using a commercially available monoclonal antibody (MCA2077, clone H4; Serotech, Raleigh, NC, USA). Antigen retrieval was carried out using the Tris–ethylenediaminetetraacetic acid buffer at pH 8.0. The reaction was revealed using the streptavidin–biotin method. The presence of aromatase expression was rated either as positive if there was any detectable staining reaction or negative when no reaction was observed. Placental tissue and an atrophic endometrial sample were used as positive and negative controls, respectively, in all immunostaining reactions for aromatase p450. Statistical analysis was carried out using the StatsDirect software program, version 2.3.8 (StatsDirect Ltd, Cheshire, UK, 2004). The χ^2 and Fisher's exact tests with significance established at $p < 0.05$ were used to compare proportions of aromatase expression in the endometrium among the three groups: untreated, symptomatic endometriosis patients ($n = 24$); OC users with endometriosis ($n = 16$); and asymptomatic patients with normal findings at TVS ($n = 23$). Patients included in the present study

gave their informed consent for the immunohistochemical studies to be performed on the biopsy specimens.

Results

The proportion of patients with peritoneal and ovarian forms of endometriosis was the same in both OC-treated and untreated patients, as shown in Table I. However, in all patients previously treated with gestodene there was a visible reduction in inflammation and lesion vascularization, which was observed during the laparoscopic procedure. Endometrial biopsies taken at the time of hysteroscopy showed proliferative endometrium in the untreated patients with endometriosis. In patients using an OC containing 75 μg of gestodene with 30 μg of ethinyl estradiol, the most common histological feature of the endometrium was either decidual stroma in 12/16 (75%) of the biopsies or atrophy in the remaining cases.

Aromatase expression was detected by immunohistochemistry in the eutopic endometrium of untreated, symptomatic patients with endometriosis in 22/24 cases (92%) during the proliferative phase of the menstrual cycle, but was seldom detected in the endometrium of symptom-free control women (2/23, 9%). This difference was statistically significant ($p < 0.001$). In endometriosis patients not using OCs, aromatase expression in the eutopic endometrium was present solely in the stroma in 17/24 (71%) of endometrial biopsies, while the glandular epithelium was positive in 3/24 (13%) (Figure 1). In the remaining cases, aromatase expression was either detected in both gland and stroma or the endometrium was negative (Table II). The difference between stroma and glands in the percentage of positive aromatase expression was significant ($p < 0.001$).

In endometriosis patients using an OC containing gestodene at the time of laparoscopy, aromatase expression in the eutopic endometrium was detected in only one case (1/16) (6%), being negative in the rest of the patients. This aromatase-positive patient had used OCs for 4 weeks and at the time of laparoscopy she had a 7 cm hemorrhagic functional ovarian cyst and multiple scattered endometriotic lesions in her pelvis. There were also thick adhesions between the colon and the posterior part of the uterus, obliterating the Douglas pouch. The difference in endometrial aromatase expression between untreated and OC-treated patients with endometriosis was statistically significant ($p < 0.01$). However, there was no significant difference between OC users and symptom-free controls during the proliferative phase. These results are summarized in Table III.

Discussion

The present study confirmed previous observations that aromatase expression is present in the eutopic endometrium of endometriosis patients, while it is absent in the endometrium of disease-free women. These results are in agreement with recent observations that aromatase expression is detected

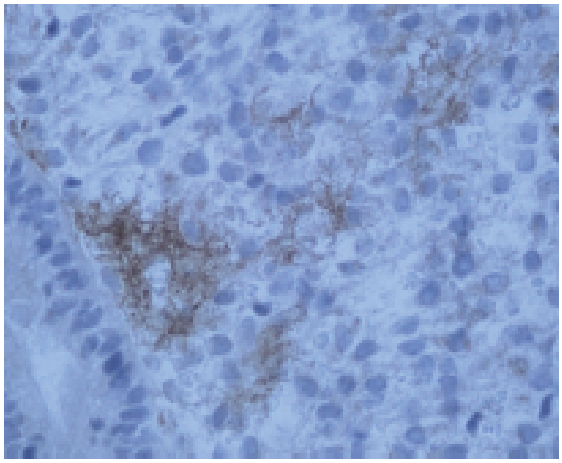

Figure 1. Aromatase expression in the endometrial stroma of a patient with endometriosis during the proliferative phase of the menstrual cycle.

Table I. Peritoneal and ovarian endometriosis in patients treated or not with oral contraceptives (OCs).

	Untreated	OC users
Peritoneal	14/24 (58)	9/16 (56)
Ovarian	10/24 (42)	7/16 (44)

Data are expressed as *n*/*N* (%).

Table II. Cellular distribution of aromatase expression in the endometrium of endometriosis patients during the proliferative phase of the menstrual cycle.

Stroma positive–gland negative	17/24 (71)*
Gland positive–stroma negative	3/24 (13)
Gland and stroma positive	2/24 (8)
Gland and stroma negative	2/24 (8)

Data are expressed as *n*/*N* (%); *significantly more than in the glandular epithelium ($p < 0.001$).

Table III. Aromatase expression in the endometrium of patients with endometriosis and disease-free patients, and the effect of oral contraceptives (OCs).

Untreated patients with endometriosis	22/24 (92)
Patients with endometriosis using OCs	1/16 (6)*
Symptom-free patients	2/23 (9)*

Data are expressed as *n*/*N* (%); *significantly less than untreated patients ($p < 0.001$).

by immunohistochemistry in endometriotic tissue and that it correlates positively with the severity of the disease [16]. Our findings that aromatase expression is detected mainly in the stroma of the eutopic endometrium is in agreement with previous studies showing a direct relationship between the amount of stroma present in endometriotic lesions and their histological appearance and hormonal responsiveness [17]. Similar to the eutopic endometrium, aromatase expression in the endometriotic foci is also present mostly in the stroma and is greatly stimulated by prostaglandin E_2 [1,2]. The stimulation of aromatase expression by inflammatory mediators is one of the key mechanisms that lead to the enhanced estrogen production in endometriosis, since aromatase expression is exponentially increased in endometrial cells upon their arrival in the peritoneal cavity and their exposure to the ensuing inflammation and its mediators [18]. One of the intriguing features of the pathogenesis of endometriosis is why macrophages have an impaired scavenger capacity to phagocyte these cells, although they continue to produce cytokines, prostaglandins and growth factors that will ultimately promote more estrogen production and angiogenesis in the endometriotic lesions [19,20]. It is possible that excessive local estrogen production may play a pivotal role in suppressing the phagocytosis of endometrial cells by activated macrophages [20]. Despite the impaired scavenger function, macrophage-conditioned media have the capacity to stimulate endometrial stromal cell proliferation *in vivo* [21]. The exponential rise in aromatase expression in the endometrial cells when they reach the peritoneal cavity through retrograde menstruation will greatly stimulate estrogen production in these cells, thereby inhibiting the scavenger function of the macrophages [1,2,4,19,20].

One may hypothesize that under these circumstances the ensuing inflammatory response triggered by the presence of menstrual debris in the peritoneal cavity, instead of being detrimental to the shed of endometrial cells, will promote their survival by stimulating an exponential rise in aromatase activity [1]. However, this will only occur in endometrial cells already displaying aromatase expression, since prostaglandin E_2, an inflammatory mediator, does not stimulate aromatase expression in normal endometrial cells [2]. It is important to emphasize that endometriosis is a disease with high recurrence rates following both medical and surgical treatment, and this may not only be due to the reactivation of preexisting lesions [22]. One additional explanation for this may lie in the fact that the eutopic endometrium of these patients is somewhat different from the endometrium of disease-free women, since it constitutively expresses the enzyme aromatase as demonstrated in this and other previous studies [1–3]. The presence of aromatase will facilitate the implantation of new cells in the peritoneum as soon as menstruation resumes, thus initiating a new cycle of inflammation and *de novo* formation of endometriotic lesions [1,2]. This explains the discrepancy between the almost universal occurrence of retrograde menstruation and the development of endometriosis in a much smaller proportion of cases [23]. The inflammation in the peritoneum triggered by the presence of menstrual debris will exacerbate aromatase expression solely in endometrial cells already expressing this enzyme, since prostaglandin E_2 is devoid of any effect in the negative endometrial stroma [2]. Therefore, the expression of aromatase will determine whether endometrial cells will be spared or destroyed in the pelvis by the immunological system in the days following menstruation. This is in agreement with our present findings that there is a high incidence of positive aromatase expression in the eutopic endometria of symptomatic women with active endometriosis lesions that is not found in the endometrium of disease-free women.

The estrogen concentration inside the endometriotic foci correlates positively with the severity of the disease, since high levels of aromatase expression are found in particularly aggressive forms of the disease [10,11,16]. The enhanced estrogen production may facilitate the progression of endometriosis through several mechanisms, although interference with macrophage phagocytosis is probably one of the most important [4,18–20]. In patients with endometriosis, there is indeed an increase in the number of activated non-adherent macrophages with a reduced surface expression of scavenger receptors and diminished capacity to destroy ectopic endometrial cells [19].

If this explanation is correct, then aromatase activity in the eutopic endometrium may be a major risk factor for the development of endometriosis, since locally produced estrogens may play a pivotal role not only in preventing macrophages from destroying these cells in the peritoneal cavity, but also in allowing their implantation and survival [1,3,5]. In this respect, the role of stroma in the development of endometriosis is crucial [17], since aromatase expression was mainly detected in this tissue not only in the eutopic endometrium but also in the endometriotic lesions [2]. Because aromatase was rarely found in eutopic endometrial glands, it is possible that stroma cells are the ones that actually implant in the peritoneum, inducing the formation of glandular-like epithelium in the peritoneal mesothelium by metaplasia. This may explain the important differences in histological features and hormonal responsiveness between the underdeveloped glands in endometriotic lesions and the fully functional ones in the ectopic endometrium [17,23]. However, this assumption cannot be proved or refuted by the

present data since this a clinical observational study that was not designed to test this hypothesis.

Our findings that OCs containing gestodene are able to suppress aromatase expression in the eutopic endometrium of patients with endometriosis may explain their effectiveness in the treatment of endometriosis [24]. Progestins may inhibit aromatase expression through several mechanisms including suppression of ovarian steroidogenesis, although a local effect on the eutopic endometrium diminishing aromatase gene transcription may be the most important one [8,25]. The ability of progestins and danazol to suppress aromatase expression in the endometrium suggests that they may play an important role in the prevention and treatment of endometriosis [8,9,25]. If aberrant aromatase expression in the eutopic endometrium plays a key role in the progression of endometriosis, then OCs should be an important line of therapy to prevent the recurrence of endometriosis [12,13,25]. OCs, when used continuously, are effective in preventing recurrences following surgical treatment. Epidemiological studies have also shown that the risk of developing endometriosis is lower during OC use; however, this protective effect disappears following discontinuation of treatment [25]. This suggests that as long as OCs are taken continuously, aromatase expression will be suppressed in the endometrium, and this may be the mechanism by which these compounds are effective in preventing the progression or recurrence of endometriosis. OCs containing gestodene also inhibit COX-2 expression in the endometrium in a similar fashion to that of endogenous progesterone, and this constitutes an additional factor contributing towards a reduction in inflammation and associated pain [14,15]. Endometriosis is an inflammatory disease and the blockade of both COX-2 and aromatase expression by OCs may explain the effectiveness of these compounds in controlling the disease [25].

One possible explanation for the failure of both medical and surgical treatments of endometriosis in the long term may be the resumption of menstruation, which in a retrograde fashion will carry aromatase-positive cells to the peritoneum. If this proves to be correct, then patients with endometriosis should not be allowed to menstruate, and one of the most cost-effective ways of accomplishing this is through the use of OCs in extended regimens.

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4 CONCLUSÕES

4.1 PAPEL DA AROMATASE P450 NO SANGRAMENTO UTERINO ANORMAL

O sangramento uterino anormal é definido como qualquer sangramento cujo volume exceda 80 ml durante a menstruação ou que ocorra fora deste período. Embora seja um sintoma muito comum na pratica ginecológica, até alguns anos atrás pouco se sabia sobre a sua etiologia. Os avanços nos métodos de diagnóstico tanto por imagem com por histeroscopia permitiram um melhor conhecimento das causas orgânicas e funcionais do sangramento uterino. Embora do ponto de vista didático se fizesse uma distinção entre estas diferenças causas, evidências recentes têm mostrado que os mecanismos responsáveis pelo sangramento uterino anormal seriam os mesmos e envolveriam alterações funcionais que ocorrem localmente no endométrio afetando tanto o metabolismo dos estrogênios como o aumento de mediadores ligados à inflamação. Esta inflamação endometrial que é ativada pela queda da progesterona atuaria através de uma cascata de mediadores, sendo que destes um dos mais estudados é a ciclooxigenase 2 (Cox-2). Esta enzima teria um papel importante na formação de várias prostaglandinas pró-inflamatórias no endométrio como a PGE2, que teria um papel importante na regulação não só da quantidade de fluxo menstrual mas também da intensidade da dismenorréia. A atividade desta enzima no endométrio é diminuída pela ação da progesterona e dos progestagênios, como a drospirenona, o dienogeste e o gestodeno. Um aumento da atividade da Cox-2, por outro lado está associado como a presença de sangramento uterino aumentado, indicando que uma maior transcrição do gene que codifica esta enzima leva ao desenvolvimento da menorragia. Por este motivo, o controle da atividade da Cox-2 no endométrio teria um papel importante no tratamento clínico do sangramento uterino anormal. O gene promotor de aromatase no endométrio pode ser ativado pelas prostaglandinas e isto cria um círculo vicioso de inflamação e de acentuada produção local de estrógeno [1-5]. A interação entre inflamação e expressão de aromatase pode ser fundamental para o desenvolvimento de patologias como endometriose, adenomiose, pólipos endometriais e miomas, já que esta enzima está presente no endométrio uterino de pacientes portadoras destas doenças. No entanto, os estímulos que desencadeiam a ativação desta enzima podem residir na constante exposição do endométrio aos mediadores inflamatórios que são ativados neste tecido após queda da produção de progesterona que antecede o início da menstruação [2,3]. Esta queda ativa a proteína NF-kappa B, que desempenha um importante papel na ativação da inflamação, na proliferação celular, apoptose, e angiogênese. A ativação persistente NF-kappa B é provavelmente um dos fatores responsáveis pelo desenvolvimento de não só de patologias estrógeno dependentes, mas também pelo sangramento uterino anormal associado com estas.

A ligação entre inflamação endometrial, sangramento uterino anormal e desenvolvimento de patologias se dá através da aromatase. Esta enzima, importante para a conversão da testosterona em estradiol, pode ser ativada em vários tecidos além do ovário, utilizando outros promotores gênicos que não o FSH. Assim por exemplo no endométrio a expressão da aromatase é ativada pela prostaglandina E2 produzida pela Cox-2. Como a transcrição do gene da ciclooxigenase-2 é ativada pelo NF-kappa B, isto estabelece uma ligação entre a exposição constante do endométrio a mediadores inflamatórios, o aparecimento da aromatase, o sangramento uterino anormal e o resultante desenvolvimento de várias patologias ginecológicas estrógeno- dependentes [3].

Vários estudos confirmaram a capacidade do endométrio de produzir estrógenos localmente, não somente em casos de endometriose, mas também na presença de outras patologias como adenomiose, pólipos endometriais e miomas, que produzem sintomas clínicos como menorragia e dismenorreia [6,7,8,9,10]. Especula-se que a produção local de estrógenos no endométrio poderia ser responsável não somente pelo desenvolvimento destas patologias, mas também pela ocorrência dos sintomas associados que podem anteceder o diagnóstico clínico [3]. Isto está de acordo com achados em primatas de que a exposição crônica do endométrio aos estrógenos locais da cavidade uterina leva ao desenvolvimento de várias patologias uterinas, incluindo adenomiose, pólipos endometriais e miomas, que estão associadas à expressão de aromatase no endométrio humano [11].

A presença aberrante de aromatases no endométrio pode aumentar a expressão de Cox-2 neste tecido, resultando desta forma em níveis mais elevados de prostaglandinas e estímulo adicional à aromatase, criando um circuito de retroalimentação positiva [1,8,12,13] e uma exacerbação de sintomas como dor e sangramento uterino. Estudos epidemiológicos mostraram que as pacientes que mais tarde desenvolvem endometriose, freqüentemente relatam uma longa história de sintomas que é anterior ao diagnóstico clínico desta patologia [8]. Isto está de acordo com a hipótese de que o desenvolvimento da patologia é o resultado de uma exposição prolongada do útero ao hiperestrogenismo local, que é conseqüência do aumento da síntese endometrial de estrógeno e de prostaglandinas pró-inflamatórias como a E2, No inicio estas alterações funcionais podem apenas causar uma exacerbação destes sintomas menstruais, porém a exposição freqüente do endométrio a este estado de inflamação aumentada pode servir de estimulo para o desenvolvimento de patologias estrogênio dependentes como a adenomiose , endometriose , miomas e pólipos endometriais [3].

4.2 PAPEL DA INFLAMAÇÃO NO DESENVOLVIMENTO DA ADENOMIOSE

A adenomiose ou endometriose interna do útero é uma patologia freqüente, associada com sintomas como dor e sangramento excessivo durante a menstruação. Nesta patologia, o defeito básico é a expressão anômala de aromatase no endométrio eutópico, que mantém uma retroalimentação estrogênica positiva neste tecido, resultando em uma expressão de persistente da Cox-2 durante a fase lútea, quando no endométrio normal a atividade desta enzima está diminuída pela ação da progesterona [6]. A produção local de estrogênios exacerbaria a inflamação endometrial e provocaria uma resistência à ação antiinflamatória da progesterona no endométrio das pacientes com adenomiose, levando assim a uma produção aumentada de prostaglandinas nestas lesões [6]. No útero normal, por outro lado, a expressão de Cox-2 torna-se negativa na maior parte das glândulas endometriais durante a fase lútea tardia, embora alguma expressão possa persistir no epitélio superficial [15]. A expressão de aromatase no endométrio eutópico destas pacientes com adenomiose seria, portanto, a causa e a conseqüência deste aumento da inflamação endometrial devido a retroalimentação positiva que os estrogênios têm sobre a Cox-2 [6,16]. No endométrio, portanto, o aumento da inflamação crônica estimula a produção local de estrogênios, que por sua vez ativa mais ainda a Cox-2, levando ao sangramento uterino anormal e a dismenorréia.

Uma vez que os mediadores inflamatórios, tais como as prostaglandinas E2, são o estímulo desencadeador para a expressão de aromatase na adenomiose e outras patologias estrogênio dependente, isto criará no endométrio um círculo vicioso de aumento de inflamação uterina e de produção estrogênica local, que poderá também exercer um efeito deletério sobre a fertilidade [17]. A expressão aumentada de Cox-2 na adenomiose durante a fase lútea pode ter um papel na patogênese da dismenorreia e menorragia associada a esta doença, já que a intensidade destes sintomas se correlaciona positivamente com os níveis de produção de prostaglandina [14].

Sabe-se que uma parte dos efeitos estimulantes que os estrógenos têm sobre o crescimento uterino é mediada pelas prostaglandinas e pelo aumento da inflamação, já que eles são bloqueados parcialmente pela indometacina e dexametasona [18]. O papel estimulante das prostaglandinas e da inflamação sobre o crescimento uterino pode explicar parcialmente a miohipertrofia observada na adenomiose, já que Cox-2 e aromatase são expressadas continuamente nesta patologia [6]. A progesterona exerce um efeito anti-inflamatório sobre o endométrio, agindo através da supressão dos genes ligados à inflamação que são induzidos

pela ativação do NF-kappa B. A diminuição da produção da progesterona antes do início da menstruação levaria à ativação do NF-kappa B no endométrio que é seguida pelo aumento da produção de citocinas inflamatórias e de prostaglandinas neste tecido [2,19]. Na adenomiose, é possível que a excessiva produção estrogênica local induzida pela expressão aberrante de aromatase, no endométrio eutópico, possa parcialmente contrabalançar os efeitos anti-inflamatórios da progesterona, resultando assim na expressão persistentemente elevada da Cox-2 e do NF-kappa B, tanto nas glândulas endometriais eutópicas como nas ectópicas durante a fase lútea, enquanto no endométrio livre de doença, a inflamação é mantida a um nível mínimo, devido ao efeito anti-inflamatório da progesterona [2,6,15]. Estas alterações inflamatórias e no metabolismo local dos estrogênios explicariam o sangramento uterino anormal, a dismenorréia e a miohipertrofia associada com a adenomiose [20]. No entanto toda esta cascata de eventos é iniciada pela ativação do NF-kappa e o seu posterior deslocamento do citoplasma para o núcleo celular, onde ele se liga ao DNA,e inicia a transcrição dos genes ligados à cascata inflamatória [2]. A progesterona parece agir inibindo este mecanismo, já que a queda nos níveis de progesterona, que antecede o início do sangramento menstrual, leva à inativação da proteína inibitória NF-kappa B no citoplasma e à liberação de NF-kappa B ativo que migra para os núcleos celulares [2,19]. Por isso, a detecção de FN-kappa pelos métodos imunohistoquímicos nos núcleos das células fornece evidência de que os genes relacionados à inflamação estão sendo ativados naquele tecido. No endométrio, a presença de elevações em NF-kappa nuclear durante a fase proliferativa e lútea tardia do ciclo menstrual é mais frequentemente detectada nas pacientes com patologias uterinas como a adenomiose, do que em controles normais [21]. A maior freqüência de NF-kappa B nuclear positivo nos úteros com patologia sugere uma associação positiva entre inflamação aumentada e o desenvolvimento não só destas condições clínicas, mas também dos sintomas menstruais associados. Por causa dos efeitos antagônicos dos estrogênios e da progesterona no endométrio, tanto o predomínio estrogênico como uma diminuição da ação da progesterona podem exercer um efeito estimulante sobre o mecanismo moleculares que ativam o NF-Kappa.b resultando assim no aumento da inflamação endometrial [2]. No nosso estudo, as patologias uterinas mais freqüentemente associadas com a presença aumentada de NF-kappa B nuclear positivo antes do início da menstruação foram adenomiose, pólipos e miomas submucosos e intramurais. É importante destacar que são nessas patologias que a expressão de aromatase é detectada freqüentemente, ou no endométrio ou nas lesões [6,9]. A inflamação aumentada na adenomiose pode assim refletir a produção aumentada dos estrógenos, que por si só aumentaria a inflamação e a produção de prostaglandina no endométrio através da ativação de Cox-2 e de

NF-kappa B [1-3,12]. Esta ativação da inflamação endometrial seria também responsável pelo sangramento uterino anormal e a dismenorréia.

Os contraceptivos orais, por outro lado, devido ao seu forte efeito progestacional sobre o endométrio e seu efeito supressivo sobre a esteroidogênese ovariana, podem interromper este círculo vicioso da expressão aumentada de Cox-2 e de aromatase no endométrio, diminuindo tanto a síntese estrogênica como a inflamação. O mecanismo pelo qual os contraceptivos orais exercem um efeito supressivo sobre a expressão de aromatase no endométrio é direto e ocorre na transcrição do gene para esta enzima, conforme sugerido pelo nosso grupo em uma publicação anterior e confirmado por estudos de biologia molecular [6,22,23]. O fato desta enzima não ser detectável através de imunohistoquímica no endométrio de pacientes em uso de contraceptivos orais contendo gestodeno poderia ser explicado pela inibição da transcrição gênica por este hormônio [23]. Da mesma forma, uma redução na inflamação é também observada no endométrio com o uso de contraceptivos orais, e o mecanismo responsável por estes efeitos é a inibição da Cox-2 e do NF-Kappa.b. Em pacientes com patologias uterinas e sintomas de menorragia e dismenorreia, o uso contínuo de contraceptivos orais contendo gestodeno diminuiu significantemente a freqüência de NF-kappa B ativado no endométrio. Este efeito foi observado somente em pacientes que ficaram em amenorréia, já que a presença de sangramento irregular durante o uso do contraceptivo oral está associada com a exacerbação da inflamação com a ativação em seqüência do NF-Kappa.b e da Cox-2 [24]. Estes resultados sugerem que os efeitos anti-inflamatórios do gestodeno sobre o endométrio são mediados pela supressão da ativação do NF-kappa B, que por sua leva à inibição da Cox-2 ao mesmo tempo que suprime diretamente a transcrição do gene da aromatase. Esta ação combinada explicaria a resolução de dor e sangramento uterino anormal associados com as patologias uterinas durante o uso contínuo dos contraceptivos orais [22,25].

Os efeitos do gestodeno sobre a aromatase são similares àqueles alcançados com o uso do danazol, que também inibe a expressão de aromatase no endométrio eutópico de pacientes com adenomiose, através de uma ação direta supressora sobre a transcrição genética, reduzindo assim os níveis de RNA mensageiro [26]. Nossos achados de que contraceptivos orais contendo gestodeno são capazes de reduzir a expressão de aromatase no endométrio eutópico de pacientes com adenomiose, endometriose e miomas, podem explicar a sua eficácia quando usados para prevenir não somente recorrências em pacientes com endometriose tratadas cirurgicamente, mas também o sangramento uterino anormal e a dor associados com estas

patologias [22,25,27]. Em pacientes com adenomiose e outras patologias associadas, o uso de um dispositivo intrauterino liberador de levonorgestrel também foi eficaz em suprimir a expressão de aromatase e da Cox-2 no endométrio e nas glândulas ectópicas presentes no miométrio [28]. Isto poderia explicar a sua eficácia no tratamento da menorragia causada pela adenomiose [29]. Entretanto a eficácia do sistema uterino liberador de levonorgestrel (Mirena) aumenta quando o endométrio é removido através de um a cirurgia histeroscópica no momento da inserção do dispositivo. Em 2003 publicamos um estudo que foi confirmado posteriormente, mostrando que o uso do Mirena em pacientes com adenomiose submetidas a ablação endometrial, os índices de amenorréia eram significativamente maiores do que quando estes métodos eram utilizados isoladamente [29]. Uma das razões para a maior eficácia do Mirena nestas pacientes era de que a ablação do endométrio permitiria uma melhor difusão do levo-norgestrel para o miométrio, fazendo com os níveis miometriais deste esteróide fossem maiores do que quando o endométrio estava intacto. Isto estaria de acordo com um estudo feito anteriormente sobre os níveis de levonorgestrel no sangue, endométrio, miométrio e trompas em usuárias de Mirena submetidas a uma histerectomia. Como era de se esperar os níveis no endométrio foram extremamente elevados, porém no miométrio, sangue e outros tecidos estes eram muito mais baixos e da mesma magnitude [30]. Isto sugeriria que o endométrio intacto poderia funcionar como uma barreira impedindo a difusão de grande parte do levonorgestel para miométrio subjacente. Com isto as glândulas endometriais presentes no miométrio ficariam expostas a níveis de levonorgestrel muito inferiores a aqueles obtidos caso o endométrio fosse removido, permitindo assim um contato direto do Mirena com o miométrio. Níveis mais elevados de levonorgestrel no miométrio levariam a uma maior supressão da Cox-2 e da aromatase nestas glândulas endometriais ectópicas , tornando assim mais eficaz a ação do Mirena na adenomiose.

Recentes mostramos que em pacientes com adenomiose utilizando o Mirena a inibição da Aromatase e da Cox-2 nas glândulas endometriais era significativamente maior quando o Mirena era inserido após uma ressecção endometrial por via histeroscópica [28]. A maior supressão destas enzimas nestas pacientes explicaria também os maiores índices de amenorréia obtidos. Estes resultados mostraram que a ressecção endometrial seguida da inserção do Mirena era uma alternativa eficaz e segura à histerectomia no tratamento do sangramento uterino anormal causado pela adenomiose.

A presença da enzima aromatase nas glândulas basais no endométrio eutópico de pacientes com adenomiose aponta para um papel crucial desta camada na patogênese da

adenomiose [6]. O meio hiperestrogênico causado pela expressão aberrante de aromatase pode ser importante não só para o mecanismo de invaginação da camada basal para dentro do miométrio, mas também para a exacerbação da inflamação associada com esta patologia. A remoção de grande parte da camada basal por histeroscopia seguida da inserção do Mirena levaria a uma inibição mais eficaz destas enzimas, resultando assim em melhores índices de amenorréia. Uma observação clinica interessante é que nestas pacientes com adenomiose submetidas à ablação endometrial e inserção do Mirena é que a presença da amenorréia está associada ao desaparecimento dos sintomas de tensão pré-menstrual nestas [31]. Como o Mirena não bloqueia a ovulação nem as flutuações hormonais associadas com esta é provável que da mesma maneira que o sangramento uterino anormal, a TPM seja também conseqüência do aumento da inflamação endometrial. Em pacientes com maior grau ativação da inflamação endometrial é plausível do ponto de vista biológico supor a queda da progesterona que começa a ocorrer nos dias que antecedem a menstruação levaria a uma exacerbação deste processo inflamatório. A produção de aumentada dos mediadores inflamatórios no endométrio destas pacientes não só provocaria os sintomas menstruais locais, mas também com a passagem destes para a circulação sangüínea haveria uma interferência com o transporte de serotonina nas sinapses neuronais, provocando assim a TPM. Sabe-se, por exemplo, que as interleucinas diminuem os níveis deste neurotransmissor no cérebro através do aumento de seu transporte nas sinapses. Existem também estudos clínicos que corroboram esta nossa sugestão para o papel da inflamação endometrial na tensão pré-menstrual. Pacientes com TPM, por exemplo, têm maiores níveis sangüíneos não só da PGE2, mas também de marcadores inflamatórios sistêmicos como a proteína C reativa (PCR). Isto indica um maior grau de inflamação nestas pacientes [31]. Em pacientes submetidas a ablação endometrial sem o uso do concomitante do Mirena, a resolução da TPM só ocorreu naquelas que permaneceram em amenorréia, já que a regeneração endometrial e retorno das menstruações trouxe de volta também estes sintomas. Do ponto de vista clinico se sabia também que não existem diferenças nos níveis sangüíneos de estradiol, testosterona e progesterona entre pacientes com ou sem TPM, indicando que não são as flutuações hormonais per se a causa da TPM, mas diferenças individuais na resposta a estas mudanças hormonais. O problema é que nunca se olhou o endométrio e a inflamação associada com a menstruação como a causa destes sintomas cíclicos, mesmo quando se havia evidências que a inflamação sub-clinica está associada com a depressão, devido aos efeitos que têm as citocinas inflamatórias sobre os níveis de serotonina no cérebro [31]. A maior incidência de TPM em pacientes com sintomas físicos ligados à menstruação é também outra evidência ligando o endométrio e a inflamação associada com a menstruação aos sintomas da TPM.

4.3 EXPRESSÃO DE AROMATASE E MIOMAS

No endométrio de pacientes com miomas submucosos e intramurais, a expressão de aromatase é detectada mais freqüentemente durante a fase proliferativa do que na fase lútea do ciclo menstrual [12]. A presença da expressão de aromatase no endométrio de pacientes com miomas mostra, como esperado, uma associação com a presença de sangramento uterino anormal. Nas pacientes com miomas subserosos, mas sem menorragia, a expressão de aromatase esteve ausente no endométrio, enquanto nas pacientes com miomas submucosos, a expressão de aromatase foi freqüentemente detectada [32]. Isto está de acordo com a hipótese de que a expressão de aromatase exerce um papel de causalidade no desenvolvimento de sintomas como sangramento uterino anormal ou dor, que estão freqüentemente associados com miomas. O aumento na produção local de estrogênio no endométrio de pacientes com úteros miomatosos exacerba a inflamação e a ativação de NF-kappa B, como descrito previamente [21]. Na verdade, um aumento na produção de prostaglandina e inflamação foi observado em úteros com miomas submucosos e intramurais, mas não naqueles com miomas subserosos. Isto corrobora a hipótese de que o aumento da produção local de estrogênio aumentará a inflamação e criará um círculo vicioso que por fim levará ao sangramento uterino anormal. Embora seja inconclusivo se a expressão de aromatase no endométrio poderia ou não contribuir para a presença de sangramento menstrual anormal em pacientes com úteros miomatosos, a expressão aberrante de aromatase foi detectada na presença de outras patologias uterinas associadas com sangramento uterino anormal e dismenorréia, como adenomiose, endometriose e pólipos endometriais [6,12,32].

A associação freqüente de miomas sintomáticos com endometriose, adenomiose e pólipos endometriais também sugerem que estas patologias compartilham a mesma patogênese. Também há evidência de que a produção local de estrógeno em miomas ou no endométrio eutópico é mais importante que estrógenos sistêmicos em relação ao estímulo de crescimento, já que o crescimento de miomas não pode ser completamente restabelecido através de estrógenos exógenos em pacientes usando análogos de GnRH, devido aos efeitos supressivos dos análogos de GnRH sobre a expressão de aromatase em úteros miomatosos [33]. Estes achados estão de acordo com a hipótese de que estrógenos produzidos localmente, atuando de modo intrácrino, ou no endométrio ou no mioma, podem ter um papel crucial tanto na patogênese do sangramento menstrual anormal como no crescimento desta neoplasma [7,16,20,22].

No entanto, a taxa e a velocidade de crescimento do fibroide podem envolver outros fatores além de estímulos estrogênicos. Nos casos de miomas subserosos, que podem atingir tamanhos muito grandes sem provocar sangramentos uterinos anormais, a taxa de crescimento rápido é mais dependente de reorganização citogenética do que de fatores hormonais, porque a expressão de aromatase é negativa nesta neoplasma [22,34-36]. Além de estimular a inflamação, os estrogênios locais irão estimular a síntese dos fatores angiogênicos, tais como VEGF, no endométrio, levando finalmente à menorragia associada com a presença de miomas [7,36]. É possível que as alterações endometriais, como o aumento da inflamação e da vascularização resultantes da produção estrogênica endometrial, sejam a causa da menorragia relacionada a miomas e não o tumor propriamente dito. Isto está de acordo com o achado de que a menorragia está positivamente relacionada com a presença de expressão de aromatase no endométrio e não com o tamanho do mioma [32]. Seria uma simplificação exagerada considerar esta produção estrogênica local como sendo o único mecanismo patogênico no desenvolvimento de miomas e do sangramento uterino anormal; outros fatores podem agir sinergicamente, incluindo anormalidades citogenéticas que podem afetar as taxas de crescimento independentemente da intensidade dos sintomas menstruais [32-36].

O uso dos análogos de GnRH ou de danazol está associado não somente com a redução de sangramento utermo anormal em pacientes com miomas mas também com a inibição da expressão de aromatase no endométrio [16]. Outro benefício do uso de progestagenios é a redução do risco relativo de desenvolvimento de miomas em mulheres usando contraceptivos orais, o que poderia, da mesma forma, ser uma conseqüência semelhante à inibição da expressão de aromatase no endométrio de úteros contendo miomas [37]. A supressão da expressão de aromatase p450 no endométrio também reduzirá em última instância a formação de fatores angiogênicos como VEGF [12,13]. O bloqueio da inflamação e da angiogênese pode, desta forma, ser o mecanismo inicial pelo qual os contraceptivos orais atuam efetivamente, não só inibindo a taxa de crescimento de miomas, mas também reduzindo o volume de sangramento uterino associado com a presença destes tumores [13].

4.4 EXPRESSÃO DE AROMATASE E O DESENVOLVIMENTO DA ENDOMETRIOSE

A patogênese da endometriose ainda não está completamente esclarecida, já que permanecem as controvérsias relacionadas a vários aspectos da doença, que impedem a eficácia do tratamento em longo prazo. Clinicamente, a endometriose é uma doença

inflamatória crônica, debilitante e recorrente, e não um evento patológico isolado durante a vida reprodutiva de uma mulher que pudesse ser resolvido com uma forma específica de tratamento [1,3,4]. Por isso, é uma condição patológica que pode ser controlada, mas nunca completamente curada, uma vez que novas células endometriais são descamadas para dentro da cavidade pélvica após sucessivas menstruações [6]. Estas sucessivas de ondas de células endometriais que chegam à cavidade uterina através da menstruação retrogradam são responsáveis pela progressão e persistência da doença [3].

A implantação e o desenvolvimento de lesões endometrióticas dependem, portanto de vários mecanismos, a começar pela ocorrência repetitiva de menstruação retrógrada [38,39]. No entanto, uma questão importante que permanece sem uma explicação adequada na teoria da implantação inicialmente proposta por Sampson em 1925 é como a menstruação retrógrada que é um fenômeno quase universal não leva ao desenvolvimento da endometriose em todas as mulheres. Modificações endometriais que só ocorreriam nas pacientes que viessem a desenvolver endometriose poderiam explicar estas observações. Sob esta ótica a endometriose seria considerada uma doença inicialmente endometrial e que dependeria da ocorrência da menstruação retrograda para que estas células fossem transportadas da cavidade uterina para a pélvis. Várias alterações na expressão gênica ocorrem no endométrio das pacientes com endometriose e estas podem resultar estas alterações funcionais locais que favoreceriam a implantação e o desenvolvimento destas células fora da cavidade uterina. Uma destas modificações é a capacidade que o endométrio das pacientes com endometriose adquire de produzir estrogênios localmente [1,3]. Estas alterações no metabolismo local dos estrogênios seriam resultantes da capacidade do endométrio adquirir a expressar a enzima aromatase tanto no epitélio glandular como também no estroma. A presença desta enzima estimularia a esteroidogênese local através da conversão dos androgênios presentes em estrogênios. Estes por sua vez teriam um papel importante na progressão e no agravamento das lesões endometrióticas, cuja evolução clínica pode variar consideravelmente de paciente para paciente [40]. O início e o desenvolvimento e a agressividade clínica da endometriose podem depender da nos níveis de expressão da enzima aromatase no endométrio eutópico, e esta pode ser uma das diferenças importantes com relação à expressão gênica e enzimática entre o endométrio eutópico das pacientes com endometriose e o das que não são portadoras desta enfermidade [41,42]. Estas alterações endometriais podem realmente anteceder o estabelecimento da endometriose como uma condição patológica clinicamente reconhecível na pelve, e serem responsáveis pelos sintomas menstruais associados com esta enfermidade. Recentemente mostramos que em pacientes com sintomas de dismenorréia e infertilidade, mas que a pelve

ainda era normal na laparoscopia, era possível detectar no endométrio desta a expressão da enzima aromatase [43]. Em vez de significar um dado negativo sobre o papel da aromatase na gênese da endometriose , estes achados pelo contrario, seriam evidências de que as alterações na expressão desta enzima no endométrio poderiam anteceder o aparecimento das lesões, explicando assim o longo período relatado em estudos epidemiológicos entre o inicio dos sintomas e o diagnóstico final da endometriose [40]. Estes achados sugerem que a endometriose começaria como uma doença endometrial envolvendo alterações epigenética que se manteriam estáveis no curso da vida da paciente e que se não corrigidas levariam ao aparecimento das lesões de endometriose na pelve [3]. A paciente com endometriose teria um endométrio do ponto de vista de atividade gênica diferente daquele das pacientes normais, o que permitiria a sua sobrevivência fora do útero. A endometriose seria, portanto uma patologia que dependeria da ocorrência de menstruações que levariam de uma maneira retrograda células aromatase positiva para a pelve, onde estas se implantariam e formariam as lesões características desta doença [3,43]. Estas mesmas alterações que ocorrem no endométrio eutópico poderiam também ocorrer nos restos do canal de Muller presentes no septo reto vaginal, explicando assim o desenvolvimento das lesões profundas da endometriose. Isto levanta uma questão clínica importante para o tratamento da endometriose que seria se as terapêuticas disponíveis atualmente são realmente efetiva na cura da doença a longo prazo, já que a maioria das meta-análises realizadas indicou elevadas taxas de recorrência após tratamento cirúrgico ou após a descontinuação de medicamentos [44, 45,46]. O reinicio das menstruações parece ser um fator de risco importante para a recorrência da endometriose, pois estas alterações funcionais podem persistir ou reaparecer no endométrio após o termino do tratamento. Isto favoreceria o retorno desta doença tão logo os ciclos menstruais se restabeleçam e novas células endometriais expressando a enzima aromatase cheguem a pelve [3,43]. Por este motivo a recorrência desta patologia não deveria ser encarada como evidência da falta de eficácia das várias abordagens cirúrgicas e farmacológicas para o tratamento desta doença mas como conseqüência da persistência do defeito endometrial funcional que foi responsável pelo desenvolvimento da endometriose em primeiro lugar. Na verdade as altas taxas de recorrência mostram que se a produção local de estrogênios não for inibida a endometriose não pode ser controlada. Isto leva a um importante dilema clinico, pois a paciente com endometriose não poderia menstruar. A amenorréia prolongada seria a única maneira eficaz de controlar esta patologia, pois o defeito endometrial que levou ao aparecimento da aromatase no endométrio pode envolver alterações epigenéticas que não seriam facilmente reversíveis mantendo-se como alterações gênicas estáveis [3,10,47]. A expressão de aromatase no endométrio eutópico seria, portanto um importante fator de risco

importante para o desenvolvimento de endometriose podendo inclusive influenciar o comportamento clínico destas lesões. A ativação da inflamação no endométrio durante a menstruação seria também um importante estimulo para a ativação do gene da aromatase, devido às grandes quantidades de prostaglandinas pró inflamatórias produzidas neste tecido pela Cox-2 [3]. Existem evidências de a expressão da enzima aromatase é maior tanto no tecido endometrial como nas lesões endometrióticas mais ativas e com um curso clínico mais agressivo [1,3,47,48]. Estes achados estão também de acordo com observações de que a expressão de aromatase não é detectável no endométrio de mulheres que não têm a doença, mas está presente no endométrio de pacientes com endometriose e outras patologias como adenomiose, pólipos endometriais e miomas [3,13,49,50]. Recentemente relatamos que a intensidade de expressão da aromatase no endométrio eutópico detectada através de métodos imunohistoquimicos tinha uma relação positiva com a severidade da endometriose, sugerindo assim que o comportamento clínico da doença seria determinado por estas alterações endometriais [3,43]. Entretanto para melhor entendermos como a presença da aromatase no endométrio afeta de uma maneira positiva a progressão e o agravamento das lesões de endometriose, temos que rever qual seria o papel dos estrogênios para a sobrevivência das células endometriais nestes sítios extra-uterinos, e como a inflamação contribui para a perpetuação deste processo permitindo a sobrevivência destas lesões na pélvis. A relação entre inflamação e a produção local de estrogênios de estrogênios se dá através das protaglandinas pró inflamatórias, como a E2 produzida a partir do acido araquidônico por ação da ciclooxigenase 2 (Cox-2) [51,52]. Em pacientes com endometriose e outras patologias estrogênios dependentes existe um aumento deste estado inflamatório que não só poderia levar pela repetição frequente das menstruações à ativação da enzima aromatase mas também a uma amplificação deste processo causado pela efeito estimulante que os estrogênios tem sobre a Cox-2 [3,49,50]. Isto criaria um ciclo vicioso de aumento de inflamação e da produção local de estrogênios que permitiria não só a sobrevivência destas células endometriais na pelve mas também explicarias os sintomas de dor provocado por esta patologia [43].

A transcrição do gene da aromatase no endométrio das pacientes com endometriose depende de um promotor que é ativado pelas prostaglandinas, principalmente a prostaglandina E2, embora possivelmente outros mediadores inflamatórios possam também ativá-lo [1]. Entretanto foi observado que a prostaglandina E2 somente aumenta a transcrição do gene da aromatase nos endométrios que já têm níveis basais detectáveis desta enzima, não tendo qualquer efeito sobre os endométrios de pacientes normais que não têm nenhuma expressão basal da aromatase [1,3,49,50,51]. Isto indica que estas alterações epigenéticas exacerbam a

resposta endometrial à inflamação, levando à produção local de estrogênios. A expressão da enzima aromatase no endométrio e a sua ativação pelos mediadores inflamatórios como a prostaglandina E2, parece portanto desempenhar um papel fundamental no desenvolvimento das lesões de endometriose na cavidade abdominal e na exacerbação da inflamação associada com estas lesões [41-44]. A menstruação teria também um papel importante na gênese da endometriose não só por que transportaria de uma maneira retrógrada estas células endometriais para a pelve, mas também por que grandes quantidades de prostaglandinas são produzidas durante este processo o que estimularia a ativação do gene da aromatrase. A chegada na pelve do sangue menstrual e das células endometriais vai desencadear uma reação inflamatória intensa, trazendo um influxo de células imunologicamente ativas, incluindo macrófagos e células natural killer com a finalidade de destruir estas células recém chegadas. Em pacientes normais com células aromatase negativa este mecanismo funcionaria de maneira adequada impedindo a implantação destas no peritôneo. No entanto, como esta intensa reação inflamatória gera grandes quantidades de prostaglandinas e de outros mediadores inflamatórios, em células endometriais que já expressam a aromatase isto irá estimular exponencialmente a transcrição desta enzima por causas destas alterações epigenéticas pré existentes neste tecido [1,23,49]. O aumento na atividade de aromatase nestas células em resposta à inflamação desencadearia uma produção local de estrógeno, que por sua vez aumentaria ainda mais a síntese de prostaglandina através da ativação da enzima ciclooxigenase-2 [49]. Isto provocaria um círculo vicioso de aumento da inflamação e da expressão de aromatase fazendo com que a excessiva produção local de estrógeno facilite a implantação destas células endometriais no peritônio [54-58]. No entanto, este meio hiper-estrogeneizado localizado na cavidade pélvica somente ocorrerá se as células endometriais que chegaram recentemente já tiverem níveis basais de expressão de aromatase, já que somente elas têm a capacidade de responder à prostaglandina E2 com o aumento da expressão de aromatase [1,3,23,49].

As quantidades excessivas de estrógenos produzidas por estas células evitarão que elas sejam destruídas pelo refluxo de macrófagos ativados e de células natural killer, já que os mecanismos de fagocitose são bloqueados pelos estrógenos [57,58]. Um exemplo comum deste efeito dos estrógenos é a bem conhecida diminuição de reabsorção óssea induzida durante a menopausa, já que os osteoclastos são macrófagos modificados que respondem aos estrógenos reduzindo a fagocitose na matriz extracelular do osso. A inibição da fagocitose pelos níveis locais elevados de estrógeno nas células endometriais provavelmente constitui o mecanismo através do qual estas células são poupadas na cavidade peritonial, desta forma implantando-se

e dando origem às lesões endometriais, enquanto as células aromatase-negativas são eliminadas da cavidade pélvica nos dias que se seguem à menstruação [1,3,54-58]. Isto indica que a tanto a apresentação como a evolução clínica da endometriose vai depender da quantidade de aromatase que está expressa nas lesões, e que por sua vez depende dos níveis basais da enzima presente no endométrio eutópico. Portanto, a presença de um endométrio eutópico aromatase-positivo desempenha um papel fundamental no desenvolvimento das lesões endometrióticas e pode explicar não somente a ocorrência de altas taxas de recorrência da doença mesmo após um bem-sucedido tratamento farmacológico ou cirúrgico, mas também explicar porque algumas lesões podem regredir espontaneamente sem qualquer forma de tratamento [59]. Esta hipótese está também de acordo com as observações relatadas anteriormente de que a agressividade da endometriose se correlaciona de uma maneira positiva com a intensidade da expressão da aromatase tanto no endométrio eutópico como nas lesões.

A hipótese de que a endometriose é uma doença crônica que depende da quantidade de expressão de aromatase no endométrio eutópico para progredir, prediz que a evolução clínica da doença pode depender do grau de inflamação ao qual o endométrio eutópico é exposto durante o ciclo menstrual, já que o gene promotor para esta enzima responde às prostaglandinas [1,3]. Se esta hipótese está correta, isto também poderia explicar as altas taxas de insucesso em longo prazo de todas as formas de tratamento de endometriose, já que o defeito primário é a expressão aberrante de aromatase no endométrio eutópico, o que pode retornar mediante a suspensão do tratamento e o reinício da menstruação e seu fluxo retrógrado.

A expressão de aromatase no endométrio eutópico é desta forma, fundamental não somente para o desenvolvimento da endometriose, mas também para determinar o grau de agressividade da sua evolução clínica [1,3]. Neste aspecto, medicamentos que suprimam a expressão de aromatase ou modulem a atividade imunológica terão um papel importante na terapia da endometriose [60]. Publicações recentes mostraram a eficácia de inibidores de aromatase no tratamento de formas freqüentemente agressivas de endometriose associadas a níveis elevados de expressão de aromatase [61,62]. A expressão de aromatase no endométrio eutópico é, portanto, crucial para determinar a evolução clínica desta doença, incluindo a propensão que algumas lesões têm para regredir espontaneamente [3.] Isto pode ser verificado pela correlação da agressividade da lesão endometriótica com a presença da expressão de aromatase no endométrio eutópico, porém, esta associação pode não se comprovar como uma

relação de causalidade. Estudos prévios já demonstraram que a quantidade de RNAm transcritos para aromatase é significantemente mais alta nas lesões mais agressivas [47].

Recentemente, nosso grupo conduziu um estudo clínico observacional para investigar se formas mais agressivas de endometriose estavam associadas com expressões mais elevadas de aromatase no endométrio eutópico [43]. A presença desta enzima foi detectada através de métodos imunohistoquímicos. Biópsias do endométrio foram realizadas durante a laparoscopia em pacientes com endometriose, para determinar a expressão de aromatase neste tecido. A severidade, tipo, e extensão da endometriose foram registradas e comparadas com a presença de expressão de aromatase no endométrio eutópico. Os resultados publicados mostraram que a presença da expressão de aromatase no endométrio eutópico das pacientes teve uma correlação positiva com a agressividade clínica das lesões endometrióticas [3,43]. Quando estes dados são analisados em função da classificação da *American Society of Reproductive Medicine* (ASRM) nas quais a severidade da endometriose é dividida em 4 tipos, do I ao IV , a depender das extensões das lesões, foi possível observar que a expressão de aromatase no endométrio eutópico ocorria com uma freqüência significativamente maior nas pacientes que apresentavam lesões mais severas (estágio IV) do que naquelas que apresentavam lesões menos extensas na pelve (estágio I) [43]. Um estudo similar medindo a quantidade de mRNA para a aromatase mostrou que no endométrio a transcrição do gene se correlacionava de uma maneira positiva com a severidade da endometriose e da adenomiose [48].

Estes resultados, embora não estabeleçam uma relação de causalidade entre expressão de aromatase no endométrio eutópico e a progressão da endometriose, sugerem uma forte associação entre a presença desta enzima no endométrio eutópico e a detecção de lesões ativas na pelve [43,48]. As regressões da endometriose e dos seus sintomas com o uso continuam de contraceptivos orais, por outro lado, foi associada com o desaparecimento da expressão de aromatase no endométrio eutópico destas pacientes [3,43]. A evolução clínica desta doença pode, desta forma, ser ditada pela presença de aromatase no endométrio. Todos os fatores que possam interferir direta ou indiretamente com a atividade de aromatase no endométrio ou nas lesões endometrióticas, incluindo ai a modulação da inflamação, podem aumentar ou diminuir a agressividade das lesões, afetando assim a evolução da doença [3]. As observações clinicas sobre a eficácia do uso continuo dos contraceptivos orais no tratamento da endometriose podem ser explicadas também pelos efeitos supressivo que os progestagênios têm sobre a atividade da aromatase [3,12,13,16,32]. Outras agentes farmacológicos usados no tratamento da

endometriose como o danazol ou mais recentemente o dienogeste são também inibidores da expressão da aromatase e da Cox-2 [63]. Estes dados estão de acordo com a nossa hipótese de que a progressão da endometriose depende da repetição dos ciclos menstruais em pacientes cujo endométrio expressa a enzima aromatase.

5 CONSIDERAÇÕES FINAIS

A presença da expressão de aromatase no endométrio é um fator de risco para o desenvolvimento de patologias, uma vez que a produção local de estrógenos estimulará a inflamação. Este mecanismo é complexo e envolve um círculo vicioso de produção aumentada de prostaglandina E2 através da ativação de Cox-2 em resposta aos níveis aumentados de estrógenos. A ocorrência de inflamação sub-clínica no endométrio na presença de várias patologias estrógeno dependentes também pode ser inferida pela existência da expressão de FN-kappa B nos núcleos celulares, um fator de transcrição importante na cascata inflamatória. A ativação do FN-kappa B nuclear é estimulada pela exposição ao estrógeno ou pela supressão de progesterona, já que a constante exposição do endométrio aos progestínicos suprime a inflamação e inibe a translocação de FN-kappa B para os núcleos celulares. Os progestínicos, portanto são importantes hormônios anti-inflamatórios cujas ações sobre o endométrio são capazes de suprimir a ativação de FN-kappa e de reduzir a expressão de enzimas como aromatase e Cox-2. Estes efeitos são de importância fundamental para a aplicação clínica dos progestínicos no tratamento de patologias causadas pela inflamação crônica e expressão aberrante de aromatase como a endometriose. A produção aumentada de estrógenos locais, que é catalizada pela presença de aromatase no endométrio através do aumento da inflamação, e a conseqüente formação excessiva de prostaglandina, também é responsável pela exacerbação de sintomas associados à menstruação como dismenorreia e menorragia.

Acredita-se que a produção local de estrogênio seja o mecanismo de supressão dos macrófagos e células NK por células fagocíticas. O grau de severidade da endometriose correlaciona-se positivamente com a intensidade da expressão de aromatase no endométrio eutópico, corroborando desta forma a hipótese de que a progressão da endometriose depende da descamação constante das células aromatase-positivas através da menstruação retrógrada. A coexistência de outras patologias como a endometriose também está associada com a presença da expressão de aromatase no endométrio. Isto tem sido observado em casos de

miomas submucosos/intramurais, pólipos endometriais e adenomiose. Em todos estes casos, a presença de aromatase esteve associada com um aumento de inflamação no endométrio conforme determinado pela presença de NF-kappa B nos núcleos celulares. O número de núcleos positivos para NF-kappa B foi significantemente maior no endométrio de úteros portadores de patologia em comparação aos controles normais. Isto foi observado tanto durante a fase proliferativa quanto antes do início da menstruação, sugerindo assim um papel pró-inflamatório para os estrógenos e um efeito anti-inflamatório da progesterona sobre o endométrio. A progesterona endógena ou os progestínicos, como gestodeno ou levonorgestrel quando usados oralmente ou liberados localmente na cavidade uterina, são inibidores potentes da expressão de aromatase no endométrio e este efeito provavelmente envolve o silenciamento de genes através da inibição de transcrição. Na verdade, o uso contínuo de contraceptivos orais combinados contendo gestodeno ou o uso de sistemas intrauterinos liberadores de levonorgetsrel se mostraram efetivos na prevenção não só da recorrência de endometriose mas também da menorragia associada a miomas, reforçando assim a relação de causalidade entre a expressão aberrante de aromatase no endométrio, inflamação, e o desenvolvimento de patologias.

Esta diferença não estava na histologia, mas sim no padrão enzimático do tecido. Verificamos que estes endométrios não só expressavam a enzima aromatase p450 responsável pela síntese de estradiol, mas também apresentavam um maior grau de inflamação, mediada através da expressão aumentada da Cox-2 e do NF-Kappa.b . A inflamação endometrial seria o fator causal que induzia a ativação do gene da aromatase no endométrio conforme sugerimos numa publicação contendo os trabalhos apresentados no *World Congresso of Gynecological Endocrinology* em Florença em março de 2012. . Verificamos também que estas pacientes com miomas poderiam ter outras patologias associadas, como miomas, adenomiose e pólipos endometriais, e que em todas estas patologias a enzima aromatase estava também expressa no endométrio na maioria dos casos. Sugerimos nesta ocasião que a expressão da aromatase no endométrio eutópico das pacientes com endometriose era crucial para a implantação destas células fora da cavidade uterina, pois os estrogênios assim produzidos inibiriam a fagocitose destas células pelos macrófagos ativados. Outro achado importante observado por nós foi de que a expressão da aromatase do endométrio já podia ser detectada no endométrio de pacientes com sintomas menstruais, mas que ainda não apresentavam endometriose ou outras patologias. Estes achados sugeriam que a presença da aromatase neste tecido poderia anteceder o aparecimento da endometriose, o que explicaria o longo intervalo entre o aparecimento dos primeiros sintomas menstruais e o diagnóstico

definitivo desta patologia. Na verdade a interação entre a inflamação endometrial e a produção local de estrogênios parece ter um papel importante na patogênese dos sintomas menstruais e das patologias estrogênio dependentes, e esta é uma área de pesquisa que nos interessa bastante. Este estudo piloto foi amplamente confirmados por inúmeros trabalhos feitos na Europa e Ásia. Estes estudos serviram de base para o lançamento no Brasil de anticoncepcionais orais contendo gestodeno, dados de maneira contínua para o tratamento destas patologias estrogênio dependente.

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ANEXO

ITAIGARA MEMORIAL
HOSPITAL DIA

Termo de Consentimento Livre e Esclarecido

PACIENTE:
RG Nº: C.P.F.:
ENDEREÇO: CÓDIGO:
PROCEDIMENTO
ATENDIMENTO: PRONTUÁRIO:

Declaro conforme dados acima que estou de acordo com os seguintes termos:

1) Autorizo a utilização dos dados clínicos em trabalhos científicos, publicações em congressos ou outras atividade de cunho científico.

2) A presente autorização é conhecida a título gratuito, abrangendo seu uso em todo território nacional e internacional.

3) Dessa forma, declaro ser esta expressão da minha vontade, autorizando o uso acima descrito

4) Antes de Assinar este documento, eu fui suficientemente informado(a) sobre o procedimento anestésico e cir a ser realizado e conversei diretamente com meu médico e ele respondeu todas as perguntas que fiz sem deixa dúvidas. Participo voluntariamente obedecendo a resolução196/96 do Conselho Nacional de Saúde.

Salvador (BA),

C.P.F.: