Clotrimazole reduces endometriosis and the estrogen concentration by downregulating aromatase

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Abstract

This study aimed to analyse the effects of clotrimazole (CTZ) on estrogen production pathway in endometriosis progression. Experimental endometriosis was induced by autologous transplantation in female Wistar rats, and then the rats were treated with clotrimazole (200 mg/kg) or vehicle, both orally and intraperitoneally, for 15 consecutive days. Serum estrogen levels and vaginal smear analyses were performed and ER α (estrogen receptor alpha) and CYP19 (cytochrome P450 aromatase) levels in the endometriotic lesions were analysed morphologically and immunohistochemically. The clotrimazole group presented a reduction in serum estrogen levels, which were not influenced by the estrous cycle of the animals. The expression of ER α and CYP19 in endometriotic lesions was also reduced in the clotrimazole group compared to the control group. Moreover, clotrimazole treatment decreased the size of the lesions, as confirmed by histological examination, which showed glandular atrophy for both routes of administration. These results suggest that clotrimazole interferes with the estrogen production pathway by downregulating CYP19 and, therefore, reducing serum estrogen levels. Thus, the drug decreases endometriotic lesion size and consequently disease progression.

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Introduction

Endometriosis is an estrogen-dependent disease, characterized by the presence of endometrial tissue outside the uterine cavity (Giudice 2010). Its prevalence is estimated to be 10–15% in women of reproductive age (Buck Louis *et al.* 2011, Acien & Velasco 2013), and it is often associated with clinical symptoms including chronic pelvic pain, dysmenorrhea, dyspareunia and infertility (Fauconnier *et al.* 2013). The prevalence of endometriosis increases to 35–50% in infertile women and is considered one of the most common gynaecological diseases (Buck Louis *et al.* 2011, Acien & Velasco 2013).

Some risk factors such as age, family history, alcohol consumption, smoking and primarily the high level of estrogen are associated with the development of

endometriosis (Vigano *et al.* 2004, Parazzini *et al.* 2017, Hemmert *et al.* 2018). Several studies have demonstrated that lesion growth is related to the action estrogen, while hormone reduction due to menopause, pregnancy or pharmacological suppression of endogenous secretion usually reduces endometriosis (Huhtinen *et al.* 2012, Vercellini *et al.* 2016, Marie-Scemama *et al.* 2019). Naqvi *et al.* (2014) showed that a selective estrogen receptor modulator (BZA) inhibits the action of estrogen on endometrial cell proliferation in an experimental model of endometriosis, confirming that estrogen supports cellular proliferation in endometriosis (Naqvi *et al.* 2014).

One of the most important pathways responsible for the increased estrogen levels in patients with endometriosis is associated with expression of aromatase enzyme (Mori *et al.* 2018), a CYP19 gene product that is part of the

cytochrome P450 family. Cytochrome P450 aromatase (CYP19A1) catalyses the conversion of testosterone and androstenedione to estradiol (E2) and estrone (E1), respectively, increasing estrogen production and endometriosis progression (Mori *et al.* 2018).

The current pharmacological treatments for endometriosis, which include the use of gonadotropin-releasing hormone (GnRH) agonists, progesterone oral contraceptives and aromatase inhibitors, work by suppressing ovarian function (Lindsay *et al.* 2015, Vercellini *et al.* 2016). However, these treatments are associated with undesirable side effects, such as a weight gain, muscle cramps, acne, seborrhea, depression, flushing, urogenital atrophy, loss of libido and bone mass decrease (Vercellini *et al.* 2009, Lindsay *et al.* 2015, Becker *et al.* 2017). Moreover, women who want to become pregnant cannot use hormonal contraceptives for endometriosis treatment (Bedaiwy *et al.* 2017, Mori *et al.* 2018, Piccinato *et al.* 2018). Despite the drug treatment options, they are associated with many side effects, and surgery is still the gold standard therapeutic approach (Kho & Abrão 2012). Therefore, identifying effective drug therapies, alone or in combination, with fewer adverse effects is an essential step to improve endometriosis management.

Clotrimazole (CTZ) is an antifungal that is widely used in clinical practice to treat *Candida* infection and other fungal infections (Crowley & Gallagher 2014). This drug is effective in controlling tumour progression by interfering with cancer cell metabolism and proliferation (Marcondes *et al.* 2010, 2015, Furtado *et al.* 2012, 2015). Recently, our group showed that CTZ is a promising novel pharmacological treatment for endometriosis due to its anti-inflammatory and anti-angiogenic properties and that it significantly decreases the growth and size of endometriotic implants (Machado *et al.* 2018). Interestingly, CTZ has been demonstrated to inhibit CYP19A1 in humans, through its antiproliferative effects in human breast cancer (Trösken *et al.* 2004). On the other hand, the upregulation of CYP19A1 increases estrogen levels, which are correlated with increased levels of prostaglandin E2 (PGE₂) and other inflammatory mediators (Bulun *et al.* 2001) that are involved in the pathogenesis of endometriosis (Machado *et al.* 2010, 2016, 2018, Mattos *et al.* 2019).

Therefore, in the present study, we investigated the effects of CTZ on the estrogen production pathway, specifically the aromatase enzyme, and consequently on estrogen levels in an experimental model of endometriosis.

Materials and methods

Determination and cyclicity of the estrous cycle

The estrous cycle was monitored daily by vaginal smears collected using sterile swabs and stained with a Papanicolaou

kit according to the manufacturer's instructions (QEEL – Química Especializada Erich Ltda, São Paulo, SP, Brazil). The stages were identified by microscopic examination according to cytological findings as follows: proestrus, nucleated cells; estrus, cornified cells; metestrus, cornified cells plus leucocytes; and diestrus, leucocytes (Cora *et al.* 2015). Only rats that showed three consecutive 5-day estrous cycles with normal progression and synchronicity of all stages were used.

Experimental model of endometriosis

Animals were treated in accordance with protocols approved by the State University of West Zone (UEZO), with guidelines from the Ethics Commission on Animal Use (CEUA – 009/2019). Female Wistar rats (8-weeks old and 200 g) were used to test the effects of CTZ in experimental endometriosis as previously described (Machado *et al.* 2010, 2016, Machado *et al.* 2018, Mattos *et al.* 2019).

Animals were anaesthetized (ketamine and xylazine), the abdomen was opened and one uterine horn was removed; the endometrium was exposed and split longitudinally into two pieces and one piece was implanted on each side of the ventral abdominal peritoneal wall with sutures. The abdomen was closed, and the animals were allowed to recover from anaesthesia.

Clotrimazole treatment

After 15 days, ventral midline laparotomy was performed to evaluate the viability and size (length × width) of the endometrial explants. The rats were randomly divided into four groups: groups treated with CTZ (200 mg/kg body weight, clotrimazole, Sigma Chemicals 129 Co.) diluted in sunflower oil, by (1.) gavage ($n=15$) or (2.) intraperitoneal injection ($n=10$); and control groups that received sunflower oil as vehicle by (3.) gavage ($n=15$) or (4.) intraperitoneal injection ($n=10$). All treatments were administered daily for 15 consecutive days. Body weight was measured at the start of treatment (day zero, D0), on day 7 of treatment (D7) and on the last day of treatment (D15), when the animals were killed by anaesthetic overdose. The endometriotic lesions were counted, weighed, measured (length × width) and collected for histological and immunohistochemical analysis. Blood samples were collected for the analysis of serum estrogen levels.

Histology, immunohistochemistry and morphometric analysis

Peritoneal lesions were fixed in buffered formalin, embedded in paraffin and cut into 4- μ m-thick sections. Some of the sections were stained with Harris hematoxylin and eosin (HE), and the other sections were placed on silane-treated slides and incubated overnight with the following antibodies: a MAB against ER α (sc-8005, Santa Cruz Biotechnology) and a polyclonal antibody against CYP19A1 (ab-18995, Abcam discover more, Abcam) (both at 1:100 dilution). Then, the staining was visualized using a LSAB2 Kit HRP (Dako-Cytomation) with diaminobenzidine (3,3'-diaminobenzidine

tablets; Sigma) as the chromogen, and the sections were counterstained with hematoxylin. For each case, negative control slides were incubated with antibody vehicle or no immune rabbit or mouse serum.

All samples were evaluated by two blinded observers under a light microscope (Nikon) connected to a digital camera (Coolpix 990; Nikon) at a magnification of 400 $\times$. Sections immunostained (ten fields) with each antibody from each specimen were randomly chosen and captured. Using high-quality captured images (2048 $\times$ 1536 pixel buffer) and the Image Pro Plus 4.5.1 program (Media Cybernetics, Silver Spring, MD, USA), the samples were quantified. Histological scores (H) for ER α and CYP19A1 were calculated using the formula $H = \sum P_i$, where i is the intensity ranging from 0 (negative cells) to 3 (strongly stained cells) and P is the percentage of stained cells for each given i , with P values of 1, 2, 3, 4 and 5 indicating 85% and 100% positively-stained cells as previously described (Machado *et al.* 2016, 2018, Mattos *et al.* 2019). The staining results are expressed as the mean $\pm$ S.D.

Estrogen levels

Blood samples were taken at three different experimental stages (days: D-15, D0 and D15) and centrifuged at 1811 g for 10 min to obtain the serum for hormonal profile analysis. The serum estrogen levels were assayed in duplicate using an ELISA kit manufactured by Diagnostics Biochem Canada Inc. (DBC) and were calculated from standard curves generated by an automatic plate reader (Spectra Max; Molecular Devices, Sunnyvale, Calif) controlled by SoftMax software (Molecular Devices).

Statistical analysis

The data are expressed as the mean $\pm$ S.D., and statistical analyses were performed with Student's t -test. For morphometric

analysis of ER α and CYP19A1, statistical calculations were carried out using the Stat Xact-5 software program (CYTEL Software Corporation, Cambridge, MA). Significance for all statistical comparisons was set at $P < 0.05$.

Results

Serum estrogen levels are reduced by CTZ

Endometriosis model rats were orally treated with CTZ (200 mg/kg of body weight, by gavage) or the same volume of sunflower oil used as vehicle (control group) for 15 consecutive days. To characterize the estrous cycle of the animals, vaginal smear analysis was performed daily. On day 15, all rats were in the estrous phase, which was confirmed by the predominance of anucleated cornified cells (Fig. 1A and B). On days 0 (Fig. 1C and D) and 15 (Fig. 1E and F), nucleated cells and a predominance of leukocytes were observed in all animals, indicating the diestrus phase. In addition, serum estrogen levels were quantified at the different experimental stages. On days 15 (Fig. 1G) and 0 (Fig. 1H), serum estrogen levels were in the normal range in both groups (control and CTZ). However, after treatment, a significant reduction in serum estrogen levels was observed only in the CTZ group (Fig. 1I). This result allows us to conclude that the reduction in estrogen was caused by CTZ, not by the estrous cycle of the animals.

CTZ suppresses estrogen receptor α in endometriotic lesions

Estrogen upregulates the expression of estrogen receptor α (ER α) in different tissues (Liu & Shi 2015, Uebi *et al.* 2015). Therefore, we assessed the expression of ER α in endometriotic lesions to evaluate the cellular effect of

Figure 1 Estrogen is reduced by CTZ. Vaginal smears from (A, B, C, D, E and F) and estrogen serum levels (G, H and I) in rats at different times of the experiment. Anucleated cornified cells (#) were predominant on day 15, indicating the estrous phase. On days 0 and 15, the diestrus phase was confirmed by the presence of nucleated cells ($\rightarrow$) and a predominance of leukocytes (*). A reduction in the serum estrogen concentration was observed only after treatment with CTZ ($P < 0.05$, Student's t -test).

decreased estrogen levels. Indeed, treatment with CTZ (Fig. 2C and D) diminished the immunoreactivity of the endometriotic lesions to anti-ER α compared to that in controls treated with vehicle, in which prominent stromal markings were observed (Fig. 2A and B). Quantification of the images confirmed a 35% reduction in the expression of ER α in CTZ-treated samples as compared to control samples (Fig. 2E; $P < 0.05$, Student's t -test; $n = 15$).

CTZ treatment downregulates CYP19A1

This same treatment drastically downregulated the expression of CYP19A1, as analysed by immunohistochemical analysis of the endometriotic lesions (Fig. 3A and B for control group and Fig. 3C and D for CTZ group). Histomorphometric analysis of the images revealed a greater than 80% reduction in the expression of CYP19A1 in the animals upon CTZ treatment (Fig. 3E; $P < 0.05$, Student's t -test; $n = 15$).

Figure 2 CTZ suppresses the alpha estrogen receptor in endometriosis. Immunohistochemical staining for ER α in endometriotic lesions (A, B, C and D). There was greater staining for ER α in the control group (A and B), which was detected predominantly in the stroma (*), primarily around the glands CTZ treated endometriotic lesions (C and D), exhibiting a significant decrease in reaction intensity (→), which was confirmed by morphometric analysis (E).

Figure 3 Aromatase enzyme is reduced by CTZ treatment in endometriosis *in vivo*. Immunohistochemical staining for CYP19 in endometriotic lesions (A, B, C and D). There was greater labelling for CYP19 in the control group (*) (A and B) than in the group treated with CTZ (→) (C and D), as confirmed by morphometric analysis (E) ($P < 0.05$, Student's t -test).

CTZ treatment improves endometriotic lesions

After 15 days of autologous transplantation (day 0), endometriotic lesions were cystic and well vascularized in all animals (Fig. 4A, B, C and D). At the end of treatments (day 15), the animals in the control group still had cystic lesions (Fig. 4E and F). However, the maintenance, growth and implant size of the lesions were significantly decreased regardless of whether the drug was administered intraperitoneally or by gavage (Fig. 4G and H). The viability of the control group lesions was confirmed by histopathological analysis, which showed the presence of glands and stroma, which are characteristic of endometrial tissue (Fig. 4I and J). Atrophy and regression of lesions in the CTZ-treated groups were also confirmed by histological analysis (Fig. 4K and L). Furthermore, CTZ treatment significantly diminished the lesion size in the orally and intraperitoneally treated groups from day 0 to day 15 (Table 1). Moreover, there were no significant differences in endometriotic lesion growth or size between the intraperitoneally and orally treated groups (Table 1).

Figure 4 CTZ decreases endometriotic lesion growth. Endometriotic lesion controls (A, B and E, F) and models treated with CTZ (C, D and G, H) intraperitoneally or orally showed hematoxylin and eosin staining (I, J, K and L). Control endometriosis models showed the presence of endometrial glands (arrows) and stromal cells (*). In the treated animals, the maintenance of the lesions was suppressed, and prominent atrophy of the glands was observed (arrowhead). Magnification, 200x.

Discussion

Approximately 176 million women of reproductive age are affected by endometriosis worldwide, and this condition causes severe pain and disability (World Bank 2010). Although highly expensive, surgery is still the principal treatment for the disease due to several undesirable side effects of available pharmacological treatments (Kho & Abrão 2012). However, new drugs with minimal side effects are being still pursued by many groups aiming to improve patient quality of life. In this context, experimental models of endometriosis induced by autologous transplantation have been used for the evaluation of new therapies for the treatment of endometriosis (Grümmer 2006, Machado *et al.* 2010, 2016, 2018, Mattos *et al.* 2019). As endometriosis is an estrogen-dependent disease, drugs that interfere with estrogen production and/or the effects of estrogen are strong candidates for pharmacological therapy for endometriosis (Lindsay *et al.* 2015, Mori *et al.* 2018).

Azole derivatives are widely used in gynaecology to treat fungal infections, and CTZ is the most commonly used drug for this purpose (Zhou *et al.* 2016). The antifungal action of these drugs is exerted by blocking ergosterol synthesis through interference with the

fungal cytochrome P450 complex (Vanden Bossche *et al.* 1989). It has also been shown that different azole derivatives interfere with purified recombinant human CYP19A1 (Trösken *et al.* 2004), a component of the cytochrome P450 complex and the key enzyme in the biosynthetic pathway of estrogens (Mori *et al.* 2018). However, few studies have tested azole derivatives for endometriosis treatment (Lu *et al.* 2010, Jouhari *et al.* 2018, Machado *et al.* 2018), and our study is the only one that has evaluated orally administered CTZ (Machado *et al.* 2018). Oral administration is the safest and most convenient way to administer medications. In addition, the oral formulations currently available on the market are safe, easy to administer and reach desired therapeutic concentrations, making the oral route an ideal choice (Kuper 2008).

In the current work, we demonstrated that, compared to control, endometriosis model rats that received CTZ orally for 15 consecutive days showed regression of endometriotic lesions and that this effect was equivalent to that observed in rats that received intraperitoneal injection of the drug. These results confirm our previous statement that CTZ is effective in treating endometriosis (Machado *et al.* 2018) and that the oral administration of CTZ is as effective as intraperitoneally injected

Table 1 Endometriotic lesions macroscopic parameters.

Group	Weight (g)		Length (cm)		Width (cm)	
	D0	D15	P-value ^c	D0	D15	P-value ^c
Control						
Oral (n=15)	–	0.6 ± 0.35	–	0.75 ± 0.35	0.73 ± 0.32	0.871
IP (n=10)	–	0.59 ± 0.39	–	0.62 ± 0.36	0.71 ± 0.29	0.546
P-value ^a	–	0.947	–	0.378	0.875	0.818
CTZ						
Oral (n=15)	–	0.27 ± 0.18	–	0.73 ± 0.27	0.45 ± 0.26	0.007
IP (n=10)	–	0.28 ± 0.15	–	0.74 ± 0.23	0.47 ± 0.28	0.03
P-value ^b	–	0.886	–	0.924	0.857	0.823

^aStudent's *t*-test, compared control-oral to control-IP; ^bStudent's *t*-test, compared CTZ-oral to CTZ-IP; ^cStudent's *t*-test, compared D0 to D15 in the same treatment group.

CTZ. Our results also support the potential internal use of CTZ, which is widely used in gynaecology as a topic pharmaceutical, as an oral medication to treat endometriosis. Furthermore, we previously reported that there is no evidence that CTZ is toxic at the dose administered, as determined based on body weight and food consumption compared that those of control animals.

Mechanistically, we previously observed that CTZ attenuates the pro-inflammatory state in endometriotic lesions, which we believe suggests the effectiveness of the drug for treating endometriosis (Machado *et al.* 2018). In the current work, we found evidence demonstrating that CTZ downregulates CYP19A1 in endometriotic lesions. This result is in accordance with the findings that differentazole derivatives, including CTZ, inhibit CYP19A1 *in vitro* (Tröskén *et al.* 2004), and extends these findings to *in vivo* models. Targeting CYP19A1 is a desirable strategy for controlling and treating endometriosis since this enzyme is directly responsible for estrogen synthesis (Lindsay *et al.* 2015, Mori *et al.* 2018). Indeed, our findings reveal that serum estrogen levels decrease in animals upon CTZ treatment, which is a good indicator of its effectiveness against endometriosis (Zhu *et al.* 2018). Moreover, our results show that CTZ promoted a significant reduction in the expression of ER α , the primary estrogen receptor associated with the progression of endometriosis (Delvoux *et al.* 2009, Andersen *et al.* 2018). Indeed, estrogen signaling via ER α promotes the expression of this receptor in a positive feedback loop (Liu & Shi 2015, Uebi *et al.* 2015). Moreover, CYP19A1 expression is also positively modulated by estrogen via ER α (Kinoshita & Chen 2003). Although a large amount of estrogens can be supplied locally via CYP19A1, the combined action of steroid sulfatase (STS) and the enzyme estrogen sulfotransferase (SULT1E1) also represents an important pathway for estrogen production (Cornel *et al.* 2018). Clinically, patients with endometriosis present increased circulating estrogen levels, which has been associated with higher expression of ER α within endometriotic lesions, leading to disease progression (Delvoux *et al.* 2009, Huhtinen *et al.* 2012, Andersen *et al.* 2018). Therefore, we propose that the inhibition of CYP19A1 by CTZ is responsible for reducing the estrogen concentration, which, in turn, downregulates the expression of PGE₂ and ER α (Fig. 5).

Converting drug doses from animal models to humans is a challenge due to differences in pharmacokinetics, metabolism and surface intestinal area, and this is a limitation of our study. However, these dose conversions may be possible by correcting for body surface area using an exponent factor of 0.33 (Nair & Jacob 2016). In the present study, rats weighing approximately 200 g were used, which can be converted to an equivalent dose of 18 mg/kg CTZ for adult humans weighing 70 kg. This dose of CTZ (20 mg/kg) has already been administered orally

Figure 5 Proposed mechanism of action of CTZ in the estrogen pathway. In endometriosis, there is an increase in both circulating estrogen levels and estrogen levels in endometriotic lesions. This increase is due to increased expression of the aromatase enzyme, which may occur through an increase in the concentration of PGE₂. CTZ decreases the concentrations of PGE₂, which consequently decreases the expression of aromatase and estrogen production, resulting in disease regression.

in a clinical trial involving 73 patients with rheumatoid arthritis for 12 weeks and no significant undesirable effects were observed (Dennison *et al.* 1990). Thus, future investigations are necessary to better understand the adverse effects of CTZ in endometriosis treatment.

Conclusions

In conclusion, CTZ is effective in inducing the regression of endometriosis lesions. This effectiveness is due to reduced levels of local and systemic estrogen, a hormone responsible for the progression of the disease, caused specifically by the downregulation CYP19A1. We believe that, in the future, it will be possible to carry out clinical tests based on these results and thus confirm CTZ as a new strategy for the treatment of endometriosis.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

D E M, P Z and J A P performed conception and design of study. E M M, J A-P, M C C and D E M helped with all the experiments and designed the methodology. D E M, E M M, J A-P and J A P performed formal analysis and interpretation of data. D E M, E M M, J A-P and J A P wrote the original draft. L E N, M S P and P Z performed critical review and edited the manuscript for important intellectual content. D E M, P Z and J A P obtained funding and resources. All authors read and approved the final manuscript.

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