Clotrimazole is effective for the regression of endometriotic implants in a Wistar rat experimental model of endometriosis

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PII: S0303-7207(18)30124-2
DOI: 10.1016/j.mce.2018.04.005
Reference: MCE 10224

To appear in: Molecular and Cellular Endocrinology

Received Date: 5 January 2018
Revised Date: 9 April 2018
Accepted Date: 9 April 2018


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Figure 7
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The authors declare no conflict of interests

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Highlights:

- Clotrimazole promotes the regression of endometriotic lesions in a rat model
- Clotrimazole decreases inflammatory markers in endometriotic lesions
- The angiogenic markers VEGF and VEGFR-2 are decreased after clotrimazole treatment
- Regression of endometriotic lesions promoted by clotrimazole involves MAPK, Akt, AMPK and endoplasmic reticulum stress

Short title: Clotrimazole for endometriosis treatment.

Abbreviations:
ACC, acetylCoA carboxylase; Akt, protein kinase B; AMPK, AMP activated protein kinase; COX2, cyclooxygenase-2; CTZ, clotrimazole; ERK1/2, extracellular response kinase 1/2; IL-10, interleukin-10; MAPK, mitogen activated protein kinase; PERK, protein kinase R-like endoplasmic reticulum kinase; PGE2, prostaglandin E2; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α; UPR, unfolded protein response; VEGF, vascular endothelial growth factor; VEGFR-2, vascular endothelial growth factor receptor-2
ABSTRACT

The present work aimed to evaluate molecular, angiogenic and inflammatory changes induced by clotrimazole (CTZ) on endometriosis lesions. For this, thirty female Wistar rats with surgically implanted autologous endometrium were treated with CTZ or vehicle (200 mg/kg) via esophageal gavage for 15 consecutive days. CTZ treatment significantly decreased the growth and the size of the implants, and histological examination indicated regression and atrophy, with no toxicity to the animals. The levels of the angiogenic markers VEGF and VEGFR-2 were significantly decreased in CTZ group. The treatment also promotes a reduction on PGE$_2$ and TNF-$\alpha$ levels. All these effects involve the amelioration of ERK1/2, Akt, AMPK and PERK signaling upon CTZ treatment. In conclusion, CTZ promoted an overall amelioration of endometriosis in a rat model due to the anti-angiogenic properties of the drug. Therefore, our results support the proposal of a clinical trial using CTZ for the treatment of endometriosis.

Key words: clotrimazole; endometriosis treatment; angiogenesis; inflammatory.
1. Introduction

Endometriosis, an estrogen-dependent disorder, is characterized by the growth of endometrial tissue outside the uterine cavity, predominantly in the peritoneal pelvis and ovaries (Giudice and Kao, 2004). This condition is a common disorder among women of reproductive age worldwide, with a prevalence of approximately 10% within this group (Bulun, 2009). The prevalence increases up to 50% among infertile women and up to 60% among women and teenagers with pelvic pain (Giudice, 2010). Although considered a benign disease, endometriosis frequently presents characteristics of malignancy, such as cell proliferation and active angiogenesis (Kumar et al., 2011; Machado et al., 2014), and it has been reported as a risk factor for ovarian cancer (Brett M. et al., 2017; Brinton et al., 2005; Kumar et al., 2011; Viganò et al., 2007). Many of the symptoms of endometriosis including pelvic pain and infertility are strongly associated with local and systemic inflammation (Giudice, 2010). Indeed, women with diagnosed endometriosis display elevated numbers of immune cells and increased levels of cytokines in lesions and peritoneal fluid compared to healthy women (Beste et al., 2014; Hever et al., 2007; Jeung et al., 2016; Kwak et al., 2002; Schulke et al., 2009; Wu and Ho, 2003). This process leads to the increased production of reactive oxygen species (ROS), which are partially responsible for some symptoms and characteristics of endometriosis, such as cellular stress, aggravated inflammation and pain (Carvalho et al., 2012; Van Langendonckt et al., 2002).

Recently, inflammation-triggered oxidative stress has been related to increased angiogenesis in human pathologies, including heart and vascular diseases, psoriasis and cancer (Armstrong et al., 2011; Kim et al., 2013; Ushio-Fukai, 2006; Xia et al., 2007). Indeed, angiogenesis is crucial for the endometriosis development, since, in order to survive outside the uterus, endometriotic lesions have to create a novel vascular network (Laschke and Menger, 2007; Mari-Alexandre et al., 2015). In this context, not only the increased inflammation and ROS production but also the induction of vascular endothelial growth factor (VEGF) signaling via VEGFR2 plays the major transducing pathway in the endometriosis angiogenesis process (Cardoso et al., 2017; Machado et al., 2008). Therefore, studies suggest that the targeted inhibition of angiogenesis might offer an important target for the clinical treatment of endometriosis (Laschke and Menger, 2007; Mari-Alexandre et al., 2015).
Clotrimazole (CTZ) is one of the most used antimycotic drugs in gynecology (Zhou et al., 2016). It is a well-tolerated drug, presenting minor side-effects and a broad-spectrum of use (Zhou et al., 2016). Several studies have shown that CTZ also presents anticancer properties (Adinolfi et al., 2015; Furtado et al., 2015, 2012, Marcondes et al., 2015, 2010, Moreno-Sánchez et al., 2009, 2007). These properties involve different mechanisms interfering with cell proliferation, cell survival, cell metabolism, growth signals and presenting anti-inflammatory effects (Chung et al., 2015; Furtado et al., 2015, 2012, Marcondes et al., 2015, 2010). Therefore, we hypothesized that this drug might be a potential agent for treating endometriosis.

To test this hypothesis, we experimentally induced endometriosis in Wistar rats and treated them with vehicle or CTZ for 2 weeks. The treatment promoted a regression of endometriotic implants in an experimental model of endometriosis. To identify molecular changes in the endometriosis lesions promoted by the treatment with CTZ, we performed a series of Western blot analysis for molecular markers of cell biology, as well as immunohistochemistry, flow cytometry and ELISA immunoassays analyses to investigate whether CTZ modulated angiogenesis and the inflammatory process in the development of endometriosis.

2. Materials and methods

2.1. Endometriosis experimental model and CTZ treatment

Thirty female Wistar rats (200 g and 8 weeks of age) were used in the experimental induction of endometriosis, using the method described earlier (Vernon and Wilson, 1985). All experiments were conducted in accordance with the ethical guidelines from the Ethics Commission on Animal Use (CEUA), the NIH Guidelines for the Care and Use of Laboratory Animals (http://oacu.od.nih.gov/regs/index.htm. 8th Edition; 2011) and approved by the State University of West Zone (UEZO) CEUA (protocol code 002/2013). In brief, after the anesthesia with intramuscular injection of ketamine and xylazine, the animal's abdomen was opened and one uterine horn was removed, segmented and split longitudinally. One 5×5mm piece was sectioned and anchored with the endometrium side adjacent to the peritoneum of the ventral abdominal wall by nonadsorbable polypropylene sutures (6±0 Prolene, Ethicon, Piscataway, NJ). Lastly, the abdomen was closed and after fifteen days, ventral midline
A laparotomy was performed to determine the attachment, viability and the area of endometrial explants.

After one day, the animals were recovered and divided to two groups: CTZ group daily-treated with 200 mg/kg body weight CTZ (Clotrimazole, Sigma Chemicals Co., St. Louis, MO, USA) dissolved in sunflower oil; and Control group received sunflower oil only. Both treatments were administered daily by esophageal gavage for 15 consecutive days. Body weight was measured immediately before the first treatment (day zero, D0), on the seventh day of treatment (D7) and on the last day of treatment (D15), when the animals were euthanized by pentobarbital overdose. The peritoneal fluid was collected for flow cytometry and ELISA immunoassay analysis. Then, the abdomen was opened, and implantation sites were identified by the presence of a lesion or by suture alone. The surface area of each explant was measured (length × width) to the nearest 0.1 millimeter using calipers and after being excised were weighed and immediately divided for histological and Western blot analysis. In addition, the liver was weighed and blood samples were collected for biochemical and hematological analyses. To evaluate the insulin signaling in the tissues of the animals, one hour before euthanasia, eight random animals out of fifteen of each group were injected with 0.5 U/kg insulin (Humalin R, Eli Lilly, São Paulo, SP, Brazil) in the tail vein. All assessments were made without taking into account estrous stage.

2.2. Histology, immunohistochemistry and morphometric analysis

Formalin-fixed tissues were paraffin-embedded and cut into 4-micrometers-thick sections. Part of the sections were stained with Harris hematoxylin and eosin (HE) and examined microscopically at 200× magnification for the presence of histological hallmarks of endometriosis, such as endometrial glands and stroma. The other paraffin-embedded tissue sections were placed on silane-treated slides and incubated with the following antibodies: monoclonal antibody against VEGF, SC-57496 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:100 dilution and monoclonal antibody against VEGFR-2, SC-6251 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:100 dilution. Incubations were carried out overnight and then revealed using LSAB2 Kit HRP, rat (Dako-Cytonation, Carpinteria, CA, USA) with diaminobenzidine (3,3’-diaminobenzidine tablets; Sigma, St. Louis, MO, USA) as the chromogen and counterstained with hematoxylin. For each case, negative control slides consisted of
sections incubated with antibody vehicle or no immune rabbit or mouse serum. All tissues were examined by two blinded observers using a 400× magnification on light microscope (Nikon, Tokyo, Japan) connected to a digital camera (Coolpix 990; Nikon, Tokyo, Japan). Ten fields of an immunostained section (VEGF and VEGFR-2) were chosen at random and captured from each specimen. Quantification was assessed using captured high quality images (2048 × 1536 pixels buffer) using the Image Pro Plus 4.5.1 (Media Cybernetics, Silver Spring, MD, USA). Histologic scores (H) for VEGF and VEGFR-2 were calculated using the formula \( H = \sum P_i \), where \( I \) is the intensity ranging from 0 (negative cells) to 3 (deeply staining cells) and \( P \) is the percentage of staining cells for each given \( i \), with \( P \) values of 1, 2, 3, 4, and 5 indicating <15%, 15-50%, 50-85%, >85%, and 100% positive-staining cells, respectively, as previously described (Machado et al., 2016). The staining result was expressed as mean ± standard deviations.

2.3. Western blot analysis

Liquid nitrogen-frozen endometrial explants were grounded, dissolved in the appropriate buffer (Cardim Pires et al., 2017) and submitted to SDS–PAGE according to (Laemmli, 1970). The gels were transferred to polyvinylidene difluoride membrane (PVDF Imobilon-P, Millipore), and submitted to Western blot as previously described (Cardim Pires et al., 2017) (Cardim Pires, Albanese, Schwab et al., 2017). The antibodies used and their dilutions were as follows: anti-AMPKα (Cell Signaling Technology, Danvers, MA, USA, dilution 1:1000, Cell Signaling Technology Cat# 2532 RRID: AB_330331), anti-phospho-AMPKα (T172) (Cell Signaling Technology, Danvers, MA, USA, dilution 1:1000, Cell Signaling Technology Cat# 2535 RRID: AB_331250), anti-phospho-Acetyl-CoA Carboxylase (ACC) (S79) (Cell Signaling Technology, Danvers, MA, USA, dilution 1:1000, Cell Signaling Technology Cat# 3661 RRID: AB_330337), anti-Akt (Cell Signaling Technology, Danvers, MA, USA, dilution 1:1000, Cell Signaling Technology Cat# 9272 RRID: AB_328927), anti-phospho-Akt (S473) (Cell Signaling Technology, Danvers, MA, USA, dilution 1:1000, Cell Signaling Technology Cat# 9271 RRID: AB_329825), anti-ERK1/2 (Cell Signaling Technology, Danvers, MA, USA, dilution 1:1000, Cell Signaling Technology Cat# 4695 RRID:AB_390779), anti-phospho-ERK1/2 (S202/Y204) (Cell Signaling Technology, Danvers, MA, USA, dilution 1:1000, Cell Signaling Technology Cat#
9106 RRID:AB_331768), anti-ACLY (abcam, Cambridge, MA, USA, dilution 1:1000, abcam Cat# ab40793, RRID: AB_722533), anti-phospho-ACLY (S455) (abcam, Cambridge, MA, USA, dilution 1:1000, abcam Cat# ab46796, RRID: AB_867484), anti-eEF2 (Cell Signaling Technology, Danvers, MA, USA, dilution 1:1000, Cell Signaling Technology Cat# 2332 RRID:AB_10693546) and anti-actin (Cell Signaling Technology, Danvers, MA, USA, dilution 1:1000, Cell Signaling Technology Cat# 4967 RRID:AB_330288). Secondary antibodies peroxidase-affinipure goat anti-mouse IgG and peroxidase-affinipure goat anti-rabbit IgG were from Jackson Laboratories (Jackson ImmunoResearch Labs Cat# 115-035-146 RRID:AB_2307392 and Jackson ImmunoResearch Labs Cat# 111-035-144 RRID:AB_2307391), for anti-mouse and anti-rabbit, respectively and were used at the dilutions of 1:10000 and 1:20000, respectively. Immunoblotting was performed using PVDF membranes (Merck Millipore, Billerica, MA, USA, PR02531) and developed using a chemiluminescent peroxidase substrate (GE Healthcare Bio-Sciences, Pittsburg, PA, USA, RPN2124) followed by scanning using C-DiGit Blot scanner (LiCor, Lincoln, NE, USA).

2.4. ELISA Immunoassay

Peritoneal fluid was collected by rinsing the abdominal cavity with 10 mL of PBS and immediately centrifuged at 1500 rpm for 10 minutes. Supernatants were stored at -70°C until assayed for VEGF, PGE₂ and IL-10 by use of an enzyme immunoassay kit. The concentrations were calculated in triplicate from standard curves performed by an automatic plate reader (Spectra Max; Molecular Devices, Sunnyvale, Calif) controlled by SoftMax software (Molecular Devices).

2.5. Flow cytometry

Another washing of peritoneal fluid was obtained from the rat with 10 mL of PBS, pH 7.2. The cells were incubated with monoclonal antibodies PI anti-Mac-2 and FITC anti-F4/80 (Santa Cruz Biotechnology, Santa Cruz, CA). These cells were incubated with Fc blocking (clone 2.4G2) for 10min. After, the samples (10000 events per sample) were submitted to flow cytometer analysis (FACSCalibur, BD Biosciences, USA). Data analysis were performed in CellQuest (BD Biosciences, USA) and WinMDI 2.9 software packages.
2.6. Biochemical and hematological analysis

Glycemia and insulinemia were evaluated from the blood samples taken using a glucometer (Accu-chek Active Roche) and an ELISA kit for insulin (Mouse/Rat Insulin ELISA kit, Merck Millipore, MO, USA), respectively. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were evaluated using the respective kits (Doles, Goiania, GO, Brazil). The leukocyte count was performed using blood smears for differential counts of neutrophils, lymphocytes, monocytes, eosinophils and basophils. The slides were stained (Panotico Fast, Laborclin, Brazil) and viewed under a optic microscope (Nikon, Japan).

2.7. Statistical analysis

Data are expressed as mean ± standard deviations (SD) or mean ± standard error of the mean (S.E.M.), when appropriate and indicated in the legends. Statistical analyses were performed with Student’s t-test or two-way ANOVA followed by Dunnett’s post-test, when appropriate and indicated in the legends. For VEGF and VEGFR-2 morphometric analysis, statistical calculations were carried out with use of the StatXact-5 software program (CYTEL Software Corporation, Cambridge, MA). Differences were considered significant when the P values were <0.05.

3. Results

3.1. CTZ is effective in reducing endometriosis lesions

After 2 weeks of transplanting endometrial tissue, the explants formed viable cystic and well-vascularized lesions, resembling human peritoneal endometriosis, in all 30 animals. After 15 days of treatment, the growth, maintenance and implant size of the lesions were suppressed in the CTZ group (Figure 1B) as compared to the control (Figure 1A). The histopathological characterization revealed the presence of endometrial glands and stroma, which confirmed the viability of the lesions in the control group (Figure 1C), while in the CTZ group there was regression of the lesion areas and atrophy (Figure 1D). These results have been reinforced by the measurements of the weight and area of the lesions before and after treatment. Prior to treatment there were no differences between groups (weight: control: 0.59 ± 0.04 g vs CTZ: 0.61 ± 0.04 g; lesion area: control: 5.98 ± 0.07 mm² vs CTZ: 6.01 ± 0.05 mm²), but the CTZ group was significantly smaller after treatment (Figure 1E and 1F, P < 0.05, Student’s t-test).
3.2. CTZ treatment inhibits angiogenesis process

VEGF and VEGFR-2 immunoreactivity was detected in the endometriotic lesions, mainly in the stroma, in the cytoplasm of endothelial cells and around the glands (Figure 2A, 2B, 2C and 2D). The distribution of angiogenic markers (histomorphometry evaluations) significantly decreased in CTZ group compared to the control (Figure 2E). ELISA analysis revealed a decrease in VEGF concentration in the peritoneal washings (Figure 2F) and Western blot analysis (Figure 2G and 2H) demonstrated suppression of the expression of VEGF in the endometriotic lesions treated with CTZ as compared to the control group (P < 0.05, Student’s t-test). In addition, CTZ (Figure 2J) also decreased the number of macrophage positive cells (Mac-2+F4-80+) in about 50% compared to the control group in the peritoneal fluid (Figure 2I). Taken together, these results strongly suggest an important anti-angiogenic effect of CTZ in the endometriosis lesions.

3.3. CTZ interferes with growing signal

CTZ treatment significantly increased the expression of ERK1/2 (Figure 3A and 3B, P < 0.05, Student’s t-test). However, when ERK1/2 activation is evaluated, a different picture is observed. When comparing the non-stimulated phosphorylation of ERK1/2 on threonine 202 and tyrosine 204, it is clear that phosphorylation was already high in the control group, as compared to CTZ-treated group (Figure 3A and 3C, P < 0.05, two-way ANOVA followed by Dunnett’s post-test). Moreover, this phosphorylation observed in control was not augmented in animals that were injected with insulin 1 hour prior to euthanasia. On the other hand, in CTZ-treated animals, ERK1/2 phosphorylation responded to the insulin injection as expected for a healthy responsive tissue (Figure 3A and 3C, P < 0.05, two-way ANOVA followed by Dunnett’s post-test). These results are indicative that the endometriotic lesions presented a basal growth signal that was abolished by CTZ treatment.

3.4. CTZ affects cell survival and proliferation mediators

The treatment with CTZ decreased the expression of both AKT and ACLY as compared to control (Figure 4A and 4B, P < 0.05, two-way ANOVA followed by Dunnett’s post-test). The phosphorylation of AKT on serine 473, which is mediated by
mTORC2, was strongly diminished upon CTZ treatment (Figure 4B, P < 0.05, two-way ANOVA followed by Dunnett’s post-test). Consequently, phosphorylation of ACLY on serine 455, which is mediated by AKT, was also diminished by CTZ treatment (Figure 4B, P < 0.05, two-way ANOVA followed by Dunnett’s post-test).

3.5. CTZ down regulates stress markers and acts as an anti-inflammatory modulator

Treatment with CTZ decreased the expression of AMPK and its downstream mediator ACC (Figure 5A and 5B, P < 0.05, two-way ANOVA followed by Dunnett’s post-test). However, phosphorylation of these metabolic cell stress markers was improved by the treatment with CTZ (Figure 5C, P < 0.05, two-way ANOVA followed by Dunnett’s post-test). PERK, another marker of cell stress but from the unfolded protein response pathway, was also more activated (phosphorylated) upon CTZ treatment (Figure 5C, P < 0.05, two-way ANOVA followed by Dunnett’s post-test) but with no changes on its total expression (Figure 5B). The pro-inflammatory marker TNF-α was also down-regulated in the endometriotic lesions of CTZ-treated animals (Figure 5A and 5B). This is accompanied by a decrease in the concentration of PGE₂ (Figure 5D), a major signal for the development of endometriotic lesion. The levels of IL-10, an anti-inflammatory cytokine normally increased in endometriotic lesions, were also reduced in endometriotic lesions treated with CTZ as compared to the control group (Figure 5E).

3.6. No toxicity was observed in CTZ treated animals

No evidence of toxicity was noted for the CTZ dose administered based on body weight compared with controls (Figure 6A). There were no significant differences between the liver weights (Figure 6B), nor serum AST and ALT (Figure 6C), glycemia (Figure 6D) and insulinenia (Figure 6E) between the treated CTZ group and control. In addition, in the hematologic analysis with peripheral blood, we observed an accentuated lymphocytosis in control animals, while in the treated group there was a recovery in the leukocytes number with normal parameters (Figure 6F). So, the toxicity assessments used in this study did not reveal any toxic effects induced by CTZ.

4. Discussion
Endometriosis frequently produces serious effects on social and marital life, because it is often associated with infertility, and severe and incapacitating painful symptoms (Bulun, 2009; Fourquet et al., 2010). It is hoped that new approaches will be developed to improve endometriosis treatment. In the current work, we have provided evidence for the pharmacological use of CTZ for the treatment of endometriosis. The current pharmacological treatment approaches for endometriosis are largely focused on creating a hypoestrogenic or progestin dominated environment and relieve pelvic pain (Ruhland et al., 2011). However, a recent systematic review has reported that many patients gained only limited alleviation from pain symptoms (Becker et al., 2017). In addition, for all the patients, particularly those wishing to conceive, the side effects of medication treatments are unacceptable (Bedaiwy et al., 2016). Our results here indicate that CTZ treatment is able to reduce lesions size. Based on the morphological studies, we observed a reduction in the endometriotic lesion with regression and atrophy in the animals treated with CTZ and, importantly, without signs of drug toxicity. The dose of 200 mg/kg CTZ used in our study was equivalent to those reported in previous experimental models and it was without considerable adverse reaction or expressive variation in hepatic or blood parameters (De Franceschi et al., 1994; Khalid et al., 2005; Rufo et al., 1997; Takei et al., 2003; Wang et al., 2014). Moreover, we used a relatively short treatment, and it is possible that a longer treatment (plus one or two weeks) would lead to the complete reversion of the picture.

The contribution of new blood vessels is fundamental for the development and sustainability of the endometriotic lesion, drawing attention to the importance of angiogenesis that will provide a substrate for cell survival (Taylor et al., 2009). Many studies have reported the up-regulation of VEGF and VEGFR associated to endometriosis and their importance to the progression of the disease (Braza-Boïls et al., 2014; Marí-Alexandre et al., 2015; Ramm et al., 2011). Therefore, anti-angiogenic agents are discussed as possible candidates for new therapeutic approaches (Becker and D’Amato, 2007; Nap et al., 2004). In our study, VEGF and VEGFR-2 expression were downregulated in CTZ group as compared to control. This anti-angiogenic effect of CTZ had previously been described in different models of tumor growth (Belo et al., 2004; Takei et al., 2003). These observations are important because the VEGF/VEGFR-2 signal enhances endothelial cell migration and proliferation (Ferrara et al., 1992)
being essential conditions for the lesions maintenance and growth (Cardoso et al., 2017; Machado et al., 2008).

The mechanism by which endometriotic lesions up-regulate angiogenesis have been frequently associated to two different mechanisms: an increased level of pro-inflammatory cytokines that increase local inflammation and its consequent up-regulation of PGE$_2$, which directly promotes angiogenesis (Kim et al., 2013; Machado et al., 2010; Sacco et al., 2012; Szade et al., 2015). Our results here show that the treatment of the animals with CTZ reduced inflammation (evaluated by means of TNF-α levels) and PGE$_2$ levels. PGE$_2$ promotes the production of estrogen by endometriotic cells and its elevated levels are directly associated with the progression of the disease (Sacco et al., 2012). Moreover, TNF-α is normally elevated in endometriotic lesions due to its secretion by the increased infiltrated macrophages (Kurt et al., 2015). We also observed a decrease in macrophage infiltration upon the treatment with CTZ, which is consistent with the TNF-α results. Moreover, TNF-α is described to promote the expression of cyclooxygenase-2 (COX-2) in macrophages (Sacco et al., 2012). COX-2 is an enzyme responsible for the synthesis of PGE$_2$ (Sacco et al., 2012) and, thus, the decrease in macrophage infiltration promoted by CTZ might be responsible for the lower levels of TNF-α and PGE$_2$ observed here. The altered function of the local immune system cells and cytokines profile is characteristic of endometriosis (Ahn et al., 2015). Notably, macrophages are important immune cells contributing to this dysregulation because they can produce both pro-inflammatory and pro-angiogenic cytokines (Burney and Giudice, 2012; Capobianco and Rovere-Querini, 2013; Machado et al., 2016; Scheerer et al., 2016; Takebayashi et al., 2015). Therefore, the fact that the treatment of the animals with CTZ reduced in macrophage infiltration accompanied by the decrease in TNF-α and PGE$_2$ support the efficacy of the drug to treat endometriosis.

In spite of the fact that endometriosis promotes local and systemic inflammation, it has been reported the occurrence of elevated serum levels of IL-10, a markedly anti-inflammatory cytokine, in patients with endometriosis (Suen et al., 2014). The importance of this cytokine to the progress of the disease is evident since, in a rat model for endometriosis, depletion of IL-10 considerably decreased the size of the endometriotic lesions and, conversely, administration of IL-10 promoted the growth of the lesions (Suen et al., 2014). This effect might be due to the putative effect of IL-10 on the immunity of the patients preventing the immune system to control the
progression of the endometriotic lesions. Nevertheless, the fact that the treatment with CTZ reduced the levels of IL-10 substantiates the effects of the drug against endometriosis and corroborates its clinical use for the control of the disease.

Although the etiology and pathogenesis of endometriosis remain uncertain, a recent study highlighted the ERK1/2 are significant effectors on the development of the disease (Uimari et al., 2017). The current work shows that ERK1/2 expression is down-regulated in CTZ-treated rats, as compared to controls. Isolated, this result is encouraging per se. Nonetheless, we also observed that, in control rats, ERK-1/2 is over phosphorylated even in a non-stimulated condition (no insulin injected previous to the euthanasia) and that insulin does not augment this phosphorylation. This is a strong indicative that ERK1/2 is constitutively activated in endometriotic lesions and corroborate the major role of ERK1/2 on the progression of the disease. Intriguingly, the treatment of the animals with CTZ not only reduced the expression of ERK1/2 but also reduced to very low levels the unstimulated phosphorylation of the enzyme. Moreover, CTZ-treated mice recovered the responsiveness to insulin on regard of ERK1/2 phosphorylation that was not observed on control rats. This result is a strong indicator that the treatment reversed the previously reported dysregulation of the expression of ERK1/2 (Afshar et al., 2013).

Other signaling pathways were also affected by CTZ treatment, such as mTORC2/AKT signaling. Although we have not evaluated mTOR, phosphorylation of AKT on serine 473 is mediated by the mTORC2. Our results reveal that this phosphorylation of AKT is strongly attenuated upon CTZ treatment. We have previously shown that CTZ is a direct inhibitor of PI3K (Furtado et al., 2015), another upstream activator of AKT. This effect is corroborated by the phosphorylation of ACLY, which is a substrate for AKT and is involved in cell proliferation, and followed a similar pattern observed for AKT phosphorylation upon CTZ treatment. These effects are observed for non-stimulated and insulin-stimulated rats, suggesting that the whole signalization is affected by the treatment. In endometriotic lesions, mTOR is activated suppressing autophagy and decreasing endometriotic cells apoptosis (Choi et al., 2015).

Indeed, CTZ promotes cellular stress, such as revealed by the activation of the nutrient sensor AMPK and its downstream effector ACC. Moreover, the increased phosphorylation of PERK, an ultimate UPR effector, suggests that the endoplasmic reticulum stress is also triggered upon the treatment. Thus, by interfering with these
pathways, CTZ might also contribute to the induction of apoptosis of the endometriotic cells, as well as to the reduction on these cells proliferation, resulting in the reversion of the progress of the disease.

CTZ is a well-tolerated drug, majorly used to treat oral and vaginal candidiasis (Crowley and Gallagher, 2014). Presented as different formulations and brands, CTZ is one of the top pharmaceuticals of gynecological use worldwide (Crowley and Gallagher, 2014). When orally administrated, cases of elevated hepatic enzymes and irritation of the gastrointestinal tract have been reported (Ellepola and Samaranayake, 2000). Recently, we have developed a nanoformulation of CTZ aimed to circumvent these possible side effects (Marcondes et al., 2015). However, in the current study, we administered the drug orally to the rats without alterations of hepatic enzymes in the serum. This is a strong indication that CTZ is not promoting hepatic damage to the animals and support its utilization to control endometriosis.

Finally, based on the results of this and previous studies, we demonstrated that the angiogenic factor VEGF and that the AMPK, MAPK and Akt pathways are involved in the pathogenesis of endometriosis. We also propose a CTZ molecular mechanism on the reduction of the lesions in experimental endometriosis (Figure 7). We know that the molecular mechanisms are complex, but in our opinion, the macrophage plasticity and their ability to modulate essential survival and invasion pathways is the key to a better understanding of the malignant behavior of endometriosis. In the endometriotic microenvironment, the macrophage polarization signals are essential to promote the angiogenesis process, inflammation and the growth because it leads to upregulation of VEGF expression and the AMPK and MAPK pathways. In addition, the Akt pathway is also activated and promotes metabolism changes. On the other hand, CTZ decreases the number of activated macrophages on the lesions resulting in the suppression of these target signals of growth, metabolism, inflammation and angiogenesis. These actions interfere in the survival and invasion of endometriotic lesions.

In conclusion, we demonstrated that CTZ has antiangiogenic and anti-inflammatory activities, which produced the regression of endometriotic lesions. The main CTZ mechanism of action was to decrease the presence of the activated macrophages in the lesions leading to the reduction of VEGF expression, as well as the downregulation of proliferative and survival signaling pathways. In addition, no toxicity
was observed in the animals treated with CTZ, a relevant fact for a possible clinical treatment for endometriosis patients. The results of this study suggest the use of CTZ as an effective pharmacological treatment for endometriosis, and we are optimistic that these effects will be reproducible in clinical tests.

Acknowledgements

Authors wish to express their gratitude to Dr. Patricia L. Mitchell for English editing of the manuscript. The current work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

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Legends to the Figures

Figure 1: CTZ suppresses endometriosis growth in vivo. In the end of the treatment,
endometriotic lesions were evaluated in all the animals by means of direct visualization
(panels A and B, as representative images of control and CTZ groups, respectively) and
by HE staining and histological analysis (panels C and D, as representative images of
control and CTZ groups, respectively). In the control group (A), the observed lesions
were cystic and well-vascularized (circle). In the CTZ group (B), was observed a drastic
reduction on the implant size and growth of the lesions. The histological analysis
showed the presence of the endometrial glands (arrow) and stromal cells (asterisks) in
the control group, characterizing the ectopic endometrial tissue. In the treated group
(D), an atrophy and regression of the lesions were visualized (arrowheads).
Measurements of the lesion weight (E) and area (F) are expressed as mean ± standard
deviation (n=15). * indicates P<0.05 as compared to control (Student’s t-test). The
individual values that generated panels E and F are presented in supplementary material
(Table S1).

Figure 2: Anti-angiogenic effect of CTZ on endometriotic lesions. Ten samples of
each group, control and CTZ, were randomly chosen and immunostained for VEGF
(panels A and C, as representative images of control and CTZ groups, respectively) and
VEGFR-2 (panels B and D, as representative images of control and CTZ groups,
respectively). The immunodistribution of angiogenesis markers VEGF (A and C) and
VEGFR-2 (B and D) was more detected in the control group (arrow) than in CTZ group
(arrowheads), being confirmed by the morphometric analysis (panel E; n=10, * indicate
P<0.05 as compared to control, two-way ANOVA followed by Dunnett’s t-test). VEGF
concentration was evaluated by ELISA immunoassay in the peritoneal washing of eight
randomly selected animals from each group (panel F; n=8, * indicate P<0.05 as
compared to control, Student’s t-test). For Western blots, five randomly selected
samples from each group were used. Panel G: representative Western blot analysis of
the effects of CTZ treatment on the expression of VEGF protein. Panel H: quantification of the Western blots represented on panel G (n=5, * indicate P<0.05 as
compared to control, Student’s t-test). FACS analysis showed a reduction of the
macrophage phenotype (Mac-2+F4-80+) in the treated group (J) than the control group
population in the peritoneal fluid (I). The individual values that generated panels E, F
and H are presented in supplementary material (Table S1).

Figure 3: Effects of CTZ treatment on ERK1/2 expression and phosphorylation.
Protein levels and phosphorylation of ERK1/2 were evaluated by Western blot analysis
of 5 randomly selected samples from each group. Panel A shows a representative
Western blot of total and phosphorylated ERK1/2. Panel B: quantification of total
ERK1/2 expression relative to ß-actin used as load control (n=5, * indicate P<0.05 as
compared to control, Student’s t-test). Panel C: quantification of the levels of
phosphorylated ERK1/2 relative to total ERK1/2 staining (n=5, * indicate P<0.05 as
compared to control, two-way ANOVA followed by Dunnett’s t-test). The individual
values that generated panels B and C are presented in supplementary material (Table
S1).
Figure 4: Effects of CTZ treatment on AKT and ACLY expression and phosphorylation. Protein levels and phosphorylation of AKT and ACLY were evaluated by Western blot analysis of 5 randomly selected samples from each group. In panel A is shown a representative Western blot of total and phosphorylated ERK1/2. Panel B: quantification of total expression relative to β-actin used as load control and phosphorylation relative to total protein of AKT and ACLY (n=5, * indicate P<0.05 between the bars indicated by the brackets, two-way ANOVA followed by Dunnett’s t-test). The individual values that generated panel B are presented in supplementary material (Table S1).

Figure 5: CTZ induces intracellular stress and acts as an anti-inflammatory modulator. Protein expression of AMPK, ACC, PERK and TNF-α, and phosphorylation of AMPK, ACC and PERK were evaluated by Western blot analysis of 5 randomly selected samples from each group. A representative result is shown in panel A. Panel B: quantification of total expression of the proteins relative to β-actin used as load control (n=5, * indicate P<0.05 as compared to controls, two-way ANOVA followed by Dunnett’s t-test). Panel C: quantification of the levels of phosphorylated proteins relative to total proteins staining (n=5, * indicate P<0.05 as compared to control, two-way ANOVA followed by Dunnett’s t-test). Panels D and E: quantification of PGE$_2$ and IL-10, respectively in the serum (n=7, * indicate P<0.05 as compared to control, Student’s t-test). The individual values that generated panels B, C, D and E are presented in supplementary material (Table S1).

Figure 6: No toxicity was observed in CTZ treated animals. No evidence of toxicity was noted between the treated CTZ group and the control based on body weight (A), liver weight (B), serum AST and ALT (C), glycemia (D) and insulina (E). In the hematologic analysis using peripheral blood, an accentuated lymphocytosis on the control animals was noted, while in the treated group there was a recovery in the leukocytes number with normal parameters (F). All the measurements were performed in all the animals (n=15).
Figure 7: CTZ signaling pathways in endometriotic microenvironment. In the endometriotic microenvironment, the macrophages polarization signals are essential to promote the angiogenesis process, inflammation and the growth because it leads to upregulation the VEGF, AMPK and MAPK pathways. Besides that, the AKT pathways are also activate and promotes metabolisms changes. CTZ downregulating these pathways and decrease the number of activated macrophages resulting in the suppresses of the target signals and interfering in the survival and growth of endometriotic lesion.
**Control**

**CTZ**

**Lesion weight**

**Lesion area**
A  

- insulin injection  
  - CTZ  
  - P-ERK1/2 (T202/Y204)  
  - ERK1/2  
  - β-actin

B  

- ERK1/2  
  - relative expression (ERK1/2/β-actin)  
  - Control  
  - CTZ

C  

- ERK1/2 phosphorylation  
  - relative phosphorylation (phosphorylated/total)  
  - saline  
  - insulin
A

CTZ - +
P-AMPKα (T172)  
AMPKα  
P-ACC (S79)  
ACC  
P-PERK (T980)  
PERK  
TNF-α  
β-actin

B

![Bar graph showing relative expression (target/β-actin) for different conditions]

C

![Bar graph showing relative phosphorylation (phosphorylated/total) for different conditions]

D

![Bar graph showing PCE concentration (pg/mL) for different conditions]

E

![Bar graph showing L-10 concentration (pg/mL) for different conditions]
Dr Somasundaram,

attached, you will find the Figure 7.

Please let me know if you need any other thing.

Best regards,

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Highlights:

- Clotrimazole promotes the regression of endometriotic lesions in a rat model
- Clotrimazole decreases inflammatory markers in endometriotic lesions
- The angiogenic markers VEGF and VEGFR-2 are decreased after clotrimazole treatment
- Regression of endometriotic lesions promoted by clotrimazole involves MAPK, Akt, AMPK and endoplasmic reticulum stress