Anastrozole and celecoxib for endometriosis treatment, good to keep them apart?

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Short title: **Aromatase and COX-2 inhibitors in endometriosis**

Abstract

Endometriosis is a benign gynecological disease. Cyclooxygenase (COX)-2 and aromatase proteins have been shown to be overexpressed in eutopic endometrium from women suffering from this disease compared to disease-free women. Furthermore, inhibiting these molecules individually was demonstrated to have antiproliferative and proapoptotic effects both *in vitro* and *in vivo* in several models. In this study, the effect of combining celecoxib, a selective COX-2 inhibitor, and anastrozole, an aromatase inhibitor, on the implantation and growth of endometriotic like lesions in a murine model of endometriosis was evaluated. Endometriosis was surgically induced in female BALB/c mice. After 28 days of treatment with celecoxib, anastrozole or their combination, animals were sacrificed and lesions were counted, measured, excised and fixed. Immunohistochemistry for proliferating cell nuclear antigen and CD34 were performed for assessment of cell proliferation and vascularization. TdT-mediated dUTP Nick-End Labelling technique was performed for apoptosis evaluation. Celecoxib was the only treatment to significantly reduce the number of lesions established per mouse, their size and vascularized area. In addition, cell proliferation was significantly diminished and apoptosis was significantly enhanced by both individual treatments. When the therapies were combined they reversed their effects. These results confirm that celecoxib and anastrozole separately decrease endometriotic growth, but when combined they might have antagonizing effects.

Introduction

Endometriosis is a benign disease that is characterized by the presence of endometrial tissue outside the uterine cavity where it proliferates and forms new blood vessels essential for its further development (Lousse et al. 2012). As an estrogen dependent disease, treatments available up to date, aim mainly at reducing estrogen levels (Ozkan et al. 2008). Since endometriosis affects women in reproductive age and given that current available treatments mostly impede ovulation (Rocha et al. 2012), it is the goal of recent research to find alternatives to the existing treatments reducing the side effects of those now accessible.

We and others have studied the involvement of cyclooxygenase (COX)-2 in this pathology (Matsuzaki et al. 2004, Banu et al. 2008, Olivares et al. 2008, Olivares et al. 2011), which has been described to be overexpressed not only in eutopic and ectopic endometrium from endometriosis patients (Ota et al. 2001, Fagotti et al. 2004), but also in a wide variety of cancers (Mendes et al. 2009, Ghosh et al. 2010, Wang et al. 2010). Selective COX-2 inhibitors are a special class of nonsteroidal antiinflammatory drugs (NSAIDs) that were developed to treat pain and inflammation without inhibiting COX-1, thus sparing the gastrointestinal system (Wadman 2007). The COXs are the enzymes responsible for the synthesis of pain mediators including prostaglandins (PGs). Particularly, high concentration of PGE_2 has been found in the peritoneal fluid of patients with endometriosis, primarily provided by activated macrophages and the endometriotic lesions (Wu et al. 2010). Our group has demonstrated that selective inhibition of COX-2 activity with celecoxib reduces the proliferation rate of endometrial epithelial cells as well as it augments their apoptosis levels both *in vitro* and *in vivo* (Olivares et al. 2008, Olivares et al. 2011). Similar results were obtained by other investigators demonstrating that the inhibition of COX-2 activity has antiproliferative, proapoptotic and antiangiogenic effects in several *in vivo* and *in vitro* cancer models (Gupta et al. 2004, Basu et al. 2005, Dandekar et al. 2005, Barnes et al. 2007) and in other models of endometriosis (Dogan et al. 2004, Laschke et al. 2007, Machado et al. 2010). Moreover, a COX-2 inhibitor has been used in a pilot study evaluating pain response in endometriosis patients with good results (Cobellis et al. 2004).

Similarly, aromatase has also been found to be overexpressed in endometriosis with higher protein and mRNA levels in eutopic endometrium from patients compared to control women as well as in ectopic endometrium (Bulun et al. 1997, Bulun et al. 2002, Kyama et al. 2008). In this sense, two aromatase inhibitors, letrozole and anastrozole, have also been tested *in vitro* and *in vivo* in our laboratory and demonstrated to have antiproliferative and proapoptotic effects (Meresman et al. 2005, Bilotas et al. 2010).

COX-2 is overexpressed in some malignancies including carcinoma of the breast. COX-2 mRNA and protein levels have been found to correlate with aromatase levels within human breast cancer tissue. The current understanding of the role of COX-2 in breast cancer suggests that COX-2 inhibitors may have a role in chemoprevention which is based in part on the generic issues of antiangiogenesis and proapoptotic processes, and in part on a tissue-specific inhibition of estrogen synthesis (Davies et al. 2002). It has been reported that celecoxib induces a marked inhibition of aromatase protein expression, assessed by western blot, in human breast cancer cell lines (Bocca et al. 2011). In addition, treatment with COX-2 siRNAs resulted in suppression of the aromatase gene CYP19 in breast cancer cells (Brueggemeier et al. 2007). In another experimental model, Sirianni *et al.* reported that inhibition of COX-2 downregulates aromatase activity and decreases proliferation of Leydig tumor cells (Sirianni et al. 2009). In addition, previous studies had shown that aromatase and COX-2 simultaneous inhibition might have additive or even synergic effects (Bundred & Barnes 2005, Ebert et al. 2005, Chow et al. 2008, Dirix et al. 2008, Falandry et al. 2009).

Furthermore, a feed forward loop has been described between aromatase and PGE_2 in endometriosis. Aromatase synthesizes estrogens, which in turn stimulate the production of PGE_2 via COX-2, and PGE_2 stimulates aromatase activity (Bulun et al. 2002). This describes a perfect circle in this system in which high expression of aromatase will produce high concentrations of estrogens, which will stimulate COX-2 activity producing high concentrations of PGE_2, stimulating again aromatase.

Considering all these data and in sight of the feed forward loop established between these molecules in endometriosis, we decided to investigate the effect of combining a COX-2 inhibitor with an aromatase inhibitor, celecoxib and anastrozole respectively, in a murine model of endometriosis.

For this purpose we treated mice with induced endometriotic like lesions with anastrozole, celecoxib or both drugs combined, during a four week period and studied their effects on the development of the disease and on cell proliferation, apoptosis and vascularization within the lesions.

Results

Celecoxib inhibits the establishment and growth of endometriotic like lesions

After four weeks of treatment with anastrozole, celecoxib or their combination, animals were sacrificed and the abdominal cavity was explored to localize and measure the lesions developed. Figure 1 shows the results obtained for the number of lesions established, according to the treatment the animals received, as well as the volume of those developed.

When mice received celecoxib as the only treatment, not only the lesions established diminished compared to the control group ($p<0.05$; Figure 1A), but also the size of those established and developed was significantly smaller ($p<0.01$ vs. Control; Figure 1B). However, when animals received either anastrozole alone or combined with celecoxib, the number of lesions established as well as their size did not differ from those of the control group.

The COX-2 and aromatase inhibitors affect endometriotic like lesion development

Developed endometriotic like lesions, were excised and fixed for cell proliferation, apoptosis and vascularization evaluation. Cell proliferation in the epithelial fraction of the lesions was significantly diminished compared to the control group when animals were treated with celecoxib or anastrozole separately ($p<0.05$ for both groups vs. Control); when the treatments were combined, cell proliferation within the lesions as assessed by PCNA immunohistochemistry, was similar to the control group ($p>0.05$). The results are displayed in Figure 2A.

Accordingly, the number of apoptotic epithelial cells was quantified by the TUNEL technique and the results showed that the combination of the compounds had no effect on cell death where as the administration of either of them alone significantly enhanced TUNEL positive cells ($p<0.05$ for

Anastrozole or Celecoxib vs. Control; $p>0.05$ for Anastrozole+Celecoxib vs. Control). Results are displayed in Figure 2B.

When vascular density was assessed within the endometriotic like lesions by immunohistochemistry of CD34, only the treatment of celecoxib administered on its own showed an inhibitory effect ($p<0.05$ vs. Control). The aromatase inhibitor alone or in combination with the COX-2 inhibitor, had no effect on vascular density compared to the control group ($p>0.05$) (Figure 3).

COX-2 immunostaining would be enhanced by celecoxib and inhibited by anastrozole

COX-2 protein expression was evaluated in a semiquantitave fashion on developed endometriotic like lesions after treatment with celecoxib, anastrozole of their combination. When animals received celecoxib alone, COX-2 immunoreactivity was apparently stimulated while anastrozole seemed to inhibit it, in both cases, compared to the control group. When the compounds were administered in combination, the immunostaining of COX-2 was comparable to that in the lesions from control mice. However, there was no statistical significance in the changes observed. In all cases, and as reported earlier (Hayes & Rock 2002), immunoreactivity of COX-2 was evident both in the epithelial and stromal fraction, but higher in the epithelial one. Results are shown in Figure 4.

Discussion

Endometriosis affects a large number of women all over the world and great efforts are being done by researchers to give better and longer lasting answers to patients. Treatment for endometriosis is usually performed with surgery and/or medications. Up to date treatment options are poor and do not really cure this disease; they aim mainly at reducing pain and endometriotic growth. Nevertheless, the high recurrence rate of this illness is one of the most challenging problems we face nowadays. In this sense, investigations are focusing on finding new and more effective alternatives for patients.

In the present study, and taking into account previous results obtained in our laboratory (Bilotas et al. 2010, Olivares et al. 2011) and earlier promising results obtained in cancer (Chow et al. 2008,

Falandry et al. 2009), we decided to combine two inhibitors: of COX-2 and aromatase. Already some years ago, Ebert and coworkers reviewed the importance of aiming at these molecules given the abnormalities present within the eutopic and ectopic endometrium of endometriosis patients (Ebert et al. 2005) and acknowledging the positive feedback loop present between these molecules (Bulun et al. 2002).

Indeed, few studies had been conducted in breast cancer patients combining exemestane, an aromatase inhibitor, with celecoxib with somewhat inconclusive results. While some authors suggested that the addition of celecoxib to exemestane treatment might have promising benefits (Chow et al. 2008, Falandry et al. 2009), others did not find a beneficial effect from this combination (Dirix et al. 2008).

Our first results showed that celecoxib was the only treatment capable of reducing not only the size of established lesions, but also the number of lesions established. We had already seen this strong inhibitory effect of celecoxib in a previous work; at that time we had combined the treatment of celecoxib with a PPARγ agonist, rosiglitazone, obtaining no additional benefit with this combination (Olivares et al. 2011). In the present study, the treatment with the aromatase inhibitor and the combination of both inhibitors did not reduce the number of lesions established nor their size compared to the control animals.

Even though aromatase and COX-2 inhibition had been postulated to have additive or even synergic effects in breast cancer (Goss & Strasser-Weippl 2004, Chow et al. 2008) some authors have not found a clear beneficial effect from this combination (Dirix et al. 2008, Falandry et al. 2009). The results we are presenting are more in agreement with the latter.

We then investigated the rate of cell proliferation and apoptosis in the lesions developed in all groups. We observed that cell proliferation in the epithelial fraction of the lesions was significantly diminished and apoptotic levels were significantly augmented, when animals were treated with celecoxib or anastrozole separately compared to the control group. It is important to note that even though the rate of cell proliferation was seen significantly reduced in both of these groups, the incidence of this decrease was only evidenced macroscopically, in the celecoxib treated animals at the doses and period of time tested. It was also observed that the administration of anastrozole and

167 celecoxib together reversed the effects of either of them alone. Based on these data, we could
168 speculate that these two compounds, against all odds, were having an antagonistic effect, but further
169 studies are needed to test this hypothesis.

170 On the other hand, we only obtained a significant reduction in vascularization with the treatment with
171 celecoxib alone. In accordance with our results, treatment with another selective COX-2 inhibitor had
172 been reported to reduce microvessel density in a SCID mouse model of endometriosis (Ozawa et al.
173 2006). Furthermore another study in a xenograft model of breast cancer showed that the use of
174 letrozole, another aromatase inhibitor did not reduce the number of CD31 stained vessels, another
175 well established vascularization marker (Banerjee et al. 2010).

176 In contrast, Hull *et al.* found that nimesulide does not reduce lesion size nor blood vessel development
177 in an estrogen-supplemented nude mouse model of endometriosis. The authors suggest that the effect
178 of COX-2 inhibition may be obscured by iatrogenically administered estrogen (Hull et al. 2005).
179 Nevertheless, other authors have demonstrated COX-2 inhibitors to be effective in ovariectomized
180 endometriosis mouse models (Efstathiou et al. 2005, Ozawa et al. 2006).

181 In this work, we have also examined COX-2 immunoreactivity in the developed endometriotic
182 lesions. Although we did not obtain a statistical significant difference in the changes of COX-2
183 immunostaining, we observed a tendency to be enhanced when the mice received celecoxib while the
184 aromatase inhibitor reduced it, both compared to the control group. These results are in agreement
185 with the reports that have established the existence of a negative feedback loop between the product
186 from the activity of COX-2 and its protein levels. When the activity of the enzyme is inhibited, this
187 loop disappears and its protein levels augment (Basu et al. 2005, Ohneseit et al. 2007). On the other
188 hand, it is known that the product from aromatase activity stimulates mRNA and protein levels, as
189 well as the activity, of COX-2 (Bulun et al. 2002). With the support from previous reports, the
190 observed reduction in COX-2 immunostaining after anastrozole treatment is consistent with the
191 interaction between these two enzymes and their products. Furthermore, even though it has been
192 reported that the inhibition of COX-2 activity also inhibits the protein expression of aromatase in

193 breast cancer cell lines *in vitro* (Bocca et al. 2011), when exemestane was combined with celecoxib
194 the protein levels of aromatase were seen unaltered in breast cancer patients (Lustberg et al. 2011).

195 There are no studies published in the field of endometriosis where the combination of anastrozole
196 with celecoxib, or any other aromatase or COX-2 inhibitors, has been tested. In breast cancer
197 research, the combination of celecoxib has been evaluated with exemestane. Exemestane and
198 anastrozole, both well known and thoroughly studied third generation aromatase inhibitors, do have
199 distinct ways of action. Anastrozole, as well as letrozole, is a reversible nonsteroidal inhibitor;
200 whereas exemestane is an irreversible steroidal inhibitor (Geisler 2011). Maybe it is on this difference
201 where it resides the contrasting results we have obtained.

202 Furthermore, we have performed this study using the mentioned mouse model of endometriosis and it
203 has to be considered the possibility that women's endometrium might not have the same response to
204 the treatment with these compounds. The tissue implanted in the mouse peritoneum in this particular
205 model consists not only of endometrial cells but it also contains myometrial tissue, this is not the case
206 in the endometriotic lesions developed in women. Moreover, women's endometriotic lesions are
207 exposed to the natural hormonal fluctuations due to the menstrual cycle rather than the much shorter
208 and more rapidly changing estrous cycle of the mouse, which may further affect the tissue developing
209 in the peritoneal cavity. Further studies should be held in humans as to being able to state the certainty
210 of the results here presented. This is an approach and a modelization of a very complicated human
211 disease and the results should be interpreted in this sense. Nevertheless, this is a well established and
212 accepted model of endometriosis which has been thoroughly used not only in our laboratory (Bilotas
213 et al. 2010, Olivares et al. 2011, Ricci et al. 2011) but by other investigators too (Fang et al. 2002,
214 Becker et al. 2006, Grummer 2006, Pelch et al. 2010).

215 To the best of our knowledge this is the first study to investigate the combination of these two
216 compounds in endometriosis research. More studies should be addressed to study the pharmacology
217 of these compounds as to evaluate if they might be having antagonizing effects. In the light of the
218 results presented, it may be a possibility. Our previous and present works have undoubtedly

demonstrated the efficacy of celecoxib and anastrozole as monotherapies; and although theoretically their combination should benefit the patient, we cannot state it so far.

Materials and Methods

Animals

In this study, 40 two months old female BALB/c mice were used. All procedures were performed according to NIH Guidelines for the Care and Use of Laboratory Animals and approved by the Ethics and Research Committee from the Instituto de Biología y Medicina Experimental (IBYME, Buenos Aires, Argentina). A total of six animals died or were sacrificed between 2-3 after surgery because they did not fully recover from the procedure.

Surgical induction of endometriosis and treatment

Endometriosis-like lesions were induced through transplantation of one of the uterine horns to the bowel mesentery as previously described (Bilotas et al. 2010, Olivares et al. 2011, Ricci et al. 2011). Briefly, animals were deeply anaesthetized with an intraperitoneal injection of ketamine (100mg/kg) (Holliday Scott, Buenos Aires, Argentina) and xylazine (10mg/kg) (Richmond, Buenos Aires, Argentina). Mice underwent laparotomy by midventral incision to expose the uterus and intestine. The right uterine horn was removed, opened longitudinally and cut into square pieces measuring approximately $4mm^2$. Three equal pieces of tissue were then sutured onto serosal layer with a single 6-0 nylon suture (Supralon, Ethicon, NJ, USA) with endometrial tissue facing the serosa. The abdomen was then closed with a 5-0 nylon suture.

Animals were assigned into four different treatment groups: Control: 150µl vehicle; Celecoxib: 1500ppm of celecoxib (Pfizer, USA) in chow (+ 150µl vehicle); Anastrozole (0.5mg/kg subcutaneous injection, anastrozole was reconstituted in physiological solution) (AztraZeneca, London, UK) and Celecoxib + Anastrozole, received the treatments combined. All treatments were administered daily, started in post-operative day 1 and continued during 28 days. The amount of celecoxib consumed by each animal was estimated weighing the chow one day and the next, this difference was divided by

the number of animals per cage; the chow was replaced and weighed every day. Each animal consumed 4.65 ± 0.17 mg of celecoxib per day. No evidence of toxicity was noted at the doses administered based on body weight, food consumption, grooming behavior or activity levels compared with controls.

Endometriotic like lesions evaluation

After 4 weeks of treatment, animals were sacrificed by cervical dislocation. The abdomen was opened by ventral midline incision. Implantation sites were localized by the presence of a lesion or by suture alone. Lesions were counted and measured for volume determination using the formula: $V = (4/3)\pi r^2 R$ (where r and R are the radiuses, $r < R$) (Brodie et al. 2003). Then lesions were excised, fixed and paraffin-embedded. Specimens were cut into 5μm serial sections. Four to five non-contiguous sections from each specimen were stained with haematoxylin-eosin and examined microscopically for the presence of histological hallmarks (glands and stroma) of endometriosis.

Immunohistochemistry for PCNA, CD34 and COX-2

Serial sections of endometriotic lesions were subjected to standard immunohistochemistry. Tissue sections were incubated overnight with the primary antibody (rabbit anti-mouse PCNA polyclonal, 1:300, FL-261, Santa Cruz Biotechnology, CA, USA; rat anti-mouse CD34 monoclonal, 1:50, ab8158 Abcam, MA, USA; or rabbit anti-COX-2 polyclonal, 1:200, sc-1747, Santa Cruz Biotechnology) at 4ºC. After that, sections were treated for 60 min with the corresponding secondary biotinylated antibody (goat anti-rabbit IgG, 1:200, B7389; or goat anti-rat IgG 1:500, B7139; both from Sigma-Aldrich, MO, USA) followed by incubation with streptavidin-peroxidase (LSAB+ System, Dako, Carpinteria CA, USA). Binding was visualized incubating sections with DAB and lightly counterstaining with haematoxylin, prior to permanent mounting.

The number of cells expressing immunoreactivity for PCNA was established using a standard light microscope. A total of 300 epithelial cells were counted and the percentage of PCNA positive cells was calculated. Any nuclear staining was regarded as positive.

For determining the percentage of vascularized area lesions were analyzed with ImageJ 1.33u software (NIH). The area positive for CD34 was visualized and marked or delimited by the usage of this software. For each animal evaluated, ten fields were micrographed; for each micrograph all positive area/s were delimited, added and then divided by the total area of the micrograph, obtaining a percentage of vascularized area per micrograph. This process was done for every set of ten micrographs per animal. Then the media for the ten micrographs was calculated, obtaining the media percentage of vascularized area per animal and the media per treatment was calculated (Olivares et al. 2011, Ricci et al. 2011).

A semiquantitative analysis of cells expressing COX-2 immunoreactivty was done using a standard light microscope. Briefly, slides were evaluated blinded to treatment by two independent observers. Ten fields were evaluated, overall staining was recorded (0, absence of staining; 1, mild staining; 2, moderate staining; 3, marked).

TUNEL assay

For apoptosis quantification, sections were processed for in-situ immunohistochemical localization of nuclei exhibiting DNA fragmentation using the apoptosis detection kit Apoptag Plus (Chemicon International, CA, USA). Sections were treated according to the manufacturer's instructions as previously described (Meresman et al. 2000). The number of cells positive for TUNEL stain was established using a standard light microscope at 400X magnification. A total of 300 epithelial cells were counted and the percentage of TUNEL positive cells was calculated.

Statistical analysis

Statistical analyses were performed using GraphPad Instat V4.0 software (for Windows, GraphPad Software, CA, USA). Statistical comparisons between groups were performed using non parametric Kruskal-Wallis test with Dunn’s multiple comparison post test. Results were expressed as median (minimum-maximum) or as mean ± S.E.M. In all cases, statistical significance was considered when $p<0.05$.

300

301 **Declaration of interest**

302 The authors declare there is no conflict of interest.

303

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Figure 1

Celecoxib reduces the number and size of established lesions. Mice underwent surgery for endometriosis induction. After 28 days of treatment with vehicle, celecoxib, anastrozole or both drugs simultaneously, mice were sacrificed and the number of lesions established was counted and measured. **A-** Celecoxib significantly reduced the ~~mean~~ number of lesions established per mouse. Scatter plot for lesion establishment; median, minimum and maximum are shown. Control: 2 (1-3); Celecoxib: 0 (0-2); Anastrozole: 2 (0-3); Anas + Cele: 2 (0-2). **B-** Celecoxib had a significant effect reducing endometriotic like lesion size. Scatter plot for the development of lesions in each group; median, minimum and maximum are shown. Control: 7.238 (2.832-32.28); Celecoxib: 0 (0-8.588); Anastrozole: 4.177 (0-25.21); Anas + Cele: 7.574 (0-16.49). n = 9 (Control), 10 (Celecoxib), 8 (Anastrozole), 7 (Anas + Cele). *$p<0.05$ vs. Control group; **$p<0.01$ vs. Control group.

Figure 2

Effect of celecoxib and anastrozole on endometriotic-like lesion development. Mice underwent surgery for endometriosis induction. After 28 days of treatment with vehicle, celecoxib, anastrozole or both drugs simultaneously, mice were sacrificed and implants were removed and fixed. Cell proliferation within the implants was evaluated by immunohistochemistry of PCNA. Apoptosis was evaluated by TUNEL technique.

Left panels: A- After treatment with celecoxib or anastrozole separately epithelial cell proliferation was significantly diminished compared to control mice. **B-** After treatment with celecoxib or anastrozole separately epithelial cell apoptosis was enhanced compared to Control group. Results are expressed as mean ± SEM. *$p<0.05$ vs. Control group. n = 5 for all groups.

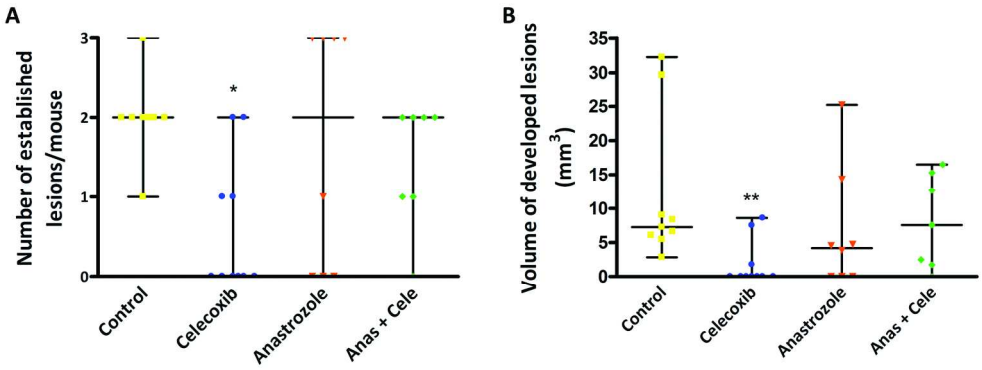
Right panels Representative micrographs of **(A)** PCNA and **(B)** TUNEL staining. (i) Control group, (ii) Celecoxib group, (iii) Anastrozole group, (iv) Celecoxib + Anastrozole group. *Insets*: negative controls, an immunoglobulin of the same immunoglobulin class and concentration as the primary antibody was used for PCNA immunohistochemistry and sectios were incubated in absence of TdT enzyme for TUNEL. Magnification 400X.

Figure 3

Effect of celecoxib on endometriotic-like lesion vascular density. Mice underwent surgery for endometriosis induction. After 28 days of treatment with vehicle, celecoxib, anastrozole or both drugs simultaneously, mice were sacrificed and implants were removed and fixed. Vascular density within the implants was evaluated performing immunohistochemistry of CD34. **Left panel:** After treatment with celecoxib vascular density was diminished compared to control mice. Results are expressed as mean ± SEM. *$p<0.05$ vs. Control group. n = 5 for all groups. **Right panel:** Representative micrographs of CD34 staining. (i) Control group, (ii) Celecoxib group, (iii) Anastrozole group, (iv) Celecoxib + Anastrozole group. *Inset*: negative control, an immunoglobulin of the same immunoglobulin class and concentration as the primary antibody was used. Magnification 400X.

Figure 4

Effect of celecoxib and anastrozole on COX-2 immunoreactivity. Mice underwent surgery for endometriosis induction. After 28 days of treatment with vehicle, celecoxib, anastrozole or both drugs simultaneously, mice were sacrificed and implants were removed and fixed. COX-2 expression was evaluated performing immunohistochemistry. **Left panel:** Semiquantification of the immunostaining of COX-2 in the developed lesions. When treated with celecoxib COX-2 immunostaining showed a tendency to be enhanced; with anastrozole, reduced; and when combined, it seemed unaltered; in all cases compared to control mice. Results are expressed as mean ± SEM. $p>0.05$ vs. Control group. n = 5 for all groups. **Right panel:** Representative micrographs of COX-2 staining. (i) Control group, (ii) Celecoxib group, (iii) Anastrozole group, (iv) Celecoxib + Anastrozole group. *Inset*: negative control, an immunoglobulin of the same immunoglobulin class and concentration as the primary antibody was used. Magnification 400X.



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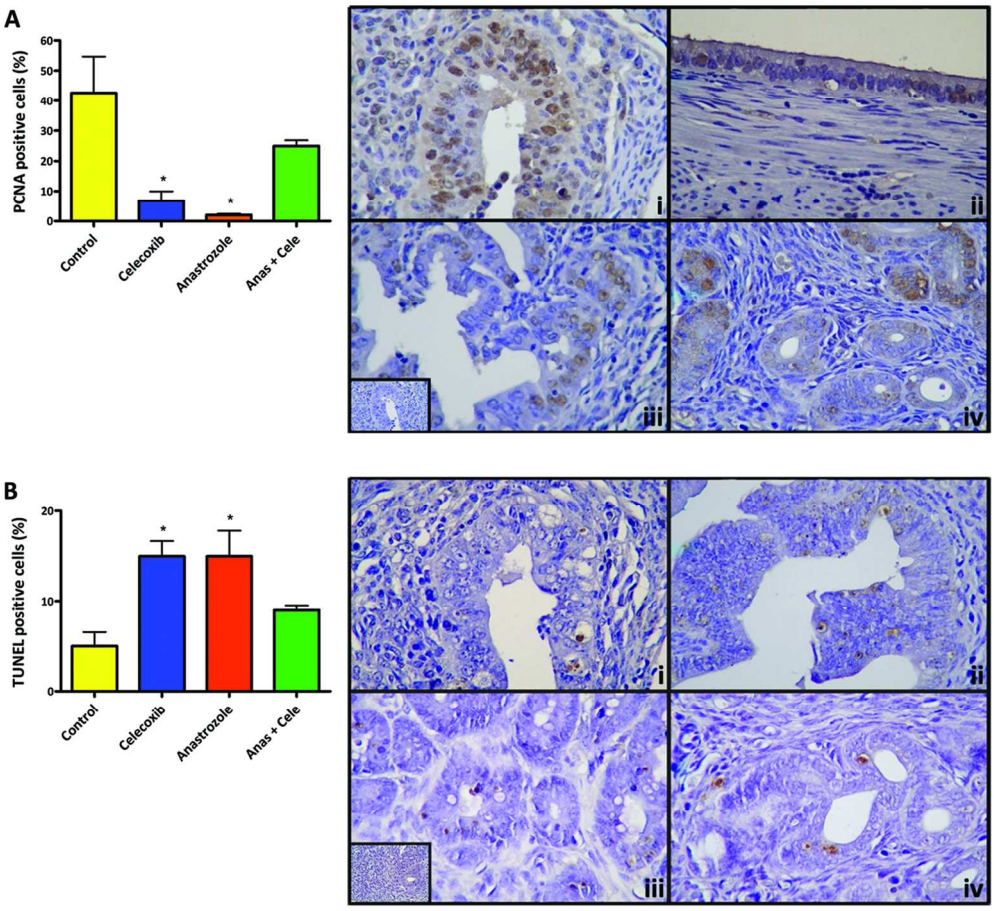

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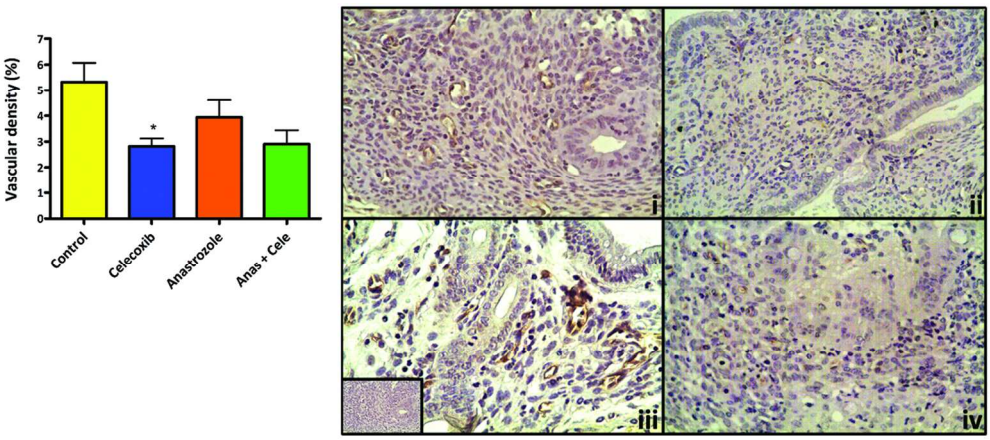

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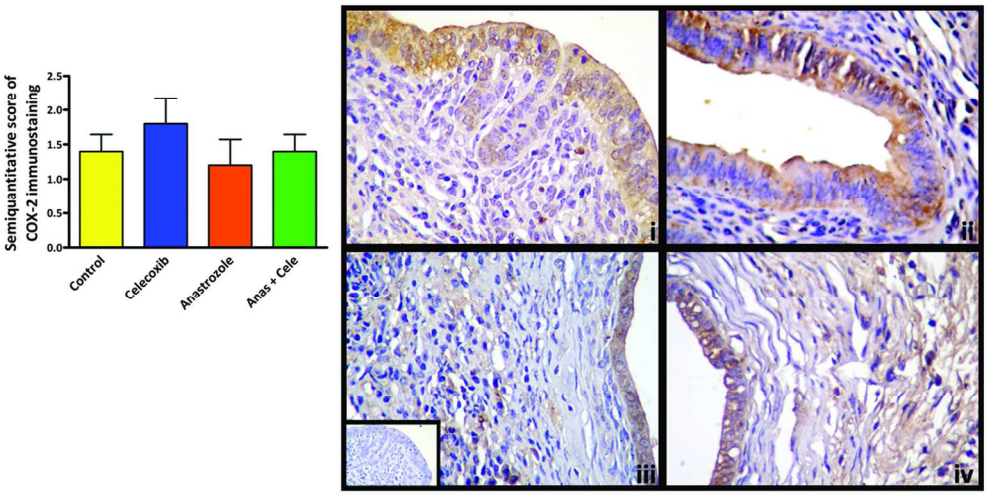

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