

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

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

17

18 Abstract

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

35

36 **Introduction**

37 Endometriosis is a benign disease that is characterized by the presence of endometrial tissue outside
38 the uterine cavity where it proliferates and forms new blood vessels essential for its further
39 development (Lousse et al. 2012). As an estrogen dependent disease, treatments available up to date,
40 aim mainly at reducing estrogen levels (Ozkan et al. 2008). Since endometriosis affects women in
41 reproductive age and given that current available treatments mostly impede ovulation (Rocha et al.
42 2012), it is the goal of recent research to find alternatives to the existing treatments reducing the side
43 effects of those now accessible.

44 We and others have studied the involvement of cyclooxygenase (COX)-2 in this pathology
45 (Matsuzaki et al. 2004, Banu et al. 2008, Olivares et al. 2008, Olivares et al. 2011), which has been
46 described to be overexpressed not only in eutopic and ectopic endometrium from endometriosis
47 patients (Ota et al. 2001, Fagotti et al. 2004), but also in a wide variety of cancers (Mendes et al.
48 2009, Ghosh et al. 2010, Wang et al. 2010). Selective COX-2 inhibitors are a special class of
49 nonsteroidal antiinflammatory drugs (NSAIDs) that were developed to treat pain and inflammation
50 without inhibiting COX-1, thus sparing the gastrointestinal system (Wadman 2007). The COXs are
51 the enzymes responsible for the synthesis of pain mediators including prostaglandins (PGs).
52 Particularly, high concentration of PGE₂ has been found in the peritoneal fluid of patients with
53 endometriosis, primarily provided by activated macrophages and the endometriotic lesions (Wu et al.
54 2010). Our group has demonstrated that selective inhibition of COX-2 activity with celecoxib reduces
55 the proliferation rate of endometrial epithelial cells as well as it augments their apoptosis levels both
56 *in vitro* and *in vivo* (Olivares et al. 2008, Olivares et al. 2011). Similar results were obtained by other
57 investigators demonstrating that the inhibition of COX-2 activity has antiproliferative, proapoptotic
58 and antiangiogenic effects in several *in vivo* and *in vitro* cancer models (Gupta et al. 2004, Basu et al.
59 2005, Dandekar et al. 2005, Barnes et al. 2007) and in other models of endometriosis (Dogan et al.
60 2004, Laschke et al. 2007, Machado et al. 2010). Moreover, a COX-2 inhibitor has been used in a
61 pilot study evaluating pain response in endometriosis patients with good results (Cobellis et al. 2004).

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

68 COX-2 is overexpressed in some malignancies including carcinoma of the breast. COX-2 mRNA and
69 protein levels have been found to correlate with aromatase levels within human breast cancer tissue.
70 The current understanding of the role of COX-2 in breast cancer suggests that COX-2 inhibitors may
71 have a role in chemoprevention which is based in part on the generic issues of antiangiogenesis and
72 proapoptotic processes, and in part on a tissue-specific inhibition of estrogen synthesis (Davies et al.
73 2002). It has been reported that celecoxib induces a marked inhibition of aromatase protein
74 expression, assessed by western blot, in human breast cancer cell lines (Bocca et al. 2011). In
75 addition, treatment with COX-2 siRNAs resulted in suppression of the aromatase gene CYP19 in
76 breast cancer cells (Brueggemeier et al. 2007). In another experimental model, Sirianni *et al.* reported
77 that inhibition of COX-2 downregulates aromatase activity and decreases proliferation of Leydig
78 tumor cells (Sirianni et al. 2009). In addition, previous studies had shown that aromatase and COX-2
79 simultaneous inhibition might have additive or even synergic effects (Bundred & Barnes 2005, Ebert
80 et al. 2005, Chow et al. 2008, Dirix et al. 2008, Falandry et al. 2009).

81 Furthermore, a feed forward loop has been described between aromatase and PGE₂ in endometriosis.
82 Aromatase synthesizes estrogens, which in turn stimulate the production of PGE₂ via COX-2, and
83 PGE₂ stimulates aromatase activity (Bulun et al. 2002). This describes a perfect circle in this system
84 in which high expression of aromatase will produce high concentrations of estrogens, which will
85 stimulate COX-2 activity producing high concentrations of PGE₂, stimulating again aromatase.

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

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

92

93 **Results**

94 *Celecoxib inhibits the establishment and growth of endometriotic like lesions*

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

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

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

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

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

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

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

120

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

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

130

131 **Discussion**

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

138 In the present study, and taking into account previous results obtained in our laboratory (Bilotas et al.
139 2010, Olivares et al. 2011) and earlier promising results obtained in cancer (Chow et al. 2008,

140 Falandry et al. 2009), we decided to combine two inhibitors: of COX-2 and aromatase. Already some
141 years ago, Ebert and coworkers reviewed the importance of aiming at these molecules given the
142 abnormalities present within the eutopic and ectopic endometrium of endometriosis patients (Ebert et
143 al. 2005) and acknowledging the positive feedback loop present between these molecules (Bulun et al.
144 2002).

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

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

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

160 We then investigated the rate of cell proliferation and apoptosis in the lesions developed in all groups.
161 We observed that cell proliferation in the epithelial fraction of the lesions was significantly
162 diminished and apoptotic levels were significantly augmented, when animals were treated with
163 celecoxib or anastrozole separately compared to the control group. It is important to note that even
164 though the rate of cell proliferation was seen significantly reduced in both of these groups, the
165 incidence of this decrease was only evidenced macroscopically, in the celecoxib treated animals at the
166 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 (Efsthathiou 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

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

221

222 **Materials and Methods**

223 *Animals*

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

229

230 *Surgical induction of endometriosis and treatment*

231 Endometriosis-like lesions were induced through transplantation of one of the uterine horns to the
232 bowel mesentery as previously described (Bilotas et al. 2010, Olivares et al. 2011, Ricci et al. 2011).
233 Briefly, animals were deeply anaesthetized with an intraperitoneal injection of ketamine (100mg/kg)
234 (Holliday Scott, Buenos Aires, Argentina) and xylazine (10mg/kg) (Richmond, Buenos Aires,
235 Argentina). Mice underwent laparotomy by midventral incision to expose the uterus and intestine. The
236 right uterine horn was removed, opened longitudinally and cut into square pieces measuring
237 approximately 4mm². Three equal pieces of tissue were then sutured onto serosal layer with a single
238 6-0 nylon suture (Supralon, Ethicon, NJ, USA) with endometrial tissue facing the serosa. The
239 abdomen was then closed with a 5-0 nylon suture.

240 Animals were assigned into four different treatment groups: Control: 150µl vehicle; Celecoxib:
241 1500ppm of celecoxib (Pfizer, USA) in chow (+ 150µl vehicle); Anastrozole (0.5mg/kg subcutaneous
242 injection, anastrozole was reconstituted in physiological solution) (AstraZeneca, London, UK) and
243 Celecoxib + Anastrozole, received the treatments combined. All treatments were administered daily,
244 started in post-operative day 1 and continued during 28 days. The amount of celecoxib consumed by
245 each animal was estimated weighing the chow one day and the next, this difference was divided by

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

250

251 *Endometriotic like lesions evaluation*

252 After 4 weeks of treatment, animals were sacrificed by cervical dislocation. The abdomen was opened
253 by ventral midline incision. Implantation sites were localized by the presence of a lesion or by suture
254 alone. Lesions were counted and measured for volume determination using the formula: $V =$
255 $(4/3)\pi r^2 R$ (where r and R are the radiuses, $r < R$) (Brodie et al. 2003). Then lesions were excised, fixed
256 and paraffin-embedded. Specimens were cut into $5\mu\text{m}$ serial sections. Four to five non-contiguous
257 sections from each specimen were stained with haematoxylin-eosin and examined microscopically for
258 the presence of histological hallmarks (glands and stroma) of endometriosis.

259

260 *Immunohistochemistry for PCNA, CD34 and COX-2*

261 Serial sections of endometriotic lesions were subjected to standard immunohistochemistry. Tissue
262 sections were incubated overnight with the primary antibody (rabbit anti-mouse PCNA polyclonal,
263 1:300, FL-261, Santa Cruz Biotechnology, CA, USA; rat anti-mouse CD34 monoclonal, 1:50, ab8158
264 Abcam, MA, USA; or rabbit anti-COX-2 polyclonal, 1:200, sc-1747, Santa Cruz Biotechnology) at
265 4°C . After that, sections were treated for 60 min with the corresponding secondary biotinylated
266 antibody (goat anti-rabbit IgG, 1:200, B7389; or goat anti-rat IgG 1:500, B7139; both from Sigma-
267 Aldrich, MO, USA) followed by incubation with streptavidin-peroxidase (LSAB+ System, Dako,
268 Carpinteria CA, USA). Binding was visualized incubating sections with DAB and lightly
269 counterstaining with haematoxylin, prior to permanent mounting.

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

273 For determining the percentage of vascularized area lesions were analyzed with ImageJ 1.33u
274 software (NIH). The area positive for CD34 was visualized and marked or delimited by the usage of
275 this software. For each animal evaluated, ten fields were micrographed; for each micrograph all
276 positive area/s were delimited, added and then divided by the total area of the micrograph, obtaining a
277 percentage of vascularized area per micrograph. This process was done for every set of ten
278 micrographs per animal. Then the media for the ten micrographs was calculated, obtaining the media
279 percentage of vascularized area per animal and the media per treatment was calculated (Olivares et al.
280 2011, Ricci et al. 2011).

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

285

286 *TUNEL assay*

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

293

294 *Statistical analysis*

295 Statistical analyses were performed using GraphPad InStat V4.0 software (for Windows, GraphPad
296 Software, CA, USA). Statistical comparisons between groups were performed using non parametric
297 Kruskal-Wallis test with Dunn's multiple comparison post test. Results were expressed as median
298 (minimum-maximum) or as mean \pm S.E.M. In all cases, statistical significance was considered when
299 $p < 0.05$.

300

301 **Declaration of interest**

302 The authors declare there is no conflict of interest.

303

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307

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445

446

447 **Figure 1**

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

458

459 **Figure 2**

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

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

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

474

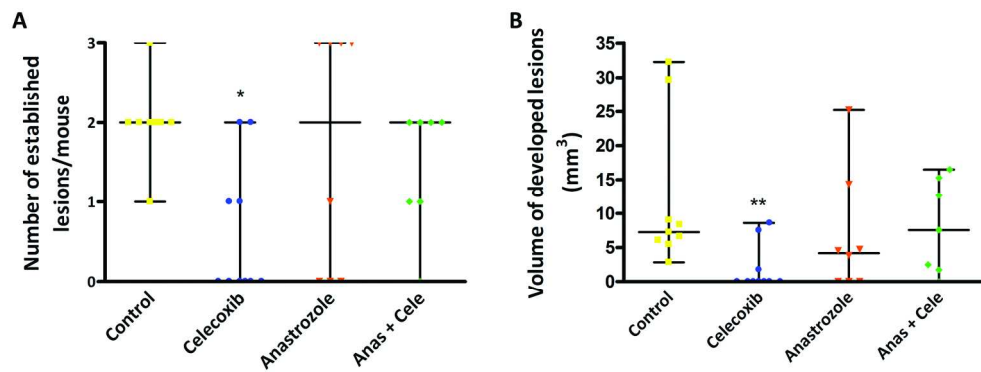
475 **Figure 3**

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

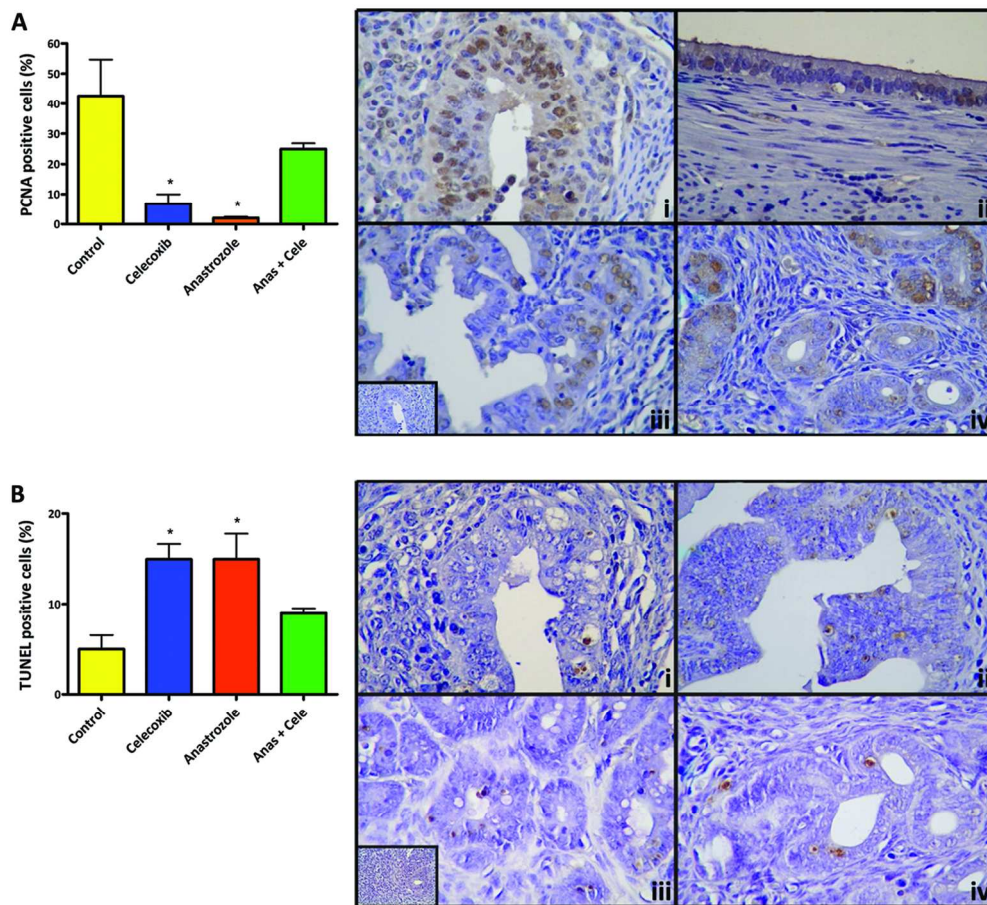
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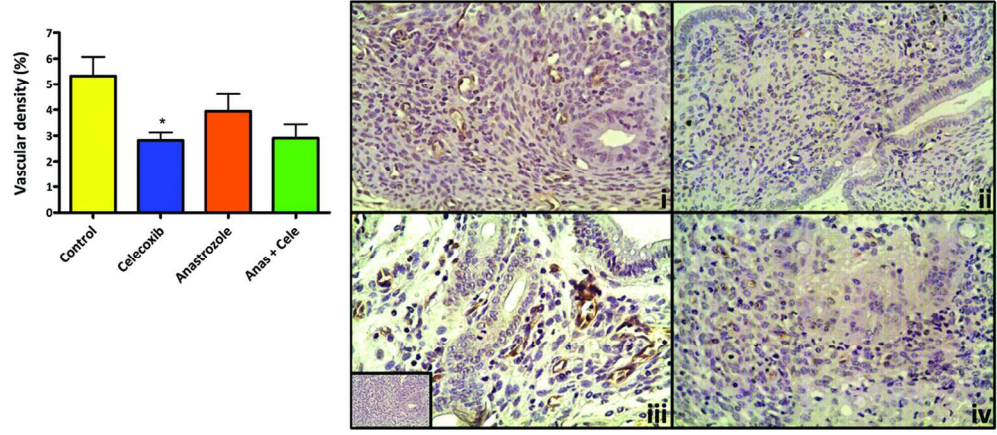
486 **Figure 4**

487 *Effect of celecoxib and anastrozole on COX-2 immunoreactivity.* Mice underwent surgery for
488 endometriosis induction. After 28 days of treatment with vehicle, celecoxib, anastrozole or both drugs
489 simultaneously, mice were sacrificed and implants were removed and fixed. COX-2 expression was
490 evaluated performing immunohistochemistry. **Left panel:** Semiquantification of the immunostaining
491 of COX-2 in the developed lesions. When treated with celecoxib COX-2 immunostaining showed a
492 tendency to be enhanced; with anastrozole, reduced; and when combined, it seemed unaltered; in all
493 cases compared to control mice. Results are expressed as mean \pm SEM. $p > 0.05$ vs. Control group. $n =$
494 5 for all groups. **Right panel:** Representative micrographs of COX-2 staining. (i) Control group, (ii)
495 Celecoxib group, (iii) Anastrozole group, (iv) Celecoxib + Anastrozole group. *Inset:* negative control,
496 an immunoglobulin of the same immunoglobulin class and concentration as the primary antibody was
497 used. Magnification 400X.

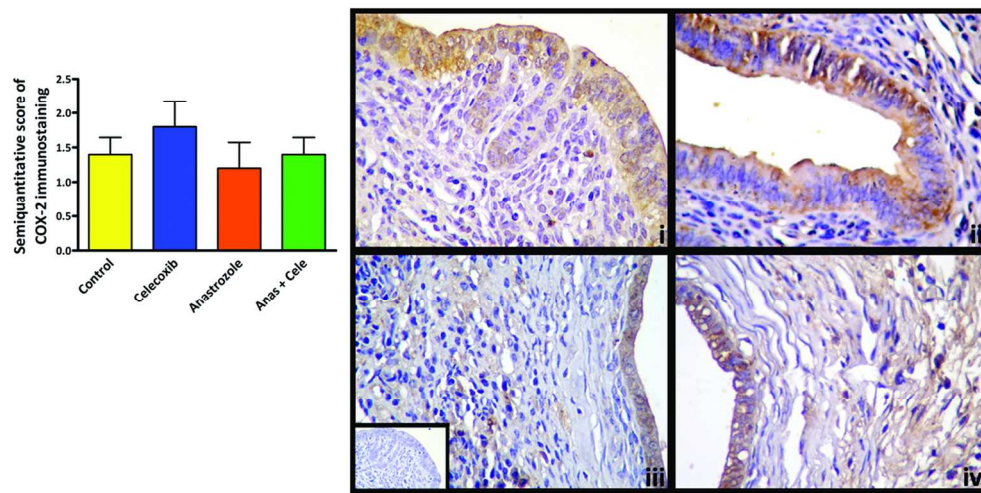


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