The inhibitory effect of celecoxib and rosiglitazone on experimental endometriosis

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Objective: To evaluate the effects of celecoxib and rosiglitazone on the implantation and growth of endometriotic-like lesions in a murine model of endometriosis.
Design: Prospective experimental study.
Setting: Animal research and laboratory facility.
Animal(s): Two-month-old female BALB/c mice.
Intervention(s): Surgically induced endometriosis in female BALB/C mice; 28 days of treatment with celecoxib, rosiglitazone, or their combination; counting, measuring, excising, and fixing lesions.
Main Outcome Measure(s): Immunohistochemical examination for proliferating cell nuclear antigen (PCNA), CD31, and CD34 to assess cell proliferation and vascularization, with the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) technique for apoptosis evaluation.
Result(s): Celecoxib and the combined treatment (celecoxib and rosiglitazone) statistically significantly reduced the mean number of lesions established per mouse, and all treatments diminished the implant volume. In addition, cell proliferation within the implants was statistically significantly reduced, and apoptosis was statistically significantly enhanced by all treatments. Also, we found that all treatments diminished the vascularized area in the lesion.
Conclusion(s): These results are promising and reveal that celecoxib and rosiglitazone, combined or separately, have a beneficial effect on overall endometriotic growth. (Fertil Steril® 2011;96:428–33. ©2011 by American Society for Reproductive Medicine.)

Key Words: Apoptosis, cell proliferation, COX-2 inhibitor, endometriosis, PPARγ agonist

Endometriosis, a common benign gynecologic disease characterized by the presence and proliferation of endometrial tissue outside the uterine cavity, affects women of reproductive age (1). Because endometriosis is an estrogen-dependent disease, the treatment options aim at maintaining a hypoestrogenic environment, which removes the opportunity for conception from women who are under treatment (2). Furthermore, the therapy's side effects limit its long-term use, and endometriosis recurrence after cessation of therapy is not uncommon (3). Therefore, developing better treatment options for patients with endometriosis is necessary.

In the past few years, COX-2, the inducible form of cyclooxygenase, has gained special attention. This enzyme, which is involved in inflammatory processes, has been demonstrated to be overexpressed in several pathologic conditions, including endometriosis (4–6). Selective COX-2 inhibitors are a special class of non-steroidal anti-inflammatory drugs (NSAIDs) that were developed to treat pain and inflammation without inhibiting COX-1 while sparing the gastrointestinal system. In previous studies, we added a selective COX-2 inhibitor to cultured epithelial endometrial human cells and found that it inhibited cell proliferation, enhanced cell apoptosis, and diminished vascular endothelial growth factor (VEGF) and prostaglandin (PG) E_2 secretion (7). These drugs have also been used in endometriosis animal models and have been demonstrated to prevent the implantation of endometrium to ectopic sites (8) and to induce the regression of endometrial explants in mice (9) and rats (10).

Peroxisome proliferator-activated receptors (PPARs) are a family of nuclear receptors that respond to endogenous ligands and chemical compounds modulating the transcription of a large number of genes (11). Three classes of PPARs have been described so far: PPARα, PPARβ/δ, and PPARγ (12).

It has been previously described that 15d-PGJ_2 is the natural ligand of PPARγ (13); the thiazolidinediones (TZDs), currently used as antidiabetic drugs, are its synthetic ligands (14). It has been reported that TZDs inhibit tumor growth both in vitro and in vivo (15–17). Panigrahy et al. (17) demonstrated that the inhibition by PPARγ ligands of tumor growth was mediated by inhibiting endothelial cell proliferation, thus diminishing angiogenesis within the tumor; some TZDs have been found to be anti-inflammatory agents (18). It has also been shown that at high concentrations COX-2 inhibitors can also act as PPARγ agonists (19).

Combining the activation of PPARγ with the inhibition of COX-2 appears to be a promising strategy in cancer. It was demonstrated that combined treatment with celecoxib and F-L-Leu, a PPARγ agonist, retards the appearance of tumors in a model of spontaneous breast cancer (20). More recently, similar results were obtained in a human pancreatic cell carcinoma model (21). In endometriosis, one study has targeted PPARγ simultaneously with another molecule, retinoid X receptor (RXR), in immortalized endometrial stromal cells, achieving a synergistic inhibition of cell proliferation (22). However,

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to our knowledge, the effects of a COX-2 inhibitor in combination with a PPARγ ligand in an endometriosis model have not yet been studied.

We examined the efficacy of celecoxib, a potent COX-2 inhibitor, and rosiglitazone, a PPARγ ligand, separately and in combination, on the establishment and growth of endometriotic-like lesions in a murine model of endometriosis. In addition, we evaluated cell proliferation and apoptosis in the epithelial cells of the endometriotic implants as well as their vascularization.

MATERIALS AND METHODS

Animals

In this study, 48 2-month-old female BALB/c mice were used. All procedures were performed according to U.S. National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Ethics and Research Committee from IBYME, Buenos Aires, Argentina. A total of seven animals died or had to be sacrificed between 2 to 3 days after surgery because they did not fully recover from the intervention.

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 elsewhere (23, 24). Briefly, the animals were deeply anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). The 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 that measured approximately 4 mm^2. Three equal pieces of tissue were then sutured onto the serosal layer with a single 6-0 nylon suture (Supralon; Ethicon) with endometrial tissue facing the serosa. The abdomen was then closed with a 5-0 nylon suture.

The animals were assigned to four different treatment groups: control (100 μL distilled water with 0.5% carboxymethylcellulose [CMC]); celecoxib (200 mg/kg in water with 0.5% CMC) (Pfizer); rosiglitazone (0.16 mg/kg) (Montpellier); or celecoxib + rosiglitazone with the drugs combined. The dosages in this study were chosen based on other in vivo reports (25, 26). The rosiglitazone dosage used is similar to that used by type 2 diabetes patients (4 or 8 mg daily), whereas the dosage of celecoxib is higher than that used in cancer patients (800 mg daily). All treatments were administered daily by esophageal gavage, starting on postoperative day 1 and continuing for 28 days. No evidence of toxicity was noted at the doses administered based on body weight, food consumption, grooming behavior, or activity levels compared with controls.

Evaluation of Endometriotic-Like Lesions

After 4 weeks of treatment, the animals were killed 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 by use of the following formula: $V = (4/3)\ \pi r2R$ (where V is volume, and r and R are the radiuses, $r < R$) (27). Then lesions were excised, fixed, and embedded in paraffin. The specimens were cut into 5-μm serial sections. Four to five noncontiguous sections from each specimen were stained with hematoxylin and eosin and were examined microscopically for the presence of the histologic hallmarks (glands and stroma) of endometriosis.

Immunohistochemistry for PCNA, CD31, and CD34

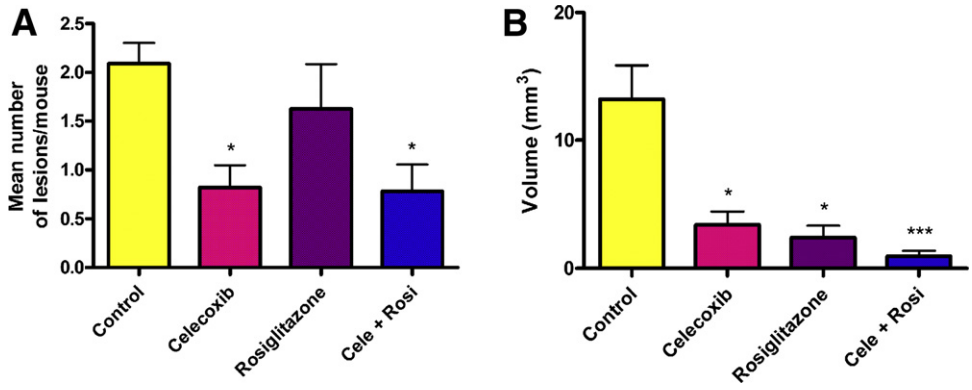
Serial sections of endometriotic lesions were subjected to standard immunohistochemical analysis. Tissue sections were incubated overnight with the primary antibody (rabbit anti-mouse proliferating cell nuclear antigen [PCNA] polyclonal, 1:300, FL-261; Santa Cruz Biotechnology; rat anti-mouse CD31 monoclonal, 1:50, ab56299; Abcam; or rat anti-mouse CD34 monoclonal, 1:50, ab8158; Abcam) at 4°C. After that, sections were treated for 60 minutes with the corresponding secondary biotinylated antibody (anti-rabbit IgG, 1:200, B7389; Sigma-Aldrich; or goat anti-rat IgG, 1:500, B7139; Sigma-Aldrich) followed by incubation with streptavidin-peroxidase (LSAB+ System; Dako). The binding was visualized by incubating sections with diaminobenzidine (DAB) and lightly counterstaining with hematoxylin before 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.

Ten fields for each slide containing all lesions were analyzed with ImageJ version 1.33u software (http://rsbweb.nih.gov/ij) for determining the percentage of the vascularized area. The positive stain for CD31 or CD34 was calculated per field and averaged per mouse. Then all averages were used to obtain the mean vascularized area per treatment group.

FIGURE 1

Effect of celecoxib and rosiglitazone on endometriotic-like lesion establishment and growth. Mice underwent surgery for endometriosis induction. After 28 days of treatment with vehicle, celecoxib, rosiglitazone, or both drugs simultaneously, the mice were sacrificed, and the number of lesions established was counted and measured. (**A**) Celecoxib and the combined treatment (Cele + Rosi) statistically significantly reduced the mean number of lesions established per mouse. (**B**) Celecoxib and rosiglitazone, separately, and the combined therapy (Cele + Rosi) statistically significantly reduced implant size. Results are expressed as mean ± standard error of the mean. *$P<.05$ versus control group. ***$P<.001$ versus control group. n = 11 (control), 12 (celecoxib), 8 (rosiglitazone), 10 (Cele + Rosi).



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TUNEL Assay

For apoptosis quantification, sections were processed for in situ immunohistochemical localization of nuclei exhibiting DNA fragmentation by use of the apoptosis detection kit Apoptag Plus (Chemicon International). Sections were treated according to the manufacturer's instructions as previously described elsewhere (28). The number of cells positive for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) stain was established by use of a standard light microscope at ×400 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 version 4.0 software (Windows version; GraphPad Software). Statistical comparisons between groups were performed by use of parametric analysis of variance (ANOVA) with Tukey's multiple comparison post test or nonparametric ANOVA with Dunn's multiple comparison post test. Results were expressed as mean ± standard error of the mean (SEM). $P<.05$ was considered statistically significant.

RESULTS

Effect of Celecoxib and Rosiglitazone on Endometriotic-Like Lesion Establishment and Size

The group treated only with celecoxib and the group receiving the combined therapy had a statistically significant reduction in the number of established lesions per animal ($P<.05$ vs. control group). The results are displayed in Figure 1A.

Regarding the volume of developed lesions, all treatment groups had a statistically significant reduction in lesion size. As seen in Figure 1B, although there is not a statistically significant difference between the treated groups, celecoxib and rosiglitazone combined tended to be more efficient in reducing the lesion volume ($P<.001$ vs. control group) than either of the treatments alone.

Celecoxib and Rosiglitazone Treatments Affect Cell Proliferation and Apoptosis in Endometriotic-Like Lesions

All treatment groups had diminished cell proliferation compared with the control group (rosiglitazone-only and celecoxib + rosiglitazone groups, $P<.001$ vs. control; celecoxib-only group, $P<.01$ vs. control). The results are displayed in Figure 2. The combined treatment appeared to be slightly more effective in decreasing cell proliferation than either of the drugs separately, but the effect was not synergistic nor additive.

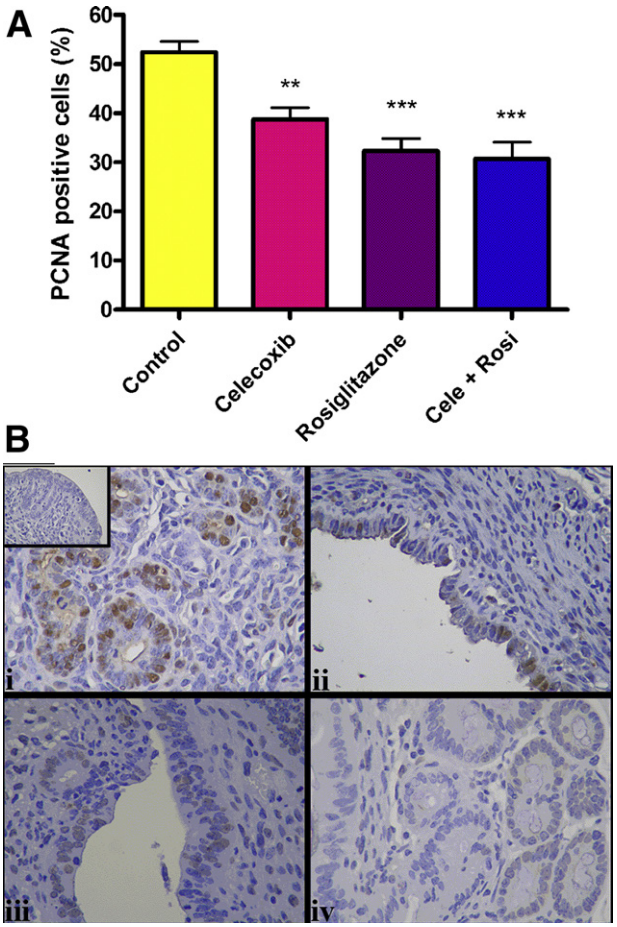
All treatments were effective enhancing apoptosis compared with the control group ($P<.05$), as seen in Figure 3. Not only did the TUNEL assay demonstrate that apoptosis was augmented in the treatment groups, but also the regression of the lesions was clearly visible in the histologic analysis of the sections (data not shown).

Inhibiting COX-2 and Activating PPARγ Decrease Vascular Density in Endometriotic-Like Lesions

Figure 4 shows the effect of the treatments on vascular density as assessed by CD31 and CD34 immunohistochemistry. As observed with CD31 staining, rosiglitazone-only and the combined treatment resulted in a statistically significant reduction of vascular density ($P<.05$ vs. control group). When the same parameter was evaluated via immunostaining with CD34, the celecoxib-only and rosiglitazone-only groups showed a statistically significant decrease in vascular density when compared with the control group ($P<.05$); even though there was no statistically significant difference between the treated groups, when the treatments were administered in combination the reduction tended to be superior ($P<.01$ vs. control group).

FIGURE 2

Effect of celecoxib and rosiglitazone on endometriotic-like lesion epithelial cell proliferation. Mice underwent surgery for endometriosis induction. After 28 days of treatment with vehicle, celecoxib, rosiglitazone, or both drugs simultaneously, the mice were sacrificed, and the implants were removed and fixed. Cell proliferation within the implants was evaluated by immunohistochemistry of proliferating cell nuclear antigen (PCNA). (**A**) After treatment with celecoxib, rosiglitazone, or both drugs simultaneously (Cele + Rosi), epithelial cell proliferation was statistically significantly diminished compared with control mice. Results are expressed as mean ± standard error of the mean. $^{**}P<.01$ versus control group; $^{***}P<.001$ versus control group. (**B**) Representative photographs of PCNA staining. Control group (i), celecoxib group (ii), rosiglitazone group (iii), celecoxib + rosiglitazone group (iv). *Inset:* negative control, an immunoglobulin of the same immunoglobulin class and concentration as the primary antibody was used. Magnification ×400. n = 8 (control), 7 (celecoxib), 5 (rosiglitazone), 5 (Cele + Rosi).



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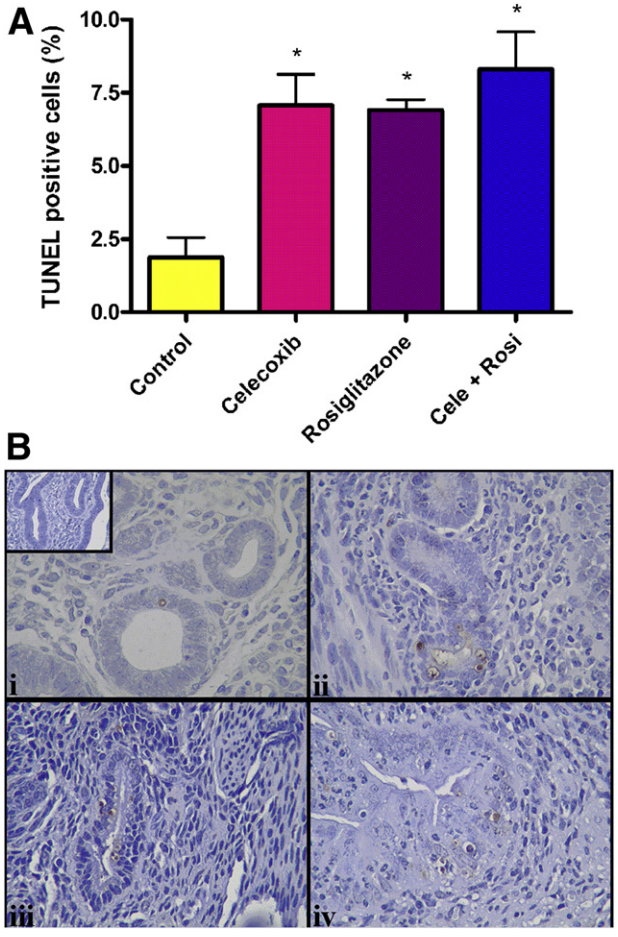
DISCUSSION

The current medical treatment of endometriosis is still associated with a high recurrence rate. The therapeutic options include surgery and hormone therapy; which are often temporarily effective but produce unwanted side effects (2, 29, 30). Given this scenario, it is of great importance to study new strategies to treat endometriosis that minimize the adverse effects and reduce the rates of recurrence.

FIGURE 3

Effect of celecoxib and rosiglitazone on endometriotic-like lesion epithelial cell apoptosis. Mice underwent surgery for endometriosis induction. After 28 days of treatment with vehicle, celecoxib, rosiglitazone, or both drugs simultaneously, the mice were sacrificed, and the implants were removed and fixed. Apoptosis within the implants was evaluated by TUNEL assay. (**A**) After treatment with celecoxib, rosiglitazone, or both drugs simultaneously (Cele + Rosi), epithelial cell apoptosis was enhanced compared with the control group. Results are expressed as mean ± standard error of the mean. *$P<.05$ versus control group. (**B**) Representative photographs of TUNEL staining. Control group (i), celecoxib group (ii), rosiglitazone group (iii), celecoxib + rosiglitazone group (iv). *Inset:* negative control, sections were incubated in absence of TdT. Magnification ×400. n = 6 (control), 7 (celecoxib), 5 (rosiglitazone), 5 (Cele + Rosi).



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In our study, we found that celecoxib treatment inhibited endometriotic-like lesion establishment and growth. To clarify the mechanisms involved in this inhibition, we assessed cell proliferation, apoptosis, and vascular density after administration of celecoxib. Cell proliferation within the lesions was diminished compared with control mice, as was the vascular density. In addition, apoptosis was augmented in the endometriosis-like lesions.

Consistent data have suggested that COX-2 inhibitors can reduce cancer risk in patients, prevent tumorigenesis, and suppress established tumor growth in animals (31). In various in vivo and in vitro cancer models, COX-2 inhibitors have proven to have antiproliferative, proapoptotic, and antiangiogenic activity (32–35). It has been previously described that COX-2 is overexpressed in the eutopic and ectopic endometrium of women with endometriosis compared with controls (5, 6), and the effects of inhibiting this enzyme in several endometriosis models have been studied (7, 9, 10). The modern selective COX-2 inhibitors have not yet been approved for use in endometriosis. However, a first promising placebo-controlled, double-blind study showed that the use of a COX-2-inhibitor was an effective, safe, and inexpensive therapeutic approach to endometriosis-associated pelvic pain (36).

In our study, we also found that rosiglitazone treatment inhibited endometriotic implant growth. Moreover, we studied the levels of apoptosis, cell proliferation, and vascularization within the endometriotic-like lesions and found that apoptosis after rosiglitazone treatment was augmented compared with controls, whereas cell proliferation and vascularization were inhibited. However, with rosiglitazone treatment alone we did not observe a reduction of endometriotic-like lesion development, as opposed to celecoxib alone or the combined treatment. We believe that this phenomenon might be due to celecoxib's more potent effect over rosiglitazone, when administered during this period of time and at the dose evaluated, on the growth of the endometriotic-like lesions. This would eventually translate into a reduced number of endometriotic-like lesions developing.

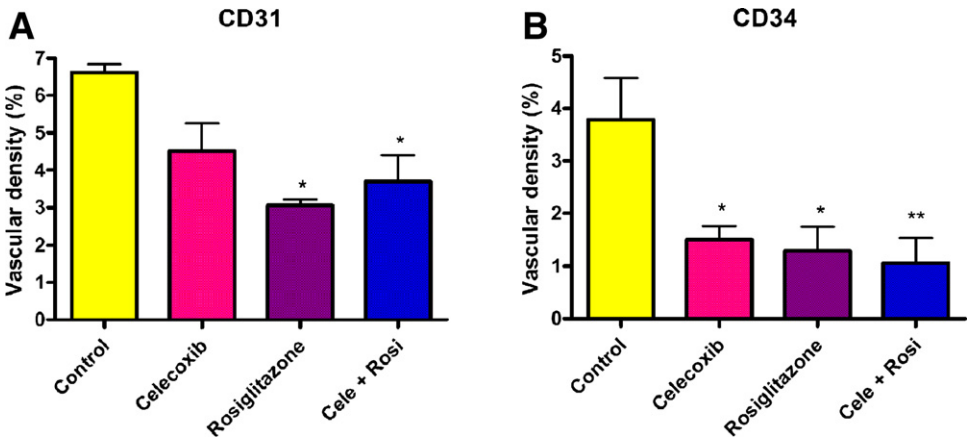
PPARs have been described in a variety of tissues, including reproductive tissues. In breast cancer, PPARγ has received focused attention because it was seen to be inactivated (37) and to mediate antitumor effects after activation by TZDs (38). In different in vivo rodent models, beneficial effects after treatment with TZDs have been described, including inhibition of tumor growth, enhancement of tumor apoptosis, and overall survival rate (16, 17).

In endometriosis, PPARγ ligands have also been used with promising results both in vitro and in vivo. Ciglitazone reduced the size of experimentally induced endometriotic lesions in a rat model of endometriosis (39), and rosiglitazone was effective at reducing endometriotic growth and causing regression of established implants in a similar animal model (40, 41). More recently, a study done on baboons evaluated the effects of rosiglitazone versus a gonadotropin-releasing hormone (GnRH) antagonist and observed that the TZD provoked implant regression (42, 43). Pioglitazone has also been described as effective in reducing adhesions in a chimeric mouse model of endometriosis (44).

In endometriosis treatment, an overall beneficial effect has been reported after administration of rosiglitazone in a reduced number of patients with endometriosis, including ameliorating endometriosis symptoms and pain (45). It is noteworthy that rosiglitazone not only does not impede ovulation (45) but also has been reported to help restore spontaneous ovulation (46), an improvement over current medical therapies for endometriosis (45). In addition, it has been reported that rosiglitazone does not cause any alteration to the mouse fetus when administered during pregnancy (25). Rosiglitazone is daily taken by type 2 diabetes patients; although it is an approved drug by the U.S. Food and Drug Administration (FDA), its safety is being questioned because of potential cardiac risks. Pioglitazone, another TZD used in the treatment of type 2 diabetes, has FDA warnings on its use as well. Several studies that have evaluated rosiglitazone's cardiovascular safety, included elderly diabetes patients with high cardiovascular risk, and when compared to pioglitazone, it seems safer than rosiglitazone (47, 48), but the matter is still controversial (49–51).

FIGURE 4

Effect of celecoxib and rosiglitazone on endometriotic-like lesion vascular density. Mice underwent surgery for endometriosis induction. After 28 days of treatment with vehicle, celecoxib, rosiglitazone, or both drugs simultaneously, the mice were sacrificed, and the implants were removed and fixed. Vascular density within the implants was evaluated by performing immunohistochemical analysis of CD31 and CD34. (**A**) *CD31:* After treatment with rosiglitazone and both drugs simultaneously (Cele + Rosi), the vascular density was diminished compared with control mice. (**B**) *CD34:* After treatment with celecoxib, rosiglitazone, or both drugs simultaneously (Cele + Rosi), the vascular density was diminished compared with control mice. Results are expressed as mean ± standard error of the mean. *$P<.05$ versus control group; **$P<.01$ versus control group. n = 5 (control), 5 (celecoxib), 5 (rosiglitazone), 5 (Cele + Rosi).



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The studies published on combination therapy with COX-2 inhibitors and PPARγ activators are mainly focused in cancer models. Both in vivo and in vitro, this treatment has accomplished beneficial results, inhibiting cell proliferation, enhancing apoptosis (21), and even increasing the overall survival rate (20).

To the best of our knowledge, ours is the first study to use combination therapy targeting PPARγ and COX-2 in an in vivo endometriosis model. This study reveals that the targeting of these molecules simultaneously has good effects on endometriotic implantation and growth, and the combination tends to show improvement over focusing on either of them alone. We conclude that the reduction in cell proliferation and vascular density and the increase in the apoptosis rate are mechanisms implicated in this effect. The reduction in the number of lesions developed, we believe, is mostly an effect of celecoxib, as with celecoxib treatment alone they developed similarly to the combination therapy.

The results presented are promising, and it is encouraging that the drugs used in this study, combined or separately, are currently being used for the treatment of other illnesses. Nevertheless, further research is necessary to ascertain whether this combination therapy is appropriate for the treatment of endometriosis.

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