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Perioperative Intervention by β-Blockade and NF-κB Suppression Reduces the Recurrence Risk of Endometriosis in Mice Due to Incomplete Excision

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Abstract

Despite the demonstrated efficacy of surgical treatment of endometriosis, recurrence after surgery still remains a formidable challenge. Surgery, especially when performed repeatedly, decreases ovarian reserve. Clearly, control of recurrence is an unmet medical need. So far nearly all efforts to control recurrence have been devoted to the identification of risk factors, biomarkers, and postoperative medication. One area that has been completely overlooked is the possibility of perioperative intervention. In this study, we tested the hypothesis that perioperative use of a nonspecific β-blocker and/or a nuclear factor-κB (NF-κB) inhibitor can retard the growth of residual endometriotic lesions that are left intact in the primary surgery. We established a mouse model of recurrence due to incomplete lesion removal by deliberately leaving residual lesions intact in the primary excision surgery. One hour before and 24 hours after the surgery, mice were either untreated or treated with andrographolide, propranolol, or both. Two weeks after the primary surgery, all mice were sacrificed and all lesions were excised and evaluated for immunohistochemistry analysis. We found that perioperative use of andrographolide and/or propranolol significantly decelerated the growth of residual lesions that were intentionally left out during the primary surgery. The perioperative intervention also significantly attenuated the generalized hyperalgesia resulting from the presence of residual lesions. It also inhibited the activation of the adrenergic receptor β2 signaling, resulting in reduced angiogenesis, epithelial–mesenchymal transition, fibroblast-to-myofibroblast transdifferentiation as well as NF-κB suppression and progesterone receptor isoform B induction. These data strongly suggest that perioperative use of β-blockers and/or NF-κB inhibitors may reduce the risk of recurrence in endometriosis.

Keywords

β-blocker, endometriosis, NF-κB inhibitor, perioperative intervention, recurrence

Introduction

For endometriosis, the efficacy of medical treatment alone is either poorly documented or of limited efficacy.[1] As such, surgery is the treatment of choice for the management of symptomatic endometriosis when all other treatment modalities fail.[2] Despite the demonstrated efficacy, however, recurrence after surgery still remains a formidable challenge: 40% to 50% of patients have relapse of the disease within 5 years after the primary surgery and would require further surgeries.[3,4] In addition, surgery, especially bilateral ovarian cystectomy and when performed repeatedly, substantially increases the risk of premature ovarian failure.[5-7] Repeat surgery also diminishes the pregnancy rate in women with infertility.[8] Clearly, minimizing or completely eliminating the risk of recurrence is an unmet clinical need that has not been adequately addressed.[9,10]

The failure to meet this pressing clinical need reflects our inability to identify patients with a high risk of recurrence and the lack of subsequent intervention, which stems from the poor understanding of the mechanisms underlying the recurrence of endometriosis. Although there is a large body of clinical studies documenting various, yet often inconsistent, risk factors for recurrence,[11,12] surprisingly and curiously only few reports on biomarkers of recurrence have been published so far.[13-15] Despite their potential utility in identification of high-risk patients, however, these putative biomarkers do not, by nature, provide any information on the completeness of surgical

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removal of endometriotic lesions, nor do they provide an immediate solution for possible intervention.

Conceivably, the recurrent endometriotic lesions could arise from residual or incompletely removed lesions, presumably small enough that elude visual detection during operation or from cell clusters disseminated through blood,[16] lymphatic vessels, or other routes. They could also arise from de novo foci. Several lines of evidence indicate that the recurring endometriotic lesions may very likely arise from minimal residual lesions (MRLs) during the primary surgery. In women who received microsurgical resection of ovarian endometrioma, the recurring lesions appear to grow from the residual foci.[17] For patients who underwent a second surgery because of endometriosis, the recurrence of deep lesions has been reported to be in the *same* area of the pelvis involved in the first operation.[18] In patients with recurrent endometriomas, the majority of the recurring cysts seem to arise from the residual loci.[19] Among patients with bowel endometriosis, it is reported that patients with positive resection margins after surgery had a 6-fold increase in recurrence risk than without.[20] A systematic review of 49 trials on the optimal approach to the management of colorectal endometriosis reports that the total recurrence rate is 3-fold higher in the full-thickness disc excision or superficially excised group than that in the bowel resection group.[21] These studies, taken together, strongly suggest that more extensive surgery, which tends to leave less residual lesions, reduces the recurrence risk.

One area that seems to be completely overlooked is whether *perioperative* intervention could be employed to reduce the recurrence risk. This is biologically plausible, since surgery, in and by itself, inevitably results in tissue damage, trauma, and stress to the body. As such, various bioactive molecules are secreted perioperatively, including catecholamines that are known to suppress cell-mediated immunity[22] and promote angiogenesis[23] and metastasis.[24] We have shown previously that surgery promotes the development of endometriosis[25] and surgical history is a risk factor for endometriosis.[26] The promotional effect of surgery is most likely through the activation of the adrenergic signaling, but such a facilitatory effect can be completely abrogated by β-blockade.[25] Perioperative combined use of β-blockers and COX-2 inhibitors has been shown to reduce the tumor metastasis in vivo[27] and also improve metastatic biomarkers in patients with breast cancer.[28] This raises the prospect of the perioperative use of β-blockers and COX-2 inhibitors may reduce the regrowth of MRLs and thus the risk of recurrence of endometriosis.

We hypothesized that perioperative use of a nonspecific β-blocker and/or a nuclear factor-κB (NF-κB) inhibitor can slow down the growth of residual endometriotic lesions that are left intact in the primary surgery. The choice of an NF-κB inhibitor was deliberate: NF-κB, which is known to play important roles in the development of endometriosis,[29,30] is on the upstream of COX-2. In addition, NF-κB has been shown to be a putative biomarker for the recurrence of endometriosis.[14] Moreover, andrographolide, the particular NF-κB inhibitor we chose in this study, is known for its excellent safety profile and also is effective in suppression of COX-2 in general[31] and in ectopic endometrium in particular.[32] To test this hypothesis, we first constructed a mouse recurrence model mimicking incomplete removal of endometriotic lesions in the primary surgery, which essentially leaves MRLs behind. With this model, we tested the hypothesis and evaluated the immunoreactivity against proteins known to be involved in recurrence in ectopic endometrium, such as NF-κB, progesterone receptor isoform B (PR-B), and vascular endothelial growth factor (VEGF, a marker for angiogenesis),[9] markers representing different developmental stage in endometriosis development, such as E-cadherin (for epithelial cells) and α-smooth muscle actin (α-SMA, a marker for myofibroblasts),[33] and in stress-induced acceleration of development of endometriosis, such as adrenergic receptor β2 (ADRB2).[25,34] We found that perioperative use of β-blockers and/or NF-κB inhibitors significantly decelerated the growth of residual lesions that were intentionally left out during the primary surgery and suppressed the activation of the ADRB2 signaling, resulting in reduced angiogenesis, epithelial–mesenchymal transition (EMT), fibroblast-to-myofibroblast transdifferentiation (FMT) as well as NF-κB suppression and PR-B induction. Thus, perioperative use of β-blockers and/or NF-κB inhibitors could reduce the recurrence risk.

Materials and Methods

Animals

A total of 69 eight-week virgin female Balb/C mice were purchased from the SLAC Experimental Animal Company (Shanghai, China) and used for this study. All mice were maintained under controlled conditions with a light/dark cycle of 12/12 hours and had access to food and water ad libitum. All experiments were performed under the guidelines of the National Research Council's *Guide for the Care and Use of Laboratory Animals*[35] and approved by the institutional experimental animals review board of Shanghai OB/GYN Hospital, Fudan University.

Chemicals

Andrographolide (Andro), which is a commercial, nonprescription drug in China, was obtained from the Andro droplets, manufactured by Tasly Pharmaceutical Co., Ltd (Tianjin, China). Propranolol (Prop) was prepared from propranolol hydrochloride tablets, manufactured by Kangpu Pharmaceutical Co., Ltd (Changzhou, China). Both droplets and the tablets were ground into powders and mixed with distilled water to make suspensions.

Induction of Endometriosis

We used an established mouse model of endometriosis by intraperitoneal (IP) injection of endometrial fragments as described[36-38] and also used in our previous studies.[25,34] This induction procedure was the basis for the recurrence model. Briefly, donor mice were initially injected with 100 μg/kg

estradiol benzoate (Animal Medicine Factory, Hangzhou, China). One week later, they were killed and their uteri were removed and harvested. The uterine tissues were seeded in a Petri dish containing warm sterile saline and split longitudinally with a pair of scissors.

Two uterine horns from each mouse were first minced into smaller fragments with scissors, ensuring that the maximal diameter of the fragment was consistently smaller than 1 mm. Then uterine fragments were intraperitoneally injected to recipient mice.

The Establishment of a Recurrence Model

Essentially, we performed an excision surgery after endometriosis was well established and the associated pain was manifested, and intentionally left small lesions intact to mimic the incomplete removal of lesions, effectively leaving MRLs behind. Briefly, mice were anesthetized with 300 mg/kg chloral hydrate and underwent a laparotomy. A 3-cm midline abdominal incision was made and all large endometriotic lesions were excised with a surgical scalpel, occasionally with a pair of scissors and a tweezer. Bleeding was stopped by ligation and pressing with a small piece of gauze. No more than 2 small lesions were left over in each mouse, and the diameter of each residual lesion was less than 0.5 mm. Once finished, the abdominal wall was closed with surgical clips. After surgery, all mice were administrated Penicillin of 40 000 U intramuscularly once daily for 3 consecutive days to prevent infection.

Experimental Design

Forty mice were randomly divided into 4 equal-sized groups: the untreated group (UNT), the Andro group (ANDRO), the Prop group (PROP), and the COMB group. The baseline bodyweight and hotplate latency (see Supplementary Information for more details) of all mice were measured and recorded. Two weeks after the induction of endometriosis, all mice had a laparotomy to excise most endometriotic lesions. Perioperatively, mice in the control or UNT group received the amount of distilled water equal to the other 3 groups, those in the ANDRO and PROP group received Andro (180 mg/kg bodyweight) or Prop (10 mg/kg), respectively, and those in the COMB group received both Andro and Prop (the same amount as the ANDRO and PROP groups). All drugs/solution and distilled water were administered intragastrically twice: 1 hour before and 24 hours after the surgery. As in real surgery, the abdominal cavity of all mice was irrigated with sterile saline after the excision of endometrial implants.

All mice were sacrificed by cervical dislocation 2 weeks after the primary excision surgery. The abdominal cavities were immediately opened up and all residual lesions left behind the primary surgery were excised and processed for quantification and immunohistochemistry analysis. For each mouse, the extent of endometriosis was evaluated by assessing the weight of all lesions. Before the excision surgery and before sacrifice, the bodyweight and hotplate latency were again evaluated for all mice.

Table 1. Information on Antibodies Used in This Study.[a]

Antibody Name	Vendor	Catalog Number	Concentration
α-SMA	Abcam	ab5694	1:100
E-cadherin	CST	#3195	1:400
p-p65	Abcam	ab86299	1:150
PR-B	CST	#3157S	1:200
VEGF	Abcam	ab52917	1:50
ADRB2	Abcam	ab61778	1:50

Abbreviations: ADRB2, adrenergic receptor β2; PR-B, progesterone receptor isoform B; α-SMA, α-smooth muscle actin; VEGF, vascular endothelial growth factor.

[a]All antibodies were purchased from Abcam (Cambridge, United Kingdom) or CST (Cell Signaling Technology, Danvers, Massachusetts).

Immunohistochemistry

Tissue samples were fixed with 10% formalin (wt/vol) and paraffin-embedded. Serial 4-μm sections were obtained from each block. All lesion samples were evaluated by hematoxylin and eosin (H&E) staining using an H&E staining kit (Sun Biotec, Shanghai, China). The typical endometriotic epithelium and stroma of the lesion were confirmed for all lesions by the H&E staining using the first resultant slide sectioned from the block. The subsequent slides stained for α-SMA (a marker for myofibroblasts), E-cadherin (for epithelial cells), phosphorylated NF-κB p65 subunit or p-p65 (the activated form of the p65 subunit), PR-B, VEGF (a marker for angiogenesis), and ADRB2.

Routine deparaffinization and rehydration procedures were performed. For antigen retrieval, the slides were heated at 98°C in a citrate buffer (pH 6.0) for a total of 30 minutes for staining for α-SMA, E-cadherin, p-p65, PR-B, VEGF, or in an EDTA buffer (pH 8.0, Shanghai Sun BioTech Company, Shanghai, China) for a total of 20 minutes for staining for ADRB2 and then cooled naturally to the room temperature. The primary antibodies against α-SMA, E-cadherin, p-p65, PR-B, VEGF, ADRB2 were diluted to 1:100, 1:400, 1:150, 1:200, 1:50, 1:50, respectively, and the sections were incubated with the primary antibody overnight at 4°C. After slides were rinsed, the HRP-labeled secondary antibody Detection Reagent (Sunpoly-HII, BioSun Technology Co, Ltd, Shanghai, China) was incubated at room temperature for 30 minutes. The bound antibody complexes were stained with diaminobenzidine for 3 to 5 minutes or until appropriate for microscopic examination and then counterstained with hematoxylin (30 seconds) and mounted. The names of primary antibodies, along with their vendor names and the concentrations used in this study, are listed in Table 1.

Images were obtained with the microscope (Olympus BX51; Olympus, Tokyo, Japan) fitted with a digital camera (Olympus DP70; Olympus). For all immunostaining markers, quantification was made through 3 to 5 randomly selected

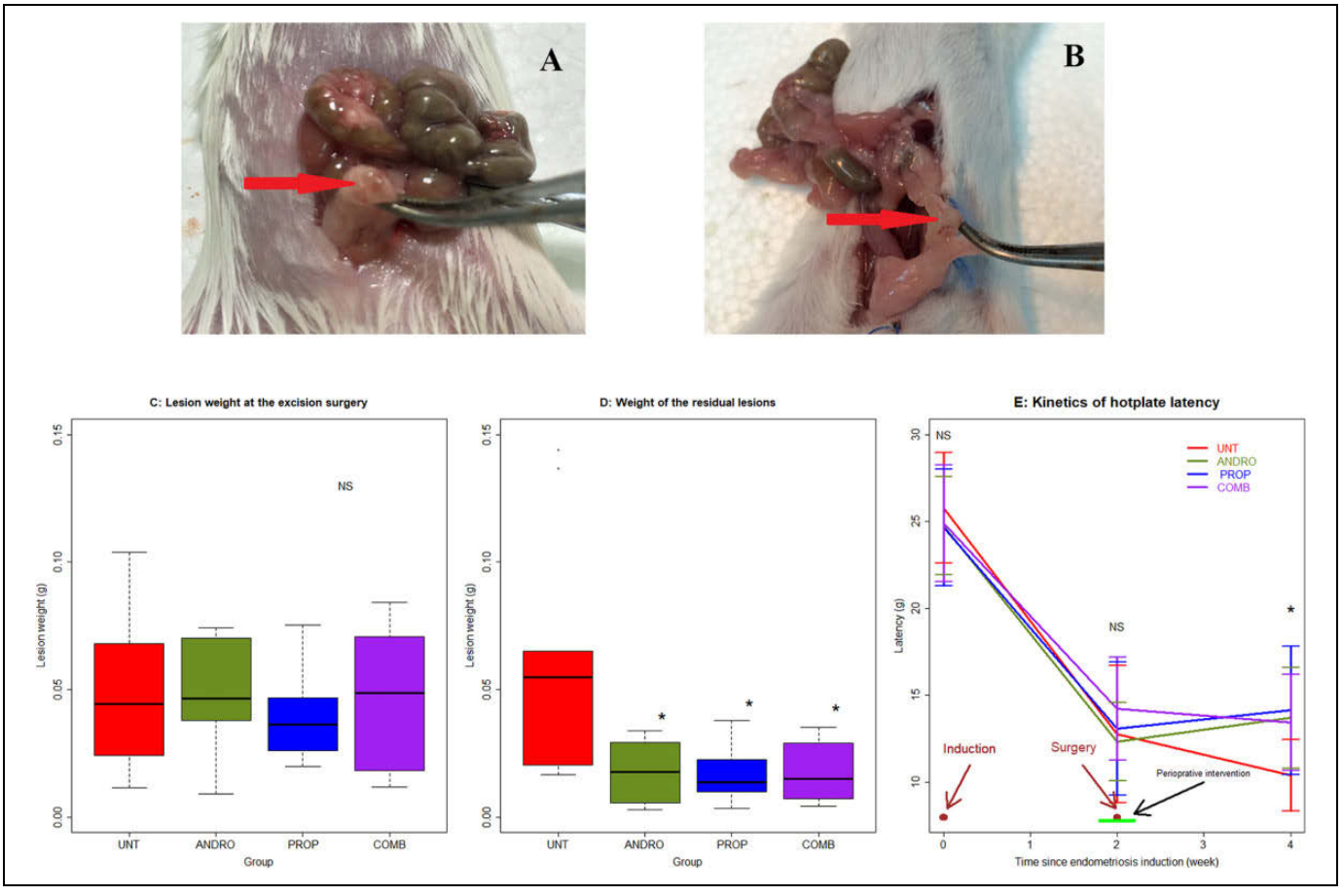

Figure 1. A, Endometriotic lesions seen at the primary excision surgery and (B) residual lesions seen 2 weeks after the excision surgery in Balb/c mice that underwent endometriosis induction via intraperitoneal injection of uterine fragments. The red arrows point to the endometriotic lesions. C, Box plot of weight of lesions excised in the primary surgery in different treatment groups. D, Box plot of weight of residual lesions excised at the end of the experiment in different treatment groups. E, The kinetic changes of hotplate latency in different groups of mice. ANDRO, mice treated with andrographolide; COMB, mice receiving combined treatment with andrographolide and propranolol; NS, not statistically significant among the 4 groups ($P > .05$); PROP, mice treated with propranolol; UNT, mice received distilled water (no treatment). *$P < .05$. In (D), the reference group is the UNT group.

images for each mouse at 400× magnification, which were taken to obtain a mean optional density value by Image Pro-Plus 6.0 (Media Cybernetics, Inc, Bethesda, Maryland), as reported previously.[25]

Tissues of human breast cancer, mouse liver, human ovarian cancer, and human brains were used as positive controls for α-SMA, E-cadherin, p-p65, PR-B, VEGF, ADRB2, respectively. For negative controls, mouse endometriotic lesion tissues were incubated with rabbit or mouse serum instead of primary antibodies (Supplementary Figure S1). To minimize potential bias, the person who evaluated the slides was blinded as to which group the slides belonged to.

Statistical Analysis

The comparison of distributions of continuous variables between or among 2 or more groups was made using the Wilcoxon and Kruskal test, respectively. Multiple linear regression was used to evaluate the effect of several factors on lesion weight or hotplate latency. *P* values of less than .05 were considered statistically significant. All computations were made with R 3.5.1.[39]

Results

Establishment of Endometriosis Recurrence Mouse Model

We first carried out a pilot experiment in an attempt to establish a recurrence model. Three Balb/c mice served as donors, and in the other 6, endometriosis was induced by IP injection. Two weeks after the induction (day 14), the recipient mice received a laparotomy to excise most endometriotic lesions and deliberately left not more than 2 small lesions behind, in order to mimic the situation when endometriotic lesions were incompletely removed, especially for small lesions. The induced endometriotic lesions appeared to be nodular or vesicular, mainly located in the peritoneum, mesentery, and omentum (Figure 1A). The total

number of lesions intentionally left intact (mimicking residual lesions) was no more than 2, and their maximum diameter was always less than 0.5 mm (and was difficult to remove by a scalpel). Two weeks after the surgical removal of lesions (day 28), all mice were sacrificed. Two mice died after the surgery, one died of intestinal injury about 1 hour after the surgery, the other died of postoperative hemorrhage 1 day after the surgery, leaving 4 (66.7%) mice at the end of the experiment. All the surviving mice had lesions in the abdominal cavity with a recurrence rate of 100% (Figure 1B).

In view of the low mortality and also the ease of induction, we used the IP induction in this study. This induction model has lower surgical stress and thus lower mortality than the surgical induction method (data not shown).

Perioperative Treatment With β-blocker and/or Andrographolide Slows Down the Regrowth of Residual Lesions

In this experiment, we started out with 40 recipient mice before the excision surgery. It can be seen from Figure 1C that there was no significant difference in weight of the lesions removed from the peritoneal cavity ($P = .96$; Figure 1C). Overall, more than 90% of the lesions were successfully removed by excision.

After the primary excision surgery, 6 mice died of excessive hemorrhage or intestinal injury during operation and postoperative hemorrhage, one each from the UNT and PROP groups, 2 each from the ANDRO and COMB groups, leaving 34 mice at the end of the experiment (n = 9 each in the UNT and PROP groups, and n = 8 each in the ANDRO and COMB groups). The survival rate was 85%, an increase from the 66.7% during the preliminary experiment. Since the deaths appeared to have occurred more or less evenly distributed among all groups, it is unlikely the death was due to any particular intervention measure. In particular, there was no difference in bodyweight and hotplate latency between the 6 dead mice and the remaining mice (all P values >.22), and since the average lesion weight in the former group was not significantly different from that of the latter (46.0 ± 49.1 mg vs 46.4 ± 25.6 mg; $P = .49$), we conclude that the deaths were not treatment related. Other than the deaths, the surviving mice appeared to be in good conditions. After sacrifice, all excised lesion tissues were carefully weighed and then processed for further analyses.

We next evaluated the lesion weight at the end of experiment and found that there was a significant difference among the 4 groups ($P = .041$; Figure 1D). Mice in all 3 groups that received perioperational intervention had significantly reduced lesion weight as compared with mice in the UNT group (all P values <.036; Figure 1D). A multiple linear regression incorporating the weight of the lesions excised at the primary surgery (which had minor, statistically not significant, variation across the 3 groups of mice) and methods of intervention as covariables indicated that the treatment with ANDRO and/or PROP significantly reduced the weight of the residual lesions (all P values <.0026, $R^2 = 0.37$), but the weight of the excised lesions at the primary surgery had no effect ($P = .10$). Interestingly, the combined use of ANDRO and PROP in fact slighted increased the lesion weight ($P = .020$) as evidenced by the positive sign of the regression coefficient. That is, the combined treatment did not yield a synergistic effect in reducing lesion weight at the second surgery. No significant difference in bodyweight among the 4 groups was found before the induction and at the primary and the second surgery (all P values >.56).

Consistent with the reduced weight of residual lesions in all groups of mice that received perioperative intervention, the hotplate latency in these groups of mice was all significantly longer than that of the UNT group (all P values <.027; Figure 1E), even though there was no significant difference before induction and before the primary excision surgery (both P values >.64). As reported previously, the latency was significantly reduced 2 weeks after the induction of endometriosis ($P = 1.2 \times 10^{-10}$; Figure 1E). A multiple linear regression incorporating the latency at the excision surgery and methods of intervention as covariables indicated that the treatment with ANDRO and/or PROP significantly increased the latency at the end of the experiment (all P values <.016, $R^2 = 0.26$), but the latency at the excision surgery had no effect ($P = .62$). Consistently, the combined use of ANDRO and PROP in fact slightly decreased the latency ($P = .046$) as evidenced by the negative sign of the regression coefficient. That is, the combined treatment did not yield a synergistic effect in increasing the hotplate latency at the end of the experiment.

Immunohistochemistry Analysis of Lesions

We next examined immunoreactivity to p-p65, PR-B, VEGF, ADRB2, E-cadherin, and α-SMA in endometriotic lesions retrieved at the end of the experiment. As shown in Figure 2, the p-p65 staining was seen primarily in glandular epithelial cells and was localized in both cell nucleus and cytoplasm. The PR-B showed a positive staining in the nuclei of epithelial cells in lesions. The VEGF immunoreactivity was seen mostly in the cytoplasm of glandular epithelial cells as well as of vascular endothelial cells. The ADRB2 immunoreactivity was seen mostly in glandular epithelial cells and was localized in the cytoplasm. E-cadherin staining was seen in the membrane of endometriotic epithelial cells. Besides the smooth muscle cells, α-SMA staining was also observed in the cytoplasm of both epithelial cells and stromal cells in lesions. Due to their locations and also due to our interest in the extent of EMT and FMT during the development of endometriosis, we evaluated the staining levels of p-p65, PR-B, VEGF, ADRB2, E-cadherin in the epithelial component, and of α-SMA in the stromal component of lesions.

The endometriotic staining levels of α-SMA, p-p65, and VEGF were significantly reduced in all treatment groups as compared with the UNT group (all P values <.0038; Figure 3). The staining levels of E-cadherin and PR-B were both elevated in all 3 treatment groups (all P values <.028; Figure 3). The staining levels of ADRB2 were reduced in all treatment groups,

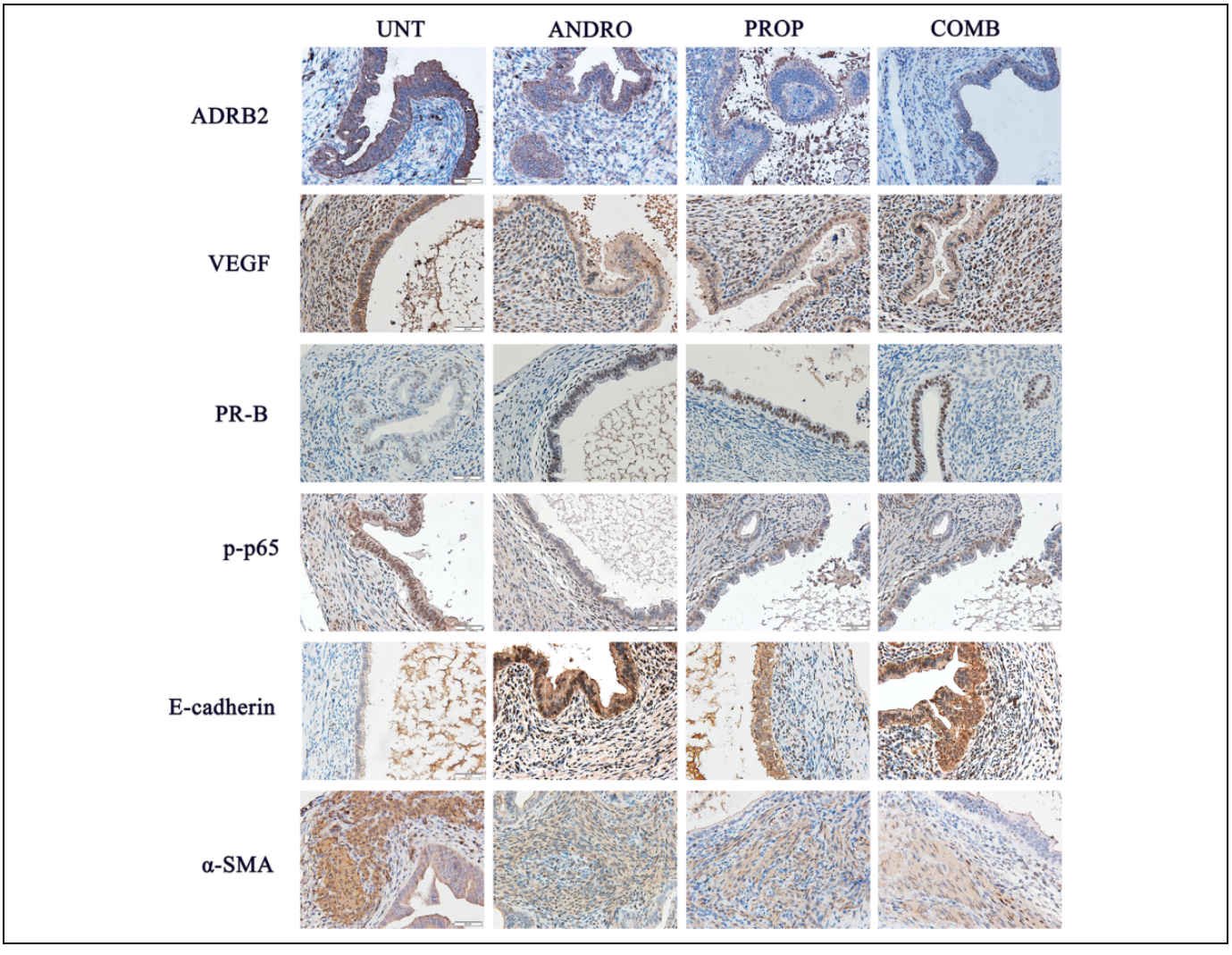

Figure 2. Immunohistochemical staining of ADRB2, VEGF, PR-B, p-p65, E-cadherin, and α-SMA in residual lesions harvested 2 weeks after the primary excision surgery. Different columns represent tissue samples taken from different groups, that is, UNT, ANDRO, PROP, and COMB groups, while different rows indicate different immunohistochemistry markers as indicated. All magnifications were ×400. The scale bar is 50 μm. ADRB2 indicates adrenergic receptor β2; PR-B, progesterone receptor isoform B; α-SMA, α-smooth muscle actin; VEGF, vascular endothelial growth factor.

especially in PROP and COMB groups, as compared with the UNT groups (all P values <.036; Figure 3).

Lesional staining levels of ADRB2 correlated positively with the lesion weight ($r = 0.51$, $P = .0019$; Figure 4). In fact, they correlated positively with the staining levels of VEGF, p-p65, and α-SMA (all r's ≥ 0.51, all P values <.0019; Figure 4). The lesion weight was found to be positively correlated with the endometriotic staining levels of VEGF ($r = 0.70$, $P = 3.4 \times 10^{-6}$), p-p65 ($r = 0.74$, $P = 4.7 \times 10^{-7}$), and α-SMA ($r = 0.61$, $P = .0001$).

Discussion

In this study, we found that perioperative use of Andro, an NF-κB inhibitor, and/or Prop, a non-selective β-blocker, significantly decelerated the growth of residual lesions that were intentionally left out during the excision surgery. The perioperative intervention also significantly attenuated the generalized hyperalgesia resulting from the presence of residual lesions. In addition, the intervention appeared to inhibit the activation of the ADRB2 signaling, resulting in reduced angiogenesis, EMT, FMT, as well as NF-κB suppression and PR-B induction. These data strongly suggest that perioperative use of β-blockers and/or NF-κB inhibitors may reduce the risk of recurrence in endometriosis.

Although surgeons may achieve negative margins when excising all visible, typically under laparoscopy, lesions and patients can go to surgeons with extensive training and skills, there is still a considerable risk of leaving MRLs behind. Given seemingly ubiquitous nature of microscopic, invisible, or occult endometriosis, that is, endometriotic foci beneath visually normal-appearing peritoneum,[40-45] removing all

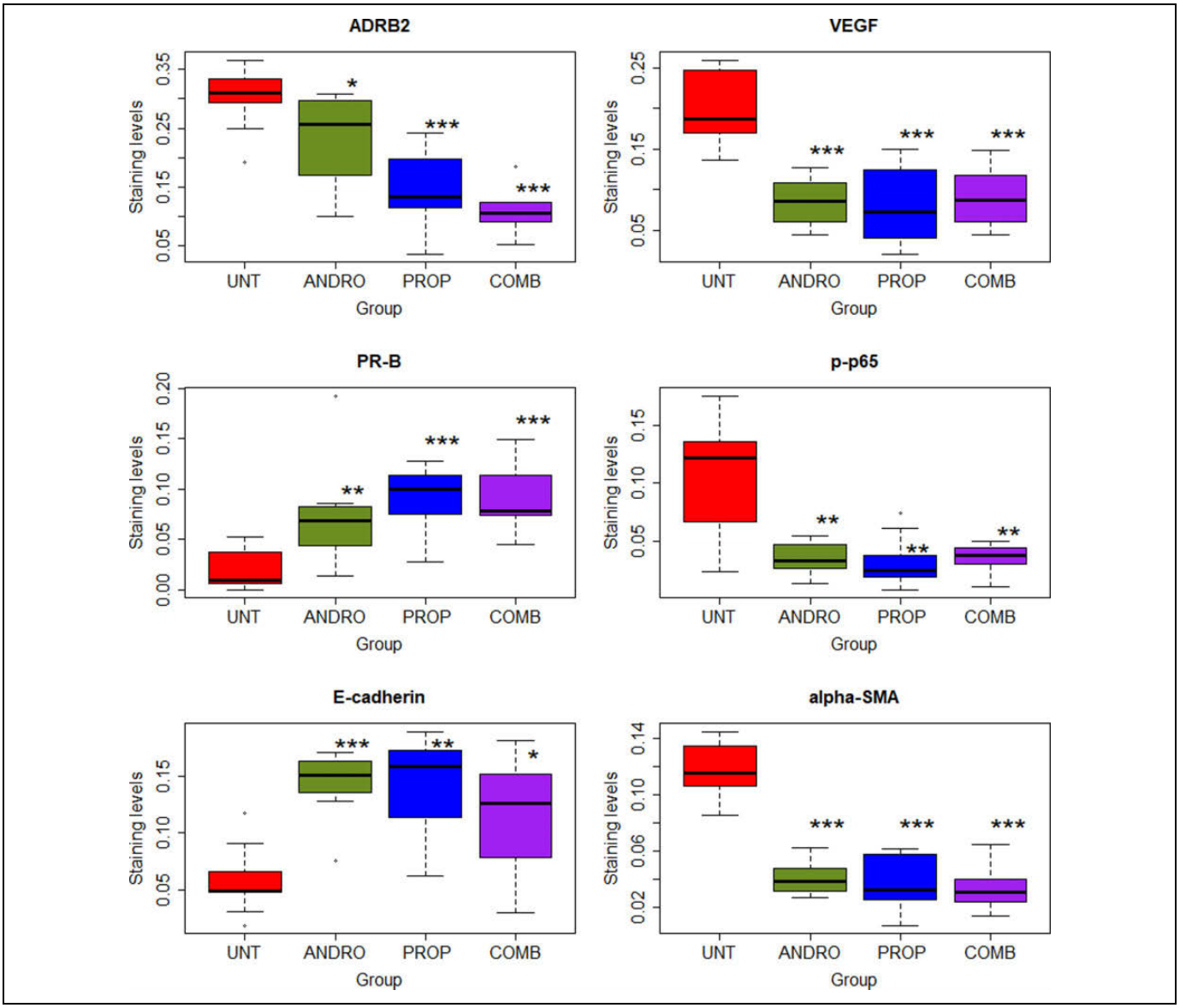

Figure 3. Boxplots of immunostaining levels of ADRB2, VEGF, PR-B, p-p65, E-cadherin, and α-SMA in residual lesions harvested 2 weeks after the primary excision surgery. Symbols for statistical significance level: NS, not statistically significant ($P > .05$); *$P < .05$; **$P < .01$; ***$P < .001$. In all plots, the reference group is the UNT group. ADRB2 indicates adrenergic receptor β2; PR-B, progesterone receptor isoform B; α-SMA, α-smooth muscle actin; VEGF, vascular endothelial growth factor.

lesions would be a great challenge. This seems to be tacitly acknowledged by the frequent use of postoperative medication, such as oral contraceptives or gonadotropin-releasing hormone agonists,[46] to suppress potential residual disease. In addition, MRLs may be present proximal to the visible foci, in the lymphatic system (within positive lymph nodes)[47,48] or vascular system,[16] or in tissues/organs distal to the visible foci, in the form of a single cell cluster or as micrometastases similar to tumors. Therefore, MRLs are very likely responsible for the recurrence of endometriosis.

There is no doubt that surgery can remove the majority of endometriotic lesions and is known to be efficacious for the management of symptomatic endometriosis.[2] However, surgery also induces stress as defined by the hormonal and metabolic changes, including the activation of the hypothalamic–pituitary–adrenal (HPA) axis, and immunological and hematological changes.[49] The activation of the HPA due to chronic stress has been shown to contribute to the progression of cancer[50] and to promote the development of endometriosis in rodents.[34,51] Extensive basic and clinical research have shown that tissue damage and the manipulations and excision/ablation of lesions and their vasculature during operation suppress cell-mediated immunity, leading to immune escape of micrometastatic disease,[52] and may conceivably increase the shedding of endometriotic cells into the blood and lymphatic circulations, to increase local and systemic levels of growth

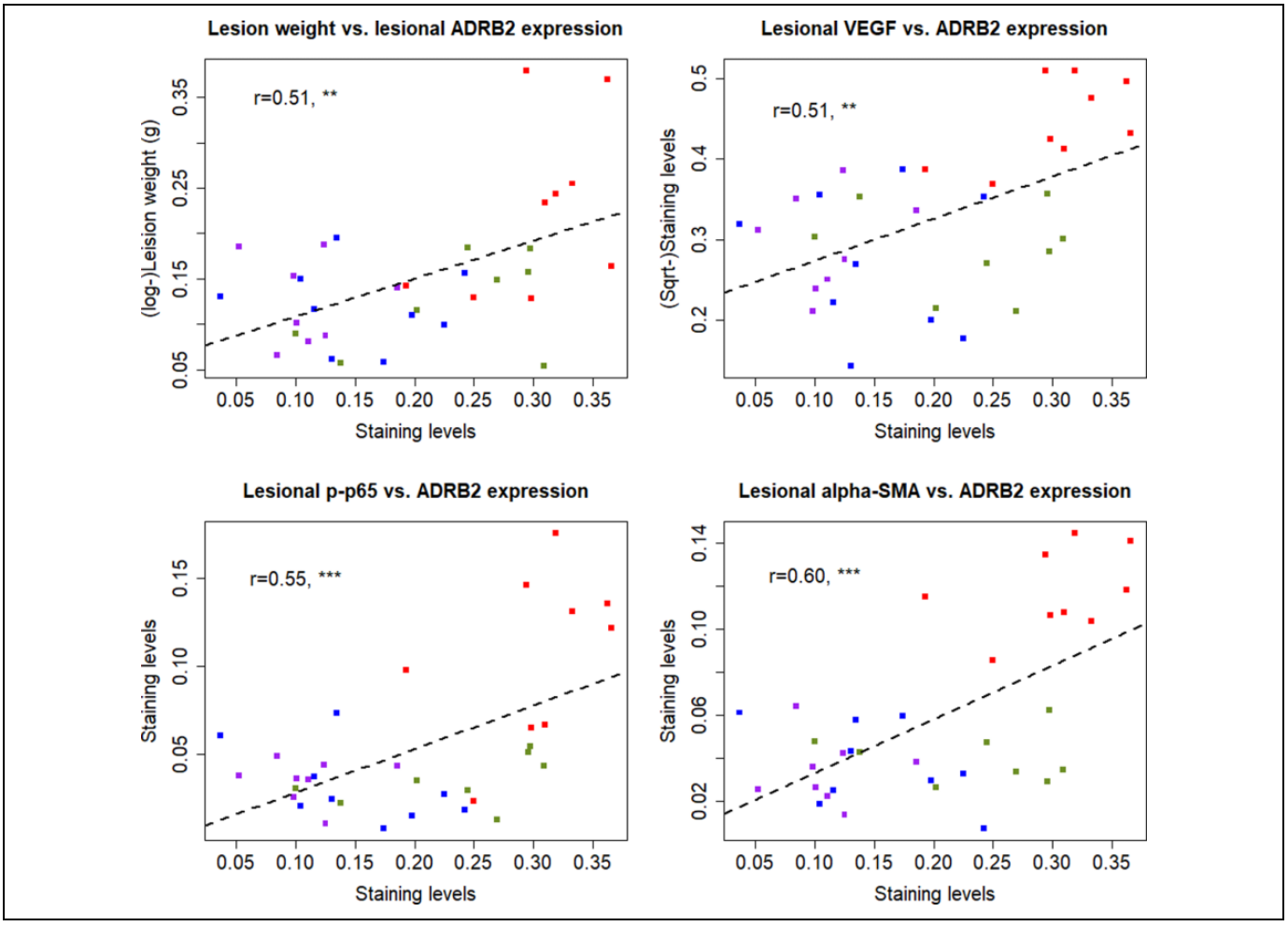

Figure 4. Scatter plots showing the relationship between the endometriotic staining levels of ADRB2 and lesion weight, endometriotic VEGF, p-p65, and α-SMA expression. ADRB2 indicates adrenergic receptor β2; α-SMA, α-smooth muscle actin; VEGF, vascular endothelial growth factor.

factors, and to decrease systemic levels of antiangiogenic factors.[53-56] In addition, nervousness, fear, and anxiety prior to the operation, blood transfusion, anesthetics, and hypothermia in the intraoperative period, and postoperative pain could also generate a microenvironment that is conducive to proliferation, angiogenesis, and invasion, thus increasing the recurrence risk in cancer[53-55] and conceivably in endometriosis as well.

Catecholamines, mostly in the form of epinephrine and norepinephrine, are copiously released perioperatively among patients who are about to undergo a major surgery because of uneasiness and anxiety and fear of the disease and the impending surgery.[56] Tissue injury resulting from surgery directly prompts the local release of prostaglandins,[57] and catecholamine secretion is a prominent neuroendocrine response to tissue injury and subsequent inflammation and pain.[58] Endometriotic lesions also produce abundant prostaglandins, with prostaglandin E2 (PGE2) playing a prominent role in endometriosis.[59,60] Since PGE2 is essentially immunosuppressive through selectively suppression of effector functions of macrophages and neutrophils and type 1 immunity (pro-inflammation),[61] the increased release of PGE2 would foster a privileged microenvironment that affords protection of residual lesions.

Considering the well-documented diagnostic delay ranging from 7 to 12 years[62-64] and presumably months, if not years, from the genesis of endometriotic lesions to the onset of symptoms, the perioperative period, defined to be days before to days after the surgical removal of lesions, is indeed a very narrow time window. Despite such a short time period, several studies have reported that this period is critical in determining the risk of postoperative metastatic diseases in cancer in animal studies.[27,65,66] Two recently published clinical trials demonstrate that perioperative inhibition of COX-2 and β-adrenergic signaling provides a safe and effective strategy for inhibiting multiple cellular and molecular pathways related to metastasis and disease recurrence in breast cancer.[28,67]

In this study, we used Andro, instead of a specific COX-2 inhibitor, simply because this drug, approved for over-the-counter medication in China for minor upper respiratory tract infection, has several desirable features. First and foremost, it

has an excellent safety profile, which is very important for a benign disease and also for patients who are about to undergo or just completed a major surgery. Andro is known to be anti-inflammatory[68] and to interfere with NF-κB binding to DNA[69] and in fact suppress NF-κB activation.[70] It is also known to be antiplatelet,[71,72] which is desirable due to the involvement of platelets in endometriosis.[33,73] Not surprisingly, it is known to exert a strong immunomodulatory effects (reviewed by Varma et al[74]) and is reported to inhibit pro-inflammatory and angiogenic mediators such as COX-2[69] and tissue factor,[75] both of which are reportedly involved in endometriosis.[76,77] Andro has also been shown to be antinociceptive in animals.[78,79] We targeted NF-κB in particular not only because NF-κB is involved in endometriosis[29,30] and upregulates COX-2 but also it is a putative biomarker for recurrence.[14] In addition, the antiplatelet capability of Andro is desirable since activated platelets may offer protection to MRLs from NK cells.[80]

Prop, a WHO-listed essential medication, is a nonselective β-blocker used to treat hypertension, tachycardia, and anxiety is inexpensive and also has an excellent safety profile with a few contraindications. Prop and metformin combination is reported to prevent cancer progression and metastasis in different breast cancer models.[81] Nonselective β-blockers have been reported to improve survival in breast cancer[82] and epithelial ovarian cancer.[83]

Our results of reduced lesion weight and VEGF immunoreactivity concomitant in lesions with reduced ADRB2 staining are consistent with previous finding that activation of the ADRB2 and cAMP responsive element-binding protein (CREB) signaling pathway yields increased angiogenesis and cellular proliferation in ectopic endometrium in mouse with induced endometriosis.[34] Similarly, the elevated PR-B staining and reduced p-p65 staining concomitant with reduced lesion weight as a result of perioperative use of the β-blocker and NF-κB inhibitor are consistent with the report that both PR-B and p-p65 are putative biomarkers for recurrence in endometriosis.[14] The elevated E-cadherin staining in endometriotic epithelial component and reduced α-SMA staining in the stromal component resulting from the perioperative intervention suggest that the intervention decelerates the development of residual lesions through hampering EMT and FMT, the 2 processes inherent in endometriosis progression toward fibrosis.[33,84]

This study has several strengths. First, this study focused on an area that is important yet conspicuously underresearched. Second, we established the first mouse model for recurrence due to incomplete removal of endometriotic lesions or MRLs.

This study also has several limitations. First, the mouse model that we established still has ample rooms for improvement. Most deaths seemed to be caused by postoperative hemorrhage or infection. We used a scalpel, scissors, and a tweezer to excise lesions and used ligation and pressing with gauze to stop intraoperative bleeding. Although the results were satisfactory, the use of mono- or bipolar coagulation instruments should be able to substantially reduce bleeding and thus the risk of postoperative hemorrhage. In addition, through more training, we may be able to further shorten the operating time and thus the extent of trauma, leading to reduced mortality in this mouse model. Second, the mouse model may not fully recapitulate its human counterpart, and vast differences exist between Homo sapiens and rodents. Hence, whether findings from this study can be automatically extrapolated to human remains to be investigated. Third, the excision surgery was performed 2 weeks after induction, which may not be long enough for fibrosis to be fully developed as commonly seen in humans.[85] However, given the significant reduction in hotplate latency at the time of surgery (Figure 2), it is safe to speculate that the pain symptom has already been manifested at the time of surgery. It is unclear as whether the presence of fibrosis may render surgical excision more difficult in this setting, which is currently our research focus. Third, the natural history of endometriotic lesions seems to be quite clear and ends with fibrosis through EMT, FMT, and smooth muscle metaplasia[86]; we did not evaluate how the extent of fibrosis in ectopic endometrium would make any difference in the context of perioperative intervention. Future studies are needed to clarify this issue. Fourth, we did not evaluate any changes in the eutopic endometrium as a result of perioperative intervention, which could have been also interesting. Lee et al have shown that previously normal endometrium in mice with induced endometriosis display aberrant gene expression and methylation.[87] Naqvi et al later showed that this change in eutopic endometrium seems to depend on the physical distance to the ectopic endometrium.[88] Again, future studies are warranted. Lastly, while this study investigated a few aspects of endometriosis development such as proliferation, angiogenesis, and adrenergic signaling through immunohistochemistry analysis, the findings are far from conclusive due to the study design and the methods we used. Nonetheless, the results presented here open up a new vista to see the possible potential of perioperative intervention to reduce the risk of recurrence and, as such, should be able to stimulate more interest in this aspect for control of recurrence and perhaps devising appropriate interventional procedures accordingly.

In summary, the perioperative use of β-blockers and/or NF-κB inhibitors significantly hinders the development of endometriosis resulting from MRLs left behind in the primary surgery, suggesting that the recurrence risk due to MRLs may be safely and effectively mitigated through simple pharmacological intervention perioperatively.

Authors' Note

Q.L. and H.Z. contributed equally to this work.

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Supplemental Material

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