Scutellarin Suppresses Platelet Aggregation and Stalls Lesional Progression in Mouse With Induced Endometriosis

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Abstract
Platelets play an important role in the development of endometriosis. Scutellarin is a flavonoid isolated from a medicinal herb traditionally used as a potent antiplatelet agent. In this study, we sought to evaluate its potential therapeutic effect, if any, in mice with induced endometriosis. Endometriosis was induced in 27 female Balb/c mice by intraperitoneal injection of uterine fragments. Two weeks after the induction, the 27 mice were randomly divided in equal sizes into 3 groups: untreated, which received only vehicle, and low-dose and high-dose groups, which received low- and high dose of scutellarin treatment. Hotplate test was administrated to all mice before endometriosis induction, and before and after the scutellarin treatment. Two weeks after the treatment, a blood sample was drawn before sacrifice and all lesions were harvested. The peripheral platelet activation rate and total lesion weight were assessed, and immunohistochemistry and histochemistry analyses were performed to evaluate the extent of proliferation, angiogenesis, fibroblast-to-myofibroblast transdifferentiation (FMT), and fibrosis in lesions. Compared with untreated mice, mice in both low-dose and high-dose groups had significantly reduced lesion weight and improved hyperalgesia. Scutellarin also reduced the peripheral-activated platelets rate and resulted in significantly reduced platelet aggregation, cellular proliferation, angiogenesis, the extent of FMT, and the extent of fibrosis in lesions. Thus, we conclude that scutellarin is efficacious in treating endometriosis in vivo by suppressing platelet aggregation, inhibiting proliferation, angiogenesis, and fibrogenesis, resulting in reduced lesion size and improved pain behavior. As such, scutellarin may be a potentially promising therapeutics for the treatment of endometriosis.

Keywords
endometriosis, fibrogenesis, hyperalgesia, mouse, platelet, scutellarin

Introduction
Endometriosis, defined as the deposition of endometrial-like tissues outside the uterine cavity, is a common gynecologic disease affecting 6% to 10% of reproductive-aged women.¹ It is the major contributing cause of dysmenorrhea, chronic pelvic pain, and infertility, negatively impacting on the quality of life of affected patients. Due mainly to the lack of understanding of its pathogenesis and pathophysiology, its clinical management is challenging.¹ While surgery is proven efficacious in relieving endometriosis-associated pain,² the high recurrence risk³,⁴ and the increased risk of premature ovarian failure due to repeated surgery render medical treatment a viable option.⁵,⁷ The current medical treatment relies on inhibition of ovulation and reduction of estradiol levels through hormonal manipulation, but their efficacy is limited by short duration.⁸ The development of nonhormonal drugs has been painfully slow.⁹,¹⁰ One defining feature of ectopic endometrium is cyclic bleeding.¹¹ Accumulating data have shown, indeed, that endometriotic lesions are essentially wounds that undergo repeated tissue injury and repair.¹²-¹⁴ As such, platelets are found to be aggregated in endometriotic lesions, which play a critical role in the development and progression of endometriosis.¹³ Activated platelets upregulate vascular endothelial growth factor (VEGF) and matrix metallopeptidase 9 and induce angiogenesis.¹³ Through the release of transforming growth factor β1 (TGF-β1) and the induction of TGF-β/Smad signaling pathway, activated platelets also drive epithelial—mesenchymal transition (EMT), fibroblast-to-myofibroblast transdifferentiation (FMT), and smooth muscle metaplasia

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(SMM) in endometriotic lesions, resulting ultimately in fibrosis.\textsuperscript{15,16} Activated platelets are also shown to be responsible for increased estrogen receptor β expression\textsuperscript{17} and reduced natural killer cell cytotoxicity in endometriosis.\textsuperscript{18} Consistent with the important roles of platelets in lesional development, women with endometriosis have been shown to be in a hypercoagulable state.\textsuperscript{19,20} Consequently, platelet depletion and antiplatelet treatment effectively suppress lesion growth in mice with induced endometriosis.\textsuperscript{13,21,22}

The drug research and development (R&D) has always been a winding, arduous, tortuous, costly, and often precarious endeavor. From discovery to the successful regulatory approval for marketing, there is an astonishingly high attrition rate: Over 99% of drugs do not make to the final end of the R&D pipeline.\textsuperscript{23} Many compounds failed simply because of unacceptable safety profiles or inferior efficacy or both. Given the difficulty in drug R&D, one seemingly shortcut would be the screening of compounds derived from herbal medicine, which are often known to have a good safety profile.

Scutellarin (4’,5,6-trihydroxyflavone-7-O-glucuronide) is a flavonoid isolated from the plant \textit{Erigeron brevicaespis} (Vant.) Hand.-Mazz., a Chinese herbal medicine used over a thousand years.\textsuperscript{24} It is the major active component of breviscapine, which is the total flavonoid extract of \textit{E. brevicaespis} containing ≥90\% scutellarin and ≤10\% apigenin-7-O-glucuronide in content. Breviscapine is a prescription drug in China for the treatment of cardiovascular and cerebrovascular diseases with an excellent safety profile.\textsuperscript{25} Scutellarin has been demonstrated to have multiple pharmacological effects, having antioxidant, antiplatelet, and anti-inflammatory properties.\textsuperscript{26-28} Scutellarin has also been shown to exhibit anticancer activities by suppressing proliferation, invasion, metastasis, and angiogenesis on various types of cancer.\textsuperscript{29-32} It was also reported that scutellarin could enhance cisplatin-induced apoptosis and autophagy to overcome cisplatin resistance in non-small cell lung cancer via extracellular regulated protein kinases (ERK)/p53 and c-met/AKT signaling pathways.\textsuperscript{33} Moreover, scutellarin has been shown to be antifibrotic.\textsuperscript{34} Since coagulation, ERK/p53, c-met/AKT, and fibrogenesis have all been reported to be involved in endometriosis,\textsuperscript{14,35-37} and since scutellarin has an excellent safety profile, naturally one may wonder whether scutellarin could have any therapeutic potential. Unfortunately, to our best knowledge, there has been no report on its use in endometriosis.

In this study, we sought to investigate the therapeutic potential, if any, of scutellarin in mice with induced endometriosis by examining the lesion growth, peripheral platelet activation, and the expression of molecules involved in the development of endometriosis.

Materials and Methods

\textbf{Mice}

Forty-one virgin female Balb/c mice, 7 weeks old and about 18 to 20 g in weight, were purchased from Shanghai BiKai Laboratory Animal Center (Shanghai, China) and used for this study. They were housed individually in cages, maintained under controlled conditions with a light/dark cycle of 12/12-hour, and had access to chow and water ad libitum. All experiments in this study were performed under the guidelines of the National Research Council’s \textit{Guide for the Care and Use of Laboratory Animals},\textsuperscript{38} and approved by the institutional experimental animals review board of Shanghai OB/GYN Hospital, Fudan University (on file). Among the 41 mice, 14 were randomly selected as donors, while the rest were designated as recipient mice.

\textbf{Induction of Endometriosis and the Experiment Protocol}

We used an established mouse model of endometriosis by intraperitoneal (ip) injection of endometrial fragments\textsuperscript{39} as used in our previous studies.\textsuperscript{13,40} Briefly, donor mice were initially injected intramuscular with estradiol benzoate (3 \textmu g/mouse, Xinyi Chemistry, Shanghai, China). Seven days later, mice were sacrificed and their uteri were harvested, seeded in a Petri dish containing sterile saline, and split longitudinally with a pair of scissors. To minimize any potential bias, 3 uterine horns, one from one mouse and the other pair from another mouse, were identically processed, minced together, mixed well, then divide them into 3 roughly equal parts, with each part injected into one each recipient mouse from the 3 groups: UT (for untreated), LS (for low-dose scutellarin), and HS (for high-dose scutellarin). The baseline body weight was measured and hotplate test was administrated before induction to all mice.

Two weeks after the induction of endometriosis, hotplate test and body weight measurement were again administrated to all mice. Before induction, the 27 recipient mice were randomly divided into 3 equal-sized groups: Mice in group HS received ip injections of scutellarin (Sigma, Sigma-Aldrich Co, St. Louis, Missouri) 15 mg/kg/mouse in 300 \textmu L sterile saline, group LS received ip injections of scutellarin (7.5 mg/kg/mouse in 300 \textmu L sterile saline), and group UT received ip injections of just 300 \textmu L sterile saline, the solvent for scutellarin solvent. All ip injections were repeated every 2 days for 2 weeks.

The choice of scutellarin doses was based on the conversion of breviscapine (Shineway Pharmaceutical Company, Shijiazhuang, Hebei, China) for human usage to mouse, assuming that 90\% of breviscapine is scutellarin. Given the usual dosage of 20 mg/60 kg/d or 0.333 mg/kg/d of breviscapine or 0.300 mg/kg/d of scutellarin for intravenous (iv) injection (Shineway Pharmaceutical Company), the body surface area–based conversion from human to mouse\textsuperscript{41} would be 0.300 \times 12.3 = 3.68 mg/kg/d scutellarin by iv injection or about 3.68 \times 1.1 = 4.05 to 3.68 \times 1.25 = 4.6 mg/kg/d for ip injection. As we used injection every 2 days, the dosage was doubled to about 8.10 to 9.2 mg/kg for one ip injection. Of course, this was a very crude estimation. Given that the dosage used orally for mouse ranged from 20 mg/kg\textsuperscript{42} to as high as 60 mg/kg\textsuperscript{43} which amounts to approximately 5 to 20 mg/kg by iv injection, we decided to have the low- and high-dose set to be 7.5 and 15 mg/kg, respectively.
Two weeks after the treatment started, the final hotplate test and body weight measurement were administrated to all mice, and before sacrifice by cervical dislocation a blood sample (about 0.8-1 mL) was drawn through right orbit and mixed with 3.2% citric acid for anticoagulation purpose. The abdominal cavity was immediately opened up and examined very carefully, and all visible lesions were excised and processed for disease assessment or immunohistochemistry evaluation. The extent of endometriosis was evaluated by assessing the total weight of all excised lesions from each mouse.

**Hotplate Test**

The hotplate test was employed to assess the extent of endometriosis-associated hyperalgesia. The hotplate latency evaluated with a commercially available Hot Plate Analgesia Meter (Model BME-480, Institute of Biomedical Engineering, Chinese Academy of Medical Sciences, Tianjin, China) consisting of a metal plate of 25 cm by 25 cm in size, which can be heated to a constant temperature of 55.0°C ± 0.18°C, on which a plastic cylinder (20 cm in diameter, 18 cm in height) was placed. Mice were brought to the testing room and allowed to acclimatize for 10 minutes before the test began. The latency to respond to thermal stimulus, defined as the time (in second) elapsed from the moment when the mouse was inserted inside the cylinder to the time when it licked or flicked its hind paws, or jolted or jumped off the hot plate. Each animal was tested only once in one session. The latency was calculated as the mean of 2 readings recorded at intervals of 24 hours.

**Assessment of Platelet Activation Rate by Flow Cytometry**

The platelet activation rate was evaluated by flow cytometry as previously described. Briefly, the blood samples were centrifuged at room temperature immediately after collection to avoid activation of platelets as much as possible. After platelets were isolated, they were incubated at room temperature with allophycocyanin-conjugated anti-mouse CD61 antibody (eBioscience, San Diego, California) labeling total mouse platelets and fluorescein isothiocyanate-conjugated anti-mouse CD62p (P-selectin) antibody (eBioscience) labeling activated mouse platelets and kept from light for 30 minutes. Platelets were washed by phosphate-buffered saline and analyzed by flow-cytometry cell sorting (BD FACS Calibur, San Jose, California).

**Histologic Analysis and Masson Trichrome Staining**

All lesion samples were fixed for 24 hours at room temperature with 10% formalin neutral buffer solution. After fixation, the tissues were placed in 70% ethanol overnight at 4°C, then embedded in paraffin and sectioned in 4-mm thickness. Each tissue sample was evaluated by hematoxylin and eosin (H&E) staining using an H&E staining kit (SunBiotec, Shanghai, China) according to the manufacturer’s instructions. To stain cell nuclei, sections were dipped into hematoxylin for 10 minutes, rinsed by tap water, then dipped into 0.1% HCl thrice, washed by tap water thrice, dipped into 0.1% NH₄OH thrice, and washed by tap water thrice. To stain cytoplasm, sections were dipped into eosin for 3 minutes. Slides were dehydrated, mounted, and examined under an Olympus microscope (Olympus, Tokyo, Japan) at ×200 magnification to confirm the establishment of the endometriotic lesion by typical epithelial and stromal components.

Masson trichrome staining was used to detect the collagen fibers in tissue samples. Tissue sections were deparaffinized in xylene and rehydrated in a graded alcohol series and then were immersed in Bouin solution at 37°C for 2 hours. Bouin solution was made with 75 mL of saturated picric acid, 25 mL of 10% formalin (w/v) solution, and 5 mL of acetic acid. The tissue sections were stained using Masson trichrome staining kit (Baso, Wuhan, China) following the manufacturer’s instructions. Slides were then mounted and evaluated under an Olympus microscope (Olympus) at ×200 magnification, capturing 4 to 5 different fields of sections. The areas of the collagen fiber layer stained in blue in proportion to the entire field of the ectopic implants were calculated by the Image Pro-Plus 6.0 (Media Cybernetics, Inc). Masson staining parameters assessed in the area detected included (1) integrated optical density (IOD), (2) total stained area (S), and (3) mean optical density (MOD), which was defined as MOD = IOD/S and used as the extent of lesion fibrosis.

**Immunohistochemistry**

Serial 4-μm sections from the mouse experimental ectopic lesions (see below) were obtained from each paraffin-embedded tissue block, with the first resultant slide being stained for H&E to confirm pathologic diagnosis, and the subsequent slides stained for CD41 (a marker for platelets), proliferating cell nuclear antigen (PCNA, a marker for cellular proliferation), VEGF, CD31 (for counting microvessel density or MVD), collagen I, α-smooth muscle actin (α-SMA), and lysyl oxidase (LOX). Routine deparaffinization and rehydration procedures were performed following published protocols.

For antigen retrieval, the slides were heated at 98°C in the ethylenediaminetetraacetic acid buffer (pH 8.0) or the citric acid solution (pH 6.0; according to the primary antibody) for a total of 30 minutes and cooled naturally at room temperature. The rabbit anti-mouse CD41 (1:200, Abcam, Cambridge, Massachusetts), rabbit polyclonal antibodies against PCNA (1:100, Thermo Littleton, Waltham, Massachusetts), VEGF (1:50, Santa Cruz, Dallas, Texas), rabbit anti-mouse CD31 (1:50, Abcam), rabbit anti-mouse α-SMA (1:100, Abcam), rabbit anti-mouse collagen I (1:100, Abcam), and rabbit anti-mouse LOX (1:100, LifeSpan BioScience, Seattle, Washington) were used as primary antibodies.

The slides were incubated with the primary antibodies overnight at 4°C. After the slides were rinsed, the horseradish peroxidase-labeled secondary anti-rabbit or anti-mouse antibody detection reagent (Shanghai SunBiotec Company) was incubated at room temperature for 30 minutes. The bound antibody complexes were stained for 1 minute or until appropriate
for microscopic examination with diaminobenzidine and then counterstained with hematoxylin (30 seconds) and mounted. Human invasive breast cancer or mice spleen tissue samples were used as positive controls. For negative controls, the endometriotic tissue samples from mouse were incubated with rabbit serum instead of the primary antibody (Supplementary Information).

Immunostaining results for CD41, PCNA, VEGF, CD31, collagen I, α-SMA, and LOX were evaluated using a semiquantitative scoring system. Briefly, the number and intensity of positive cells were counted by Image Pro-Plus 6.0 (Media Cybernetics) without prior knowledge of any information regarding which group the mouse was in. A series of 5 random images on several sections were taken for each immunostained parameter to obtain a mean value. Staining was defined via color intensity, and a color mask was made. The mask was then applied equally to all images, and measurement readings were obtained. Immunohistochemical parameters assessed in the area detected included (1) integrated optical density (IOD), (2) total stained area (S), and (3) mean optical density (MOD), which was defined as MOD = IOD/S, equivalent to the intensity of stain in all positive cells. The numbers of CD31-labeled MVD were counted under ×400 microscopic magnification.

Statistical Analysis

The comparison of distributions of continuous variables between or among 2 or more groups was made using the Wilcoxon and Kruskal tests, respectively, and the paired Wilcoxon test was used when the before-after comparison was made for the same group of participants. A Pearson or Spearman rank correlation coefficient was used when evaluating correlations between 2 variables when both variables were continuous or when at least one variable was ordinal. Multiple linear regression analysis was used to identify which factor(s) were associated with the lesion weight or immunoreactivity measure (all squared-root or log-transformed to improve normality, where appropriate).

The P values of less than .05 were considered statistically significant. All computations were made with R 3.5.1 (www.r-project.org).

Results

No mouse died during the entire experimental period. Scutellarin appeared to be well tolerated in treated mice. As previously reported, endometriosis was successfully induced in all mice and the induced endometriotic lesions were histologically confirmed.

Scutellarin Treatment Reduces Lesion Weight and Improves Hyperalgesia

The body weight of all groups of mice increased gradually after induction of endometriosis induction (P = .0002; Figure 1A), but no significant difference in body weight among the 3 groups was found before and 2 weeks after the induction, and at the end of the experiment (Ps > .72; Figure 1A).

Mice that received LS and HS treatment had significantly lower lesion weight than the UT mice (P = .0019 and P = .017, respectively; Figure 1B). The average weight in LS and HS groups of mice was reduced by 67.0% and 42.2%, respectively, as compared to that of the UT mice (Figure 1B), suggesting that the scutellarin treatment suppresses lesion growth. While LS appeared to resulted in more suppressive effect than that the HS, the difference in lesion weight between the 2 groups was not statistically significant (P = .14; Figure 1B).

As expected, there was no difference in hotplate latency prior to the induction of endometriosis as well as prior to the scutellarin treatment (both P values > .75; Figure 1C). However, there was a significant reduction in latency 2 weeks after the endometriosis induction (P = 5.9 × 10⁻⁶; Figure 1C), consistent with what we reported previously.22,46 Two weeks after treatment, the difference in hotplate latency among the 3 groups was highly significant (P = .0026; Figure 1C). In fact, mice treated with either LS or HS had significant improvement in hotplate latency (P = .004 and P = .027, respectively; Figure 1C). In contrast, UT mice had worsened latency (P = .008; Figure 1C). No significant difference in latency between the LS and HS groups (P = .23; Figure 1C). A multiple linear regression analysis incorporating the latency before treatment, dose and body weight indicated that before-treatment latency and whether or not treated with scutellarin were significantly and positively associated with the latency after treatment (P = 9.0 × 10⁻⁶ and P = 7.4 × 10⁻⁸, respectively; R² = 0.79).

Scutellarin Suppresses Platelet Activation Rate in the Peripheral Blood

We also evaluated the platelet activation rate in the peripheral blood from all groups of mice. We found that the platelet activation rate in both the LS and HS groups was significantly lower than that of the UT group (both P values < .0037; Figure 1D). The rate in the HS group was marginally significantly lower than that of the LS group (P = .072; Figure 1D). A multiple linear regression analysis incorporating the lesion weight and dose indicated that the dose was significantly and negatively associated with the activation rate (P = 3.9 × 10⁻⁵; R² = 0.56). These data indicate that scutellarin treatment suppresses platelet activation dose dependently.

Scutellarin Suppresses Platelet Aggregation, Angiogenesis, Proliferation, and Fibrogenesis in Lesions

To gain insight into the possible mechanisms underlying the scutellarin suppressive effect, we also performed an immunohistochemistry analysis of CD41 (for platelet aggregation), VEGF, CD31 (for MVD), PCNA, α-SMA, collagen I, and LOX in ectopic endometrium. In addition, we evaluated the extent of lesional fibrosis by Masson trichrome staining. We found that PCNA and LOX staining was seen in cellular nuclei in both the stromal and epithelial cells of the ectopic endometrium, while
VEGF immunoreactivity was seen mostly in the cytoplasm of glandular epithelial cells as well as of vascular endothelial cells (Figure 2). The CD31 staining was seen mostly in vascular endothelial cells. CD41-labeled platelets, \( \alpha \)-SMA, and collagen I staining were seen in the stroma of the endometriotic lesions (Figure 2).

We found that in mice treated with scutellarin, the extent of platelet aggregation in lesions was significantly reduced as compared to that of the UT mice (both \( P \) values < .0017; Figures 2 and 3A), but there was no difference between the LS and HS groups (\( P = .67 \); Figure 3A). Similarly, the immunoreactivity to all other marker proteins, the MVD in both the LS and HS groups were significantly reduced as compared to that of the UT group (\( P < .04 \); Figure 3B-G), but no significant difference between the LS and HS groups was found (\( P > .34 \)).

Consistent with the reduced collagen I staining levels in lesions, we found that the extent of lesional fibrosis in mice of both the HS and LS groups was significantly reduced as compared to that in the UT mice (both \( P \) values = 4.1 \( \times \) 10\(^{-5} \); Figure 3H), but no significant difference between the LS and HS groups was found (\( P = .86 \)), suggesting that platelet could help the fibrosis of the ectopic lesion and inhibition platelet function may suppress the lesion fibrosis.

The lesion weight correlated positively with the extent of platelet aggregation (\( r = 0.51, P = .007 \); Figure 4A). As expected, the lesion weight was positively correlated with the VEGF, the MVD, and PCNA staining levels (\( r's > 0.67, P < 9.6 \times 10^{-5} \), all square-root transformed to improve normality; Figure 4B-D). The lesion weight also correlated positively with the lesional staining levels of \( \alpha \)-SMA, collagen I and LOX and the extent of lesional fibrosis (Figure 4E-H).

The PCNA immunoreactivity was positively correlated with the MVD (\( r = 0.64, P = .0003 \)), which, in turn, was positively correlated with that of VEGF (\( r = 0.79, P = 8.9 \times 10^{-7} \)). Above all, the extent of platelet aggregation (ie, CD41+...
Figure 2. Representative immunoreactivity staining of CD41, VEGF, CD31-MVD, PCNA, collagen I, α-SMA, and LOX and representative Masson staining results in ectopic murine lesion in different groups of mice. Group UT, LS, HS. Left column: group UT; middle column: group LS; right column: group HS. Magnification in all IHC figures: ×400 (×200 for Masson). The scale bar represents 125 μm for all immunohistochemistry figures but 251 μm for Masson trichrome staining. VEGF indicates vascular endothelial growth factor; MVD, counting microvessel density; PCNA, proliferating cell nuclear antigen; α-SMA, α-smooth muscle actin; LOX, lysyl oxidase; UT, untreated; LS, low-dose scutellarin; HS, high-dose scutellarin.
platelets) was correlated with PCNA staining levels ($r = 0.58$, $P = .0016$), and all other proteins ($r's \geq 0.58$, $Ps < .0014$). In particular, it was positively correlated with the immunoreactivity against α-SMA, collagen I, LOX, and the extent of lesional fibrosis ($r's \geq 0.42$, $Ps < .031$).

**Discussion**

In this study, we have shown that scutellarin treatment resulted in significantly reduced lesion weight, improved hyperalgesia, and changes consistent with reduced proliferation, angiogenesis, and fibrogenesis in a mouse model of endometriosis.
Scutellarin treatment also significantly reduced the platelet activation rate in the peripheral blood. The reduced lesional staining of \(\alpha\)-SMA in the stromal component suggests decelerated FMT, which is consistent with the concomitant reduction in lesional fibrosis.

Our results are consistent with our previous findings that platelet depletion and antiplatelet treatment resulted in reduced lesion growth and improved pain behavior in mice with induced endometriosis.\textsuperscript{13,21,22} Our results are also consistent with the treatment effect of andrographolide\textsuperscript{47} and valproic acid.\textsuperscript{22}

Figure 4. Scatter plots showing the relationship between lesion weight and the extent of platelet aggregation (A), immunostaining levels of VEGF (B), microvessel density (C), PCNA (D), \(\alpha\)-SMA (E), collagen I (F), and LOX (G), and the extent of lesional fibrosis (H). The symbols in the plot indicate the treatment group from which the mouse came from, with UT being untreated, and LS and HS, as treated with low- and high-dose scutellarin, respectively. The dashed line represents the regression line. The number shown in each figure indicates the Pearson correlation coefficient, followed by the statistical significance levels: *\(P < .05\); **\(P < .01\); ***\(P < .001\). VEGF indicates vascular endothelial growth factor; MVD, counting microvessel density; PCNA, proliferating cell nuclear antigen; \(\alpha\)-SMA, \(\alpha\)-smooth muscle actin; LOX, lysyl oxidase.
acid in rodent models of endometriosis, as both have been shown to have antiplatelet capabilities. Collectively, these results demonstrate that antiplatelet therapeutics may be promising as a nonhormonal treatment for endometriosis and should be further investigated.

The herbal medicine from which scutelllarin is derived is a potent antiplatelet herb in traditional Chinese medicine that combats “blood stasis.” As in eutopic endometrium, the ectopic endometrium also undergoes cyclic bleeding and thus tissue injury. As such, platelets must be involved in the ensuing tissue repair. Once there is tissue injury or a wound, platelets are first cells (though anucleated) to go to and aggregate at the wounding site, initiating hemostasis and inaugurating the tissue repair process of inflammation, proliferation, and tissue remodeling. Activated platelets secrete a plethora of bioactive molecules, including various cytokines/chemokines, growth factors such as interleukins, VEGF, PDGF, EGF, FGF, and TGF-β1, with TGF-β1 being the most copious. In addition, endometriotic stromal cells also produce potent platelet-activating molecules such as thrombin and thromboxane A2 and collagens, which, in conjunction with increased angiogenesis and thus vascular permeability, may further lead to platelet aggregation. Thus, endometriotic lesions and platelets engage active cross-talks to maintain lesion growth and promote EMT, FMT, and SMM, resulting in fibrosis.

Aside from its antiplatelet capability, scutelllarin may also target other pathways involved in endometriosis. Indeed, scutelllarin is reported to relieve ischemia/reperfusion injury. The cardioprotective and neuroprotective effects of scutelllarin depend on the upregulation of the endothelial nitric oxide synthase (eNOS) expression and the downregulation of VEGF, basic FGF, and inducible nitric oxide synthase (iNOS). In addition, it is an efficient inhibitor of an important transcription factor nuclear factor-κB (NF-κB), for which aberrant activation in endometriosis has also been reported. Moreover, scutelllarin has been reported to be promising in treating resisting liver fibrosis, interstitial, and myocardial fibrosis in rats by inhibiting collagens I and III and TGF-β1. Although our study has shown reduced immunostaining of VEGF and collagen I in lesions, it is plausible that scutelllarin treatment may also cause suppression of NF-κB activation and TGF-β1 inhibition in lesions.

We note that fibrosis itself may facilitate lesional development by enhancing fibrogenesis through increased stiffness of extracellular matrix (ECM). In fibrotic diseases, it is known that increased myofibroblast contraction induces the release of latent TGF-β1 and mechanical stretch also activates TGF-β1, stimulating further profibrotic changes. In endometriosis, it has been shown that soft ECMs, which are associated with no fibrosis, inhibit cell proliferation and inactivate fibrotic phenotype in stromal cells derived from deep endometriotic lesions. In contrast, stiff ECM induces EMT in endometrial epithelial cells.

One puzzling finding in our study is that scutelllarin treatment did not demonstrate a dose–response relationship in suppressing lesion development. Although mice receiving HS treatment did respond to the treatment, they showed no better improvement than the LS group, even though the platelet activation rate in the peripheral blood seemed to be reduced dose dependently (Figure 1D). However, the reduction in the extent of platelet aggregation in lesions did not seem to be dose dependent, and the lesion weight in the HS group appeared to be similar to that of the LS group. It is possible that, given the known human dosage, which is near the low dosage used in this study, and the known side effects of breviscapine which include fatigue, skin itching and rashes, dizziness, nausea, and vomiting, higher dose might have increased discomfort and thus stress in HS mice, yielding somewhat diminished therapeutic effects. In addition, there is a likelihood that, given the vast difference in physiology between Homo sapiens and mouse, the effect of scutelllarin may be quite different between the 2 species. Further research is warranted to identify the optimal dose in future preclinical studies.

This study has several limitations. First, this study only employed immunohistochemistry and histochemistry analyses and did not provide molecular evidence that scutelllarin suppresses angiogenesis, proliferation, or fibrogenesis. Hence, the best we can say is that our data are highly consistent with the documented changes. Also due to the methods we used, only a few possible mechanisms were explored. Further research is warranted to clearly delineate the mechanism of action for scutelllarin. Second, the percentage of circulating activated platelets in controls as we reported is somewhat high. This is likely due to our use of centrifugation without prior fixation by paraformaldehyde. However, since we handled and analyzed all blood samples uniformly and without any bias, our finding that mice treated with scutelllarin had lower percentage of circulating activated platelets than the UT mice is still valid. Lastly, the treatment started just 2 weeks after induction, which may not be long enough for lesional fibrosis to be fully developed as commonly seen in humans. However, given the significant reduction in hotplate latency at the time of surgery (Figure 1C), it is safe to say that the pain symptom has already been manifested at the time of start of the treatment. In addition, the evidence that scutelllarin treatment did slow down the progression of lesional fibrogenesis (Figure 3E-H) seems to be unmistakable. Of course, it is possible that once lesions become highly fibrotic, the treatment efficacy might be diminished due to reduced vascularity and possible epigenetic changes. Nonetheless, the reduced extent of fibrosis in scutelllarin-treated groups demonstrates that scutelllarin can stall the progression of fibrogenesis. Future studies are needed to see whether scutelllarin is also effective in treating deep endometriosis-like disease.

In summary, scutelllarin appears to be effective in treating mouse with induced endometriosis, resulting in significantly reduced lesion weight, improved hyperalgesia, and changes consistent with reduced proliferation, angiogenesis, and fibrogenesis. As such, scutelllarin may be a promising nonhormonal therapeutic compound for treating endometriosis.
Authors’ Note
Ding Ding, MD, PhD, Xianjun Cai, MD, and Hanxi Zheng contributed equally to this work.

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