Original Article

Reproductive Sciences
1-12

DOI: 10.1177/1933719118817661
journals.sagepub.com/home/rsx
SAGE

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

Ding Ding, MD, PhD[1,3], Xianjun Cai, MD[2], Hanxi Zheng, MD[1], Sun-Wei Guo, PhD[1], and Xishi Liu, MD, PhD[1,3]

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 risk[3,4] 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

[1] Shanghai OB/GYN Hospital, Fudan University, Shanghai, China
[2] Ningbo No. 7 Hospital, Ningbo, Zhejiang, China
[3] Shanghai Key Laboratory of Female Reproductive Endocrine-Related Diseases, Fudan University, Shanghai, China

Corresponding Authors:
Sun-Wei Guo and Xishi Liu, Shanghai OB/GYN Hospital, Fudan University, 128 Shenyang Road, Shanghai 200090, China.
Emails: hoxa10@outlook.com; lxsdoc@hotmail.com

(SMM) in endometriotic lesions, resulting ultimately in fibrosis.[15,16] Activated platelets are also shown to be responsible for increased estrogen receptor β expression[17] and reduced natural killer cell cytotoxicity in endometriosis.[18] Consistent with the important roles of platelets in lesional development, women with endometriosis have been shown to be in a hypercoagulable state.[19,20] Consequently, platelet depletion and antiplatelet treatment effectively suppress lesion growth in mouse with induced endometriosis.[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 astoundingly high attrition rate: Over 99% of compounds do not make to the final end of the R&D pipeline.[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 *Erigeron breviscapus* (Vant.) Hand.-Mazz., a Chinese herbal medicine used over a thousand years.[24] It is the major active component of breviscapine, which is the total flavonoid extract of *E breviscapus* containing ≥90% scutellarin and ≤10% apigenin-7-O-glcuronide in content. Breviscapine is a prescription drug in China for the treatment of cardiovascular and cerebrovascular diseases with an excellent safety profile.[25] Scutellarin has been demonstrated to have multiple pharmacological effects, having antioxidant, antiplatelet, and anti-inflammatory properties.[26-28] Scutellarin has also been shown to exhibit anticancer activities by suppressing proliferation, invasion, metastasis, and angiogenesis on various types of cancer.[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.[33] Moreover, scutellarin has been shown to be antifibrotic.[34] Since coagulation, ERK/p53, c-met/AKT, and fibrogenesis have all been reported to be involved in endometriosis,[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 mouse 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

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 *Guide for the Care and Use of Laboratory Animals*[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.

Induction of Endometriosis and the Experiment Protocol

We used an established mouse model of endometriosis by intraperitoneal (ip) injection of endometrial fragments[39] as used in our previous studies.[13,40] Briefly, donor mice were initially injected intramuscular with estradiol benzoate (3 μg/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 μL sterile saline, group LS received ip injections of scutellarin (7.5 mg/kg/mouse in 300 μL sterile saline), and group UT received ip injections of just 300 μL 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[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[42] to as high as 60 mg/kg[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.[44] 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.[19] 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% NH_4OH 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.[16] 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 lesional fibrosis.[16]

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[45] (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 (*P*s > .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 ($P = 5.9 \times 10^{-6}$; 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 ($P = 9.0 \times 10^{-6}$ and $P = 7.4 \times 10^{-8}$, respectively; $R^2 = 0.79$).

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 ($P = 3.9 \times 10^{-5}$; $R^2 = 0.56$). 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), PNCA, 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

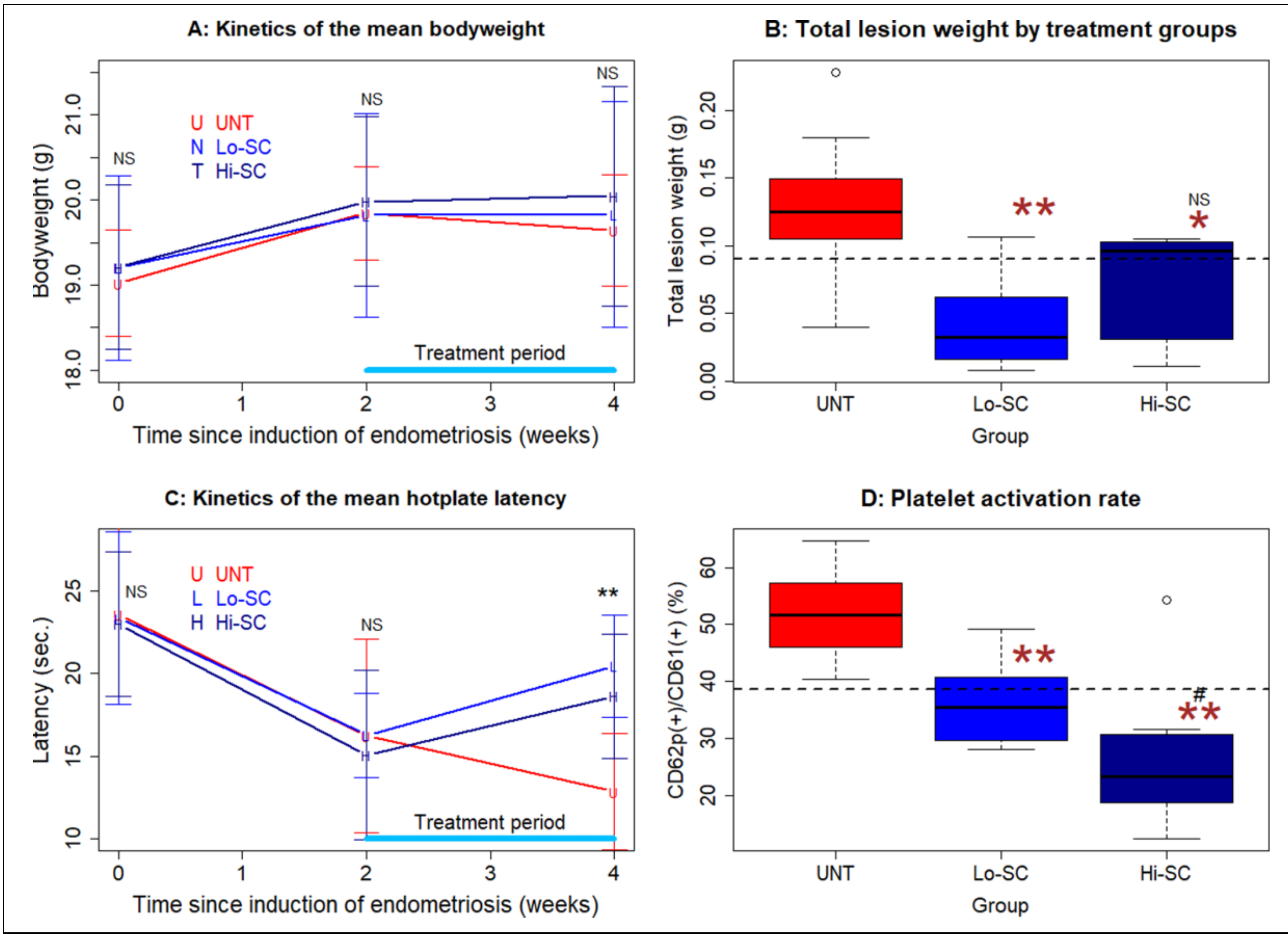

Figure 1. (A) Kinetics of changes in the mean body weight in different groups; (B) boxplot of lesion weight among different treatment groups. (C) Kinetics of changes in the mean hotplate latency in different groups; (D) boxplot of platelet activation rate in the peripheral blood among different treatment groups. NS: $P > .05$; *$P < .05$; **$P < .01$; #$P < 0.1$. In (A) and (B), Kruskal test was used. In (C) and (D), Wilcoxon test was used, and the symbols in brown indicate test using the UT group as the reference, while the black symbols indicate test using the LS as the reference. UT indicates untreated; LS, low-dose scutellarin; HS, high-dose scutellarin.

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, α-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 (Ps < .04; Figure 3B-G), but no significant difference between the LS and HS groups was found (Ps > .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 ($r = 0.51$, 0.007; Figure 4A). As expected, the lesion weight was positively correlated with the VEGF, the MVD, and PCNA staining levels (r's > 0.67, Ps < 9.6×10^{-5}, all square-root transformed to improve normality; Figure 4B-D). The lesion weight also correlated positively with the lesional staining levels of α-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+

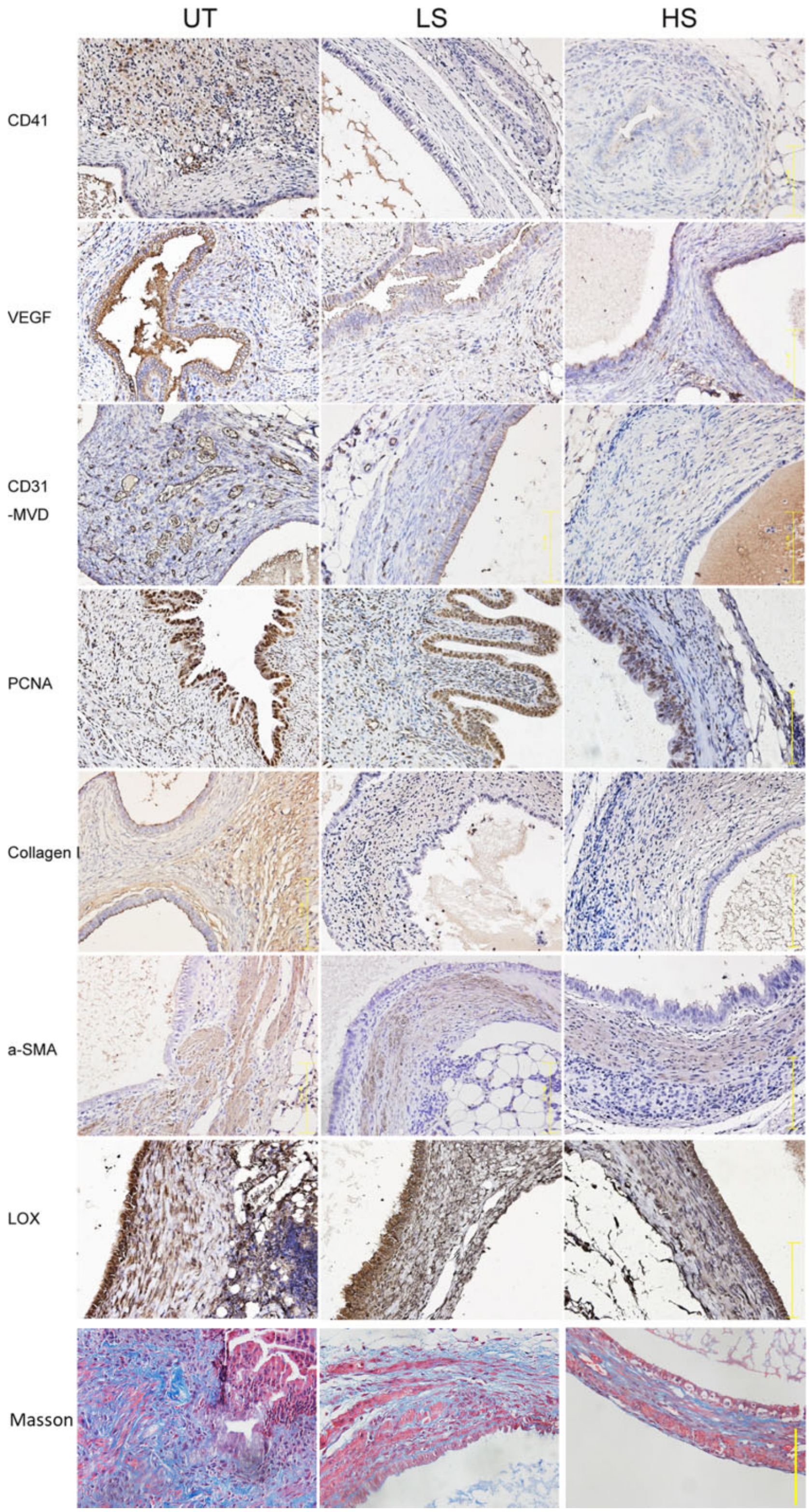

Figure 2. Representative immunoreactivity staining of CD41, VEGF, CD31-MVD, PCNA, collagen I, α-SMA, and LOX and representative Masson staining results in ectopic murine lesion in different groups of mice. Group UT, LS, HS. Left column: group UT; middle column: group LS; right column: group HS. Magnification 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.

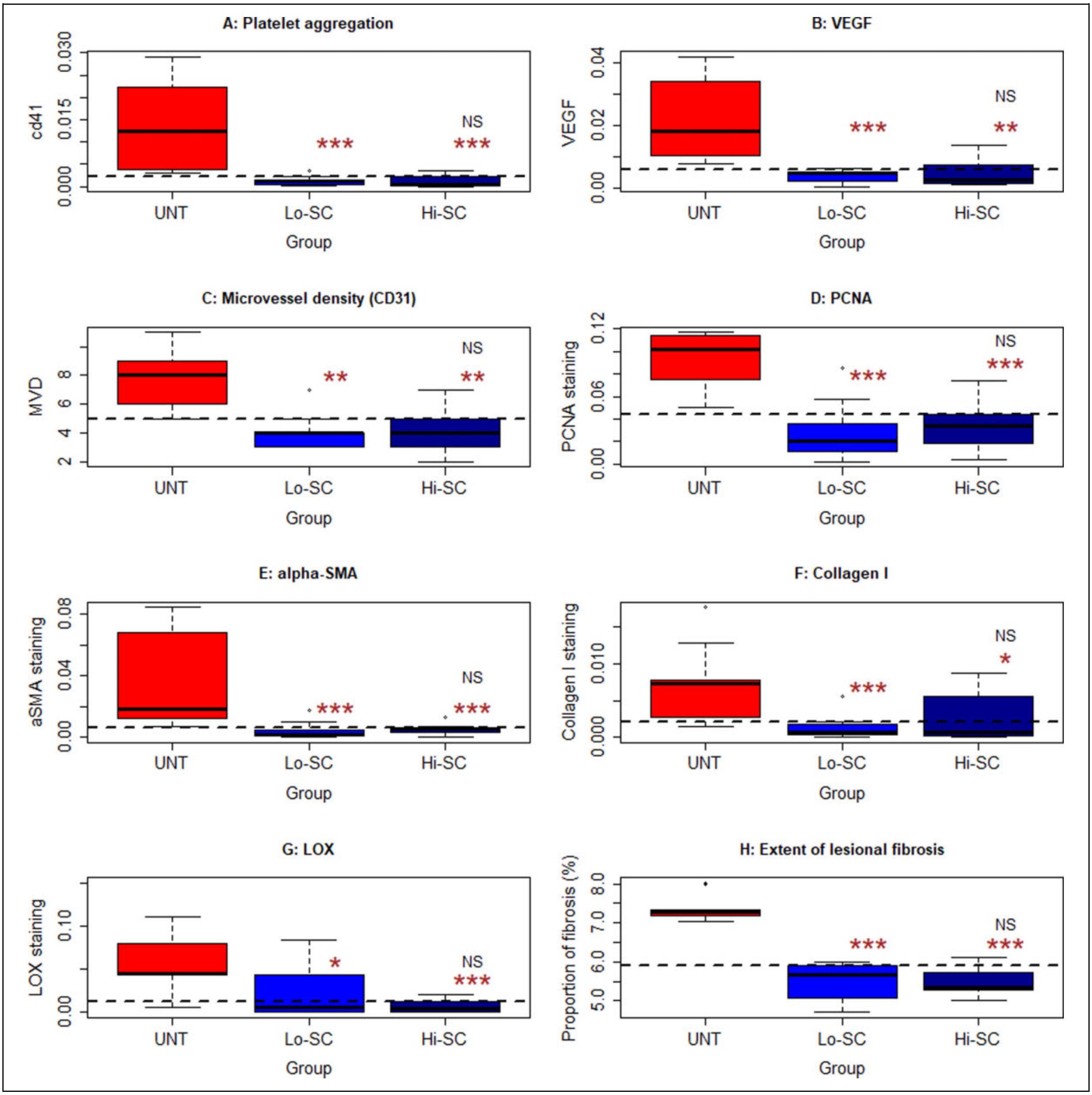

Figure 3. Boxplot summarization of the histochemistry and histochemistry staining results for platelet aggregation (A), VEGF (B), CD31-macrivessel density (C), PCNA (D), α-SMA (E), collagen I (F), LOX (G), and the extent of lesional fibrosis (H). NS: $P > .05$; *$P < .05$; **$P < .01$; ***$P < .001$. In all plots, Wilcoxon test was used, and the symbols in brown indicate test using the UT group as the reference, while the black symbols indicate test using the Lo-SC as the reference. VEGF indicates vascular endothelial growth factor; PCNA, proliferating cell nuclear antigen; α-SMA, α-smooth muscle actin; LOX, lysyl oxidase; UT: untreated; Lo-SC, low-dose scutellarin; Hi-SC, high-dose scutellarin.

platelets) was correlated with PCNA staining levels ($r = 0.58$, $P = .0016$), and all other proteins (r's ≥ 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 ≥ 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.

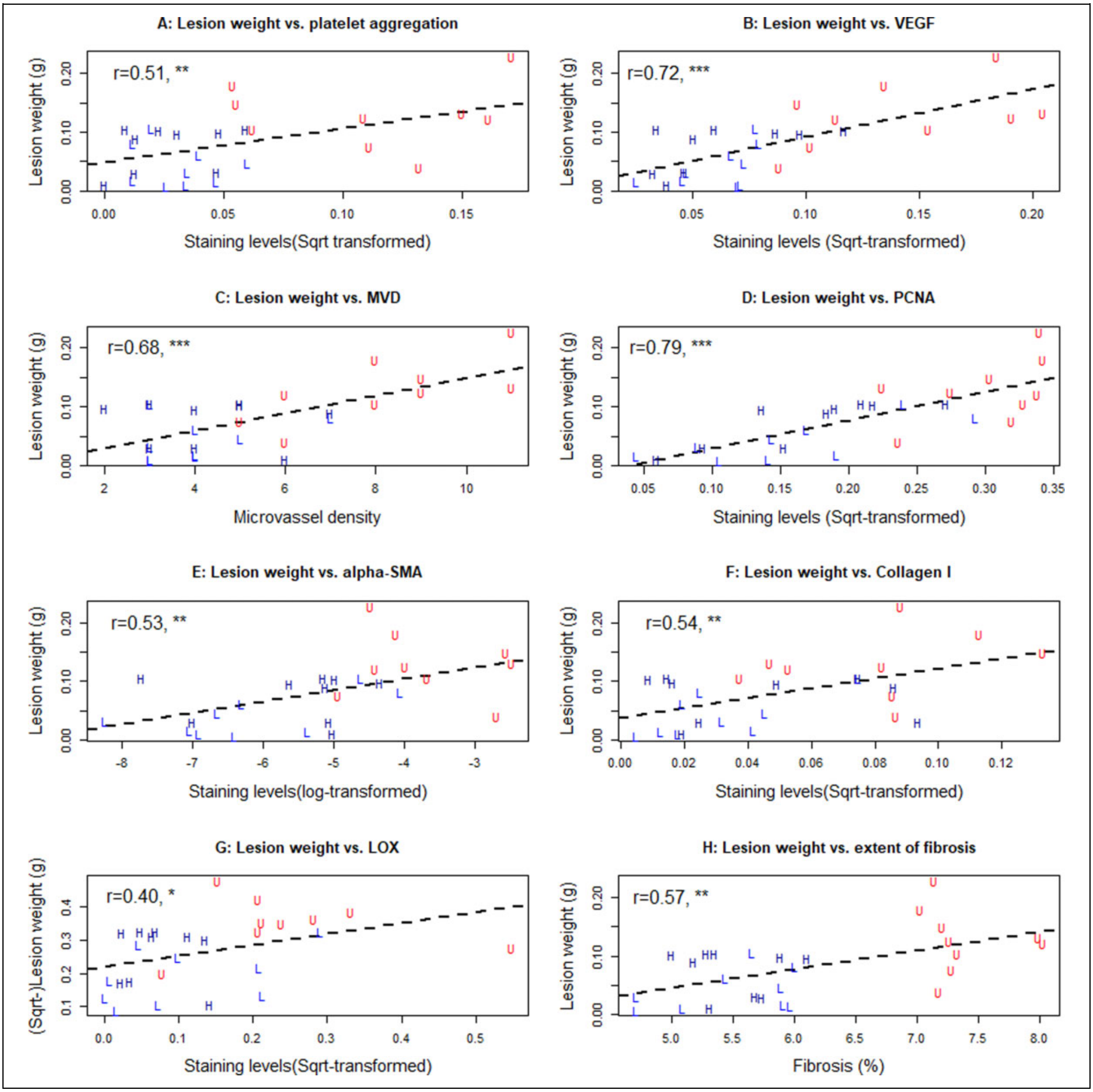

Figure 4. Scatter plots showing the relationship between lesion weight and the extent of platelet aggregation (A), immunostaining levels of VEGF (B), microvessel density (C), PCNA (D), α-SMA (E), collagen I (F), and LOX (G), and the extent of lesional fibrosis (H). The symbols in the plot indicate the 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; α-SMA, α-smooth muscle actin; LOX, lysyl oxidase.

Scutellarin treatment also significantly reduced the platelet activation rate in the peripheral blood. The reduced lesional staining of α-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.[13,21,22] Our results are also consistent with the treatment effect of andrographolide[47] and valproic acid[48] in rodent models of endometriosis, as both have been shown to have antiplatelet capabilities.[49,50] 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 scutellarin 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.[51] Activated platelets secret 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.[52] In addition, endometriotic stromal cells also produce potent platelet-activating molecules such as thrombin and thromboxane A2[53] and collagens,[15] 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.[13,15,16]

Aside from its antiplatelet capability, scutellarin may also target other pathways involved in endometriosis. Indeed, scutellarin is reported to relieve ischemia/reperfusion injury. The cardioprotective and neuroprotective effects of scutellarin depend on the upregulation of the endothelial nitric oxide synthase (eNOS) expression[54,55] 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.[56-58] Moreover, scutellarin has been reported to be promising in treating resisting liver fibrosis,[59] interstitial, and myocardial fibrosis[34,60] 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 scutellarin 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[61] and mechanical stretch also activates TGF-β1,[62] 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.[63] In contrast, stiff ECM induces EMT in endometrial epithelial cells.[64]

One puzzling finding in our study is that scutellarin 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 scutellarin 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 scutellarin 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 scutellarin. 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.[65] However, since we handled and analyzed all blood samples uniformly and without any bias, our finding that mice treated with scutellarin 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.[66] 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 scutellarin 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.[67] Nonetheless, the reduced extent of fibrosis in scutellarin-treated groups demonstrates that scutellarin can stall the progression of fibrogenesis. Future studies are needed to see whether scutellarin is also effective in treating deep endometriosis-like disease.

In summary, scutellarin 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, scutellarin 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.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This research was supported in part by grants 81471434 (SWG), 81530040 (SWG), 81771553 (SWG), 81370695 (XSL), 81671436 (XSL), and 81401183 (DD) from the National Natural Science Foundation of China, 2017A610169 (XJC) from Ningbo Municipal Commission of Science and Technology, and an Excellence in Centers of Clinical Medicine grant (2017ZZ01016) from the Science and Technology Commission of Shanghai Municipality.

Supplemental material

Supplemental material for this article is available online.

References

1. Giudice LC, Kao LC. Endometriosis. *Lancet*. 2004;364(9447):1789-1799.
2. Practice Committee of the American Society for Reproductive Medicine. Treatment of pelvic pain associated with endometriosis: a committee opinion. *Fertil Steril*. 2014;101(4):927-935.
3. Guo SW. Recurrence of endometriosis and its control. *Hum Reprod Update*. 2009;15(4):441-461.
4. Koga K, Takamura M, Fujii T, Osuga Y. Prevention of the recurrence of symptom and lesions after conservative surgery for endometriosis. *Fertil Steril*. 2015;104(4):793-801.
5. Garcia-Velasco JA, Somigliana E. Management of endometriomas in women requiring IVF: to touch or not to touch. *Hum Reprod*. 2009;24(3):496-501.
6. de Ziegler D, Borghese B, Chapron C. Endometriosis and infertility: pathophysiology and management. *Lancet*. 2010;376(9742):730-738.
7. Coccia ME, Rizzello F, Mariani G, Bulletti C, Palagiano A, Scarselli G. Ovarian surgery for bilateral endometriomas influences age at menopause. *Hum Reprod*. 2011;26(11):3000-3007.
8. Vercellini P, Buggio L, Frattaruolo MP, Borghi A, Dridi D, Somigliana E. Medical treatment of endometriosis-related pain. *Best Pract Res Clin Obstet Gynaecol*. 2018;51:68-91.
9. Vercellini P, Crosignani P, Somigliana E, Vigano P, Frattaruolo MP, Fedele L. 'Waiting for Godot': a commonsense approach to the medical treatment of endometriosis. *Hum Reprod*. 2011;26(1):3-13.
10. Guo SW, Groothuis PG. Is it time for a paradigm shift in drug research and development in endometriosis/adenomyosis? *Hum Reprod Update*. 2018;24(5):577-598.
11. Brosens IA. Endometriosis – a disease because it is characterized by bleeding. *Am J Obstet Gynecol*. 1997;176(2):263-267.
12. Guo SW, Ding D, Shen M, Liu X. Dating endometriotic ovarian cysts based on the content of cyst fluid and its potential clinical implications. *Reprod Sci*. 2015;22(7):873-883.
13. Ding D, Liu X, Duan J, Guo SW. Platelets are an unindicted culprit in the development of endometriosis: clinical and experimental evidence. *Hum Reprod*. 2015;30(4):812-832.
14. Guo SW. Fibrogenesis resulting from cyclic bleeding: the Holy Grail of the natural history of ectopic endometrium. *Hum Reprod*. 2018;33(3):353-356.
15. Zhang Q, Duan J, Liu X, Guo SW. Platelets drive smooth muscle metaplasia and fibrogenesis in endometriosis through epithelial–mesenchymal transition and fibroblast-to-myofibroblast transdifferentiation. *Mol Cell Endocrinol*. 2016;428:1-16.
16. Zhang Q, Duan J, Olson M, Fazleabas A, Guo SW. Cellular changes consistent with epithelial–mesenchymal transition and fibroblast-to-myofibroblast transdifferentiation in the progression of experimental endometriosis in baboons. *Reprod Sci*. 2016;23(10):1409-1421.
17. Zhang Q, Ding D, Liu X, Guo SW. Activated platelets induce estrogen receptor beta expression in endometriotic stromal cells. *Gynecol Obstet Invest*. 2015;80(3):187-192.
18. Du Y, Liu X, Guo SW. Platelets impair natural killer cell reactivity and function in endometriosis through multiple mechanisms. *Hum Reprod*. 2017;32(4):794-810.
19. Wu Q, Ding D, Liu X, Guo SW. Evidence for a hypercoagulable state in women with ovarian endometriomas. *Reprod Sci*. 2015;22(9):1107-1114.
20. Ding D, Liu X, Guo SW. Further evidence for hypercoagulability in women with ovarian endometriomas. *Reprod Sci*. 2018;25(11):1540-1548. doi:10.1177/1933719118799195.
21. Guo SW, Ding D, Geng JG, Wang L, Liu X. P-selectin as a potential therapeutic target for endometriosis. *Fertil Steril*. 2015;103(4):990-1000 e8.
22. Guo SW, Ding D, Liu X. Anti-platelet therapy is efficacious in treating endometriosis induced in mouse. *Reprod Biomed Online*. 2016;33(4):484-499.
23. Carnero A. High throughput screening in drug discovery. *Clin Transl Oncol*. 2006;8(7):482-490.
24. Wang L, Ma Q. Clinical benefits and pharmacology of scutellarin: a comprehensive review. *Pharm Ther*. 2018;190:105-127.
25. Liu X, Cheng J, Zhang G, et al. Engineering yeast for the production of breviscapine by genomic analysis and synthetic biology approaches. *Nat Commun*. 2018;9(1):448.
26. Luo P, Tan ZH, Zhang ZF, Zhang H, Liu XF, Mo ZJ. Scutellarin isolated from *Erigeron multiradiatus* inhibits high glucose-mediated vascular inflammation. *Yakugaku Zasshi*. 2008;128(9):1293-1299.
27. Ma JY, Jiang WW, Zhou ZT, Li JM, Wang HY. The promoting angiogenesis and anti-inflammation effect of scutellarin on polyglycolic acid scaffold of Balb/c mice model. *J Asian Nat Prod Res*. 2008;10(11-12):1147-1153.
28. Tian X, Chang L, Ma G, et al. Delineation of platelet activation pathway of scutellarein revealed its intracellular target as protein kinase C. *Biol Pharm Bullet*. 2016;39(2):181-191.
29. Hou L, Chen L, Fang L. Scutellarin inhibits proliferation, invasion, and tumorigenicity in human breast cancer cells by

regulating HIPPO-YAP signaling pathway. *Med Sci Monit*. 2017;23:5130-5138.

30. Ke Y, Bao T, Wu X, et al. Scutellarin suppresses migration and invasion of human hepatocellular carcinoma by inhibiting the STAT3/Girdin/Akt activity. *Biochem Biophys Res Commun*. 2017;483(1):509-515.
31. Li H, Huang D, Gao Z, Chen Y, Zhang L, Zheng J. Scutellarin inhibits the growth and invasion of human tongue squamous carcinoma through the inhibition of matrix metalloproteinase-2 and -9 and alphavbeta6 integrin. *Int J Oncol*. 2013;42(5):1674-1681.
32. Zhu PT, Mao M, Liu ZG, Tao L, Yan BC. Scutellarin suppresses human colorectal cancer metastasis and angiogenesis by targeting ephrinb2. *Am J Transl Res*. 2017;9(11):5094-104.
33. Sun CY, Zhu Y, Li XF, et al. Scutellarin increases cisplatin-induced apoptosis and autophagy to overcome cisplatin resistance in non-small cell lung cancer via ERK/p53 and c-met/AKT signaling pathways. *Front Pharmacol*. 2018;9:92.
34. Pan Z, Zhao W, Zhang X, et al. Scutellarin alleviates interstitial fibrosis and cardiac dysfunction of infarct rats by inhibiting TGFbeta1 expression and activation of p38-MAPK and ERK1/2. *Br J Pharmacol*. 2011;162(3):688-700.
35. Li Y, Liu YD, Chen SL, et al. Down-regulation of long non-coding RNA MALAT1 inhibits granulosa cell proliferation in endometriosis by up-regulating P21 via activation of the ERK/MAPK pathway. *Mol Hum Reprod*. 2018.
36. KhoshdelRad N, Salehi Z, Mashayekhi F, Abbasi O, Mirzajani E. Soluble c-Met expression in the peritoneal fluid and serum of patients with different stages of endometriosis. *Arch Gynecol Obstet*. 2014;289(5):1107-1112.
37. Choi J, Jo M, Lee E, Hwang S, Choi D. Aberrant PTEN expression in response to progesterone reduces endometriotic stromal cell apoptosis. *Reproduction*. 2017;153(1):11-21.
38. Council NR. *Guide for the Care and Use of Laboratory Animals*. Washington, DC: National Academies Press; 1996.
39. Somigliana E, Vigano P, Rossi G, Carinelli S, Vignali M, Panina-Bordignon P. Endometrial ability to implant in ectopic sites can be prevented by interleukin-12 in a murine model of endometriosis. *Hum Reprod*. 1999;14(12):2944-2950.
40. Long Q, Liu X, Guo SW. Surgery accelerates the development of endometriosis in mouse. *Am J Obstetr Gynecol*. 2016;215(3):320.e1-320.e15.
41. Reagan-Shaw S, Nihal M, Ahmad N. Dose translation from animal to human studies revisited. *FASEB J*. 2008;22(3):659-661.
42. Hu X, Teng S, He J, et al. Pharmacological basis for application of scutellarin in Alzheimer's disease: antioxidation and antiapoptosis. *Mol Med Rep*. 2018;18(5):4289-4296.
43. Sun C, Li C, Li X, et al. Scutellarin induces apoptosis and autophagy in NSCLC cells through ERK1/2 and AKT signaling pathways in vitro and in vivo. *J Cancer*. 2018;9(18):3247-3256.
44. Lu Y, Nie J, Liu X, Zheng Y, Guo SW. Trichostatin A, a histone deacetylase inhibitor, reduces lesion growth and hyperalgesia in experimentally induced endometriosis in mice. *Hum Reprod*. 2010;25(4):1014-1025.
45. R Development Core Team. *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing; 2016.
46. Zhao T, Liu X, Zhen X, Guo SW. Levo-tetrahydropalmatine retards the growth of ectopic endometrial implants and alleviates generalized hyperalgesia in experimentally induced endometriosis in rats. *Reprod Sci*. 2011;18(1):28-45.
47. Zheng Y, Liu X, Guo SW. Therapeutic potential of andrographolide for treating endometriosis. *Hum Reprod*. 2012;27(5):1300-1313.
48. Liu M, Liu X, Zhang Y, Guo SW. Valproic acid and progestin inhibit lesion growth and reduce hyperalgesia in experimentally induced endometriosis in rats. *Reprod Sci*. 2012;19(4):360-373.
49. Lien LM, Su CC, Hsu WH, et al. Mechanisms of andrographolide-induced platelet apoptosis in human platelets: regulatory roles of the extrinsic apoptotic pathway. *Phytother Res*. 2013;27(11):1671-1677.
50. Davidson DC, Hirschman MP, Spinelli SL, et al. Antiplatelet activity of valproic acid contributes to decreased soluble CD40 ligand production in HIV type 1-infected individuals. *J Immunol*. 2011;186(1):584-591.
51. Nurden AT, Nurden P, Sanchez M, Andia I, Anitua E. Platelets and wound healing. *Front Biosci*. 2008;13:3532-3548.
52. Assoian RK, Komoriya A, Meyers CA, Miller DM, Sporn MB. Transforming growth factor-beta in human platelets. Identification of a major storage site, purification, and characterization. *J Biol Chem*. 1983;258:7155-7160.
53. Guo SW, Du Y, Liu X. Endometriosis-derived stromal cells secrete thrombin and thromboxane A2, inducing platelet activation. *Reprod Sci*. 2016;23(8):1044-1052.
54. Hu XM, Zhou MM, Hu XM, Zeng FD. Neuroprotective effects of scutellarin on rat neuronal damage induced by cerebral ischemia/reperfusion. *Acta Pharmacol Sin*. 2005;26(12):1454-1459.
55. Yang W, Lust RM, Bofferding A, Wingard CJ. Nitric oxide and catalase-sensitive relaxation by scutellarin in the mouse thoracic aorta. *J Cardiovasc Pharmacol*. 2009;53(1):66-76.
56. Gonzalez-Ramos R, Donnez J, Defrere S, et al. Nuclear factor-kappa B is constitutively activated in peritoneal endometriosis. *Mol Hum Reprod*. 2007;13(7):503-509.
57. Gonzalez-Ramos R, Van Langendonckt A, Defrere S, et al. Involvement of the nuclear factor-kappaB pathway in the pathogenesis of endometriosis. *Fertil Steril*. 2010;94(6):1985-1994.
58. Kaponis A, Iwabe T, Taniguchi F, et al. The role of NF-kappaB in endometriosis. *Front Biosci (Schol Ed)*. 2012;4:1213-1234.
59. Wang YH, Geng L, Li H. Study on effect of scutellarin in resisting liver fibrosis in rats [in Chinese]. *Zhongguo Zhong Yao Za Zhi*. 2015;40(10):1999-2003.
60. Zhou H, Chen X, Chen L, et al. Anti-fibrosis effect of scutellarin via inhibition of endothelial–mesenchymal transition on isoprenaline-induced myocardial fibrosis in rats. *Molecules*. 2014;19(10):15611-15623.
61. Wipff PJ, Rifkin DB, Meister JJ, Hinz B. Myofibroblast contraction activates latent TGF-beta1 from the extracellular matrix. *J Cell Biol*. 2007;179(6):1311-1323.

62. Froese AR, Shimbori C, Bellaye PS, et al. Stretch-induced activation of transforming growth factor-beta1 in pulmonary fibrosis. *Am J Respir Crit Care Med*. 2016;194(1):84-96.
63. Matsuzaki S, Canis M, Pouly JL, Darcha C. Soft matrices inhibit cell proliferation and inactivate the fibrotic phenotype of deep endometriotic stromal cells in vitro. *Hum Reprod*. 2016;31(3): 541-553.
64. Matsuzaki S, Darcha C, Pouly JL, Canis M. Effects of matrix stiffness on epithelial to mesenchymal transition-like processes of endometrial epithelial cells: implications for the pathogenesis of endometriosis. *Sci Rep*. 2017;7:44616.
65. Michelson AD, Barnard MR, Krueger LA, Frelinger A III, Furman MI. Evaluation of platelet function by flow cytometry. *Methods*. 2000;21(3):259-270.
66. Zhang Q, Liu X, Guo SW. Progressive development of endometriosis and its hindrance by anti-platelet treatment in mice with induced endometriosis. *Reprod Biomed Online*. 2017;34(2): 124-136.
67. Liu X, Zhang Q, Guo SW. Histological and immunohistochemical characterization of the similarity and difference between ovarian endometriomas and deep infiltrating endometriosis. *Reprod Sci*. 2018;25(3):329-340.