BASIC SCIENCE

# Soy Isoflavone Improved Female Sexual Dysfunction of Mice Via Endothelial Nitric Oxide Synthase Pathway

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## ABSTRACT

**Introduction:** Female sexual dysfunction (FSD) is a common endocrine disease that impairs the quality of life for many women. The existing therapy strategies still have many disadvantages. It is necessary to explore new pharmacologic treatments that are effective and safe.

**Aim:** The aim of this study was to explore the effects of soy isoflavone (SI) on FSD in mice and the underlying mechanisms.

**Methods and Main Outcome Measures:** Laser Doppler flowmetry was used to determine vaginal blood flow. Serum hormone levels and histologic changes of the vagina were analyzed by enzyme-linked immunosorbent assay (ELISA) and by hematoxylin and eosin (H&E) and Masson's trichome staining. The mRNA and protein expression of endothelial nitric oxide synthase (eNOS) was then evaluated by quantitative real-time polymerase chain reaction and western blot assays.

**Results:** Vaginal blood flow was found to be remarkably lower in adult mice, and SI was shown to increase vaginal blood flow in a dose-dependent manner ($P < .05$). The results of ELISA and H&E and Masson's trichome staining suggest that SI had a positive effect on FSD, as evidenced by the levels of hormones in serum and histologic changes of the vagina, which changed consistently. In addition, the level of eNOS was positively correlated with the concentration of SI, and eNOS inhibitor was able to reverse the improvement in sexual function induced by SI.

**Conclusion:** Our study demonstrated that SI could improve sexual function by upregulating the eNOS pathway. Therefore, SI might serve as a promising candidate for the treatment of sexual dysfunction. **Zhang J, Zhu Y, Pan L, et al. Soy Isoflavone Improved Female Sexual Dysfunction of Mice Via Endothelial Nitric Oxide Synthase Pathway. Sex Med 2019; 7:345–351.**



**Key Words:** Soy isoflavone; Female sexual dysfunction; Hormone levels; Endothelial nitric oxide synthase (eNOS)

## INTRODUCTION

Female sexual dysfunction (FSD) is a common endocrine disease that impairs the quality of life for many women. FSD may present as unrelated emotional disturbances, concern about female sexual interest, or vaginal pain.[1] FSD has direct and indirect effects on the quality of life, including making it difficult to have children, and can even develop into depression or suicidality.[2] Previous studies suggest that the prevalence of FSD ranges from 30% to 70% worldwide, and FSD can affect women at any age.[3] Hormonal and neurotransmitters factors contribute to the pathophysiology of sexual dysfunction.[4] Hormonal changes alter sexual activity, and neurotransmitters such as serotonin, norepinephrine, and dopamine affect sexual responses. It has also been demonstrated that FSD is associated with age, menopause, parity, smoking, and various sociocultural elements.[5]

Over the last several decades, multiple pharmacologic and nonpharmacologic methods have been investigated to target FSD. The current study of FSD focused on female sexual hormones such as estrogen receptor and its derivatives.[6] Prior research has demonstrated improved perimenopausal symptom relief after treatment

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with hormone replacement therapy (HRT) based on estrogen and progesterone.[7] Despite the wide use of HRT, studies suggest that HRT is related to a risk of stroke, venous thrombosis, coronary heart disease, pulmonary embolism, or breast cancer.[8] Previous studies have shown a correlation between HRT and a risk of meningioma in postmenopausal women, particularly when replenishing estradiol only.[9,10] Taken together, prevention efforts as well as better therapy strategies for FSD patients are in high demand.

In vitro studies have shown that soy isoflavone (SI) can induce breast cancer cell apoptosis and inhibit the cell proliferation of cervical cancer.[11,12] As the major food-derived phytoestrogen, SI is capable of preventing cardiovascular diseases, certain tumors, and osteoporosis, in addition to alleviating menopausal syndrome.[13] SI can selectively bind to estrogen receptors, particularly the $\beta$ subtype, with distinct affinities because of structural similarities to endogenous 17$\beta$-estradiol. SI exerts estrogenic activities and serves as a natural agonist for estrogen receptor isoforms in humans and animals.[14] SI could affect the metabolism of endogenous steroids by inhibiting aromatase, an important enzyme involved in the conversion of testosterone to estrogens. It has been reported that SI suppresses cell proliferation by regulating cyclin-dependent kinases and induces cell apoptosis.[15] Early research found that SI was involved in the modulation of signaling pathways such as insulin-like growth factor 1 and epidermal growth factor signaling.[16] Our preliminary study indicated that SI at high doses (>20 mg/kg/day) caused erectile dysfunction accompanied by decreasing testosterone levels in mice.[17] However, the effect of SI on sexual function remains largely unknown.

In the present study, we investigated the effects of SI on sexual dysfunction in 12-month-old female mice, which served as our model of female perimenopause. We assessed the effects of SI on sexual dysfunction parameters such as vaginal blood flow and serum hormone levels and conducted histopathological analyses of vaginal and ovarian tissue. We hypothesized that SI would improve sexual function in perimenopausal women via an estrogen receptor/endothelial nitric oxide synthase (eNOS) pathway. Such findings would support the notion that SI represents a new therapeutic agent for impaired sexual dysfunction.

## MATERIALS AND METHODS

### Chemicals

SI (≥98% purity) and fulvestrant (ICI182780; ≥98% purity) were purchased from Sigma-Aldrich (Shanghai, China), and stilboestrol was obtained from Meryer Chemical Technology Company, LTD (Shanghai, China). For the experiments, SI was dissolved in water, and the fulvestrant was dissolved in corn oil as described in Milošević et al.[18]

### Treatment of Animals

Female wild-type C57BL/6 mice (200 to 240 g, 12 months old) were obtained from Nanjing Medical University Animals Laboratory. The animal protocols were approved by the ethics committee of Nanjing Medical University and carried out in line with criteria for the care and use of laboratory animals. All mice were kept under standard conditions (eg, a cycle of 12 hours dark/12 hours light, water and food ad libitum). Mice were randomly assigned to 10 groups of 6 each. The experimental groups were given either SI at doses of 5, 10, and 20 mg/kg/day by intragastric administration for 30 days or fulvestrant (ICI182780) suspended in a corn oil vehicle by subcutaneous injection; the positive control group was given 0.6 mg/kg stilboestrol, and the negative control group was given an equivalent amount of saline. At the end, animals were sacrificed under anesthesia with 1% sodium pentobarbital (40 mg/kg).

### Laser Doppler Flowmetry Assay

A bipolar platinum wire electrode was placed at the initial site of the pelvic nerve vaginal branch, and the other side was connected to the electrical stimulator. The pelvic nerves were stimulated at a frequency of 10 Hz with 0.8-ms pulses and a strength of 6 V by a Grass S9 stimulator (Grass Instruments; Quincy, MA). The nerves were stimulated intermittently for periods of 30 seconds alternating with 5 minutes of rest. Vaginal blood flow was recorded by laser Doppler flowmetry (Transonic Systems, Inc; Ithaca, NY). The probe was positioned inside the lower third of the vagina. Blood flow was recorded as the peak vaginal wall blood flow (PF) and area under the curve (AUC) after pelvic nerve stimulation, and the perfusion was presented as blood flow per unit.

### Biochemical Assays

At the end of the 30th day, the blood samples were collected from the caudal vein under anesthesia. The serum samples were separated by centrifugation at 1,500 rpm for 15 minutes and stored at −20°C before analysis. The serum levels of estradiol ($E_2$), follicle-stimulating hormone (FSH), luteinizing hormone (LH), progesterone, and testosterone were examined by using enzyme-linked immunosorbent assay (ELISA) kits (Sigma-Aldrich; St. Louis, MO) according to the manufacturer's instructions.

### Histopathological Evaluation

Ovarian and vaginal tissues were isolated from mice in each group for histologic evaluation as described in Carriel et al.[19] Briefly, the specimens were fixed in 4% paraformaldehyde, paraffin embedded, and sectioned at a thickness of 5 $\mu$m. The pathological changes in ovarian and vaginal tissues were analyzed by hematoxylin and eosin (H&E) staining and by Masson's trichome staining under a light microscope (Olympus; Tokyo, Japan).

### Western Blot

Total proteins were extracted from vaginal tissues by radioimmunoprecipitation assay buffer (Sigma-Aldrich), and

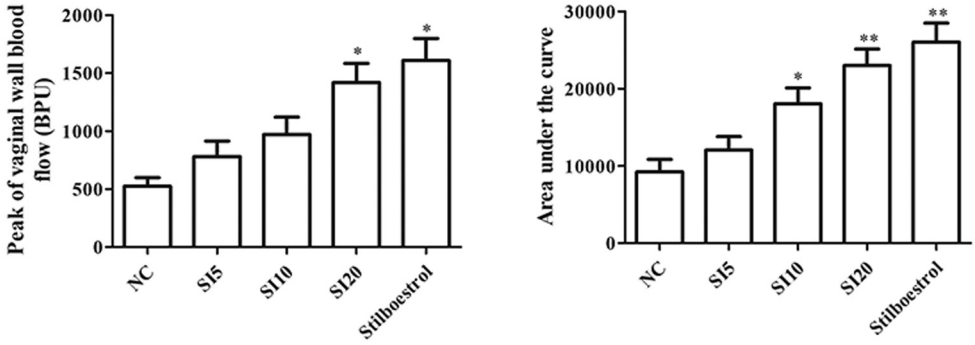


**Figure 1.** Vaginal blood flow was detected by laser Doppler flowmetry and expressed as the peak vaginal wall blood flow and area under the curve. NC = negative control. $^{*}P < .05$, $^{**}P < .01$

concentrations were determined using a BCA kit (Sigma-Aldrich). The protein samples were loaded to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene difluoride membranes (Sigma-Aldrich). The membranes were then blocked with 5% skim milk and probed with primary antibodies against phospho-eNOS (p-eNOS) (1:500, cat. no. ab76199), eNOS mouse monoclonal antibody (1:1,500; cat. no. ab76198), and $\beta$-actin (cat. no. ab8226) overnight at 4°C, followed by incubation with HRP-conjugated anti-mouse immunoglobulin G (1:2,000; cat. no. ab6789) secondary antibodies for 1 hour at room temperature. The proteins were analyzed using an enhanced chemiluminescence kit (Sigma-Aldrich) and were quantified by Image J software.

### Statistical Analysis

Data were analyzed by GraphPad 5.0 (Prism; La Jolla, CA). Quantitative data were expressed as mean ± SD. Analysis of variance (ANOVA) was employed to compare groups. A $P$ value of $<.05$ was considered statistically significant.

## RESULTS

### In Vivo Vaginal Blood Flow

The results of vaginal blood flow for different groups are shown in Figure 1. As depicted, significantly lower PF (524.89 ± 74.37) and AUC (9,207.63 ± 1,634.65) were observed in the control group, possibly indicating that the adult mice represented a menopausal depression model. Compared to the control group, all treated groups demonstrated notable increased blood flow ($P < .05$). The mice in the high-dose groups showed remarkably enhanced vaginal blood flow, as evidenced by the PF data (1,419.43 ± 165.45) and AUC (22,976.03 ± 2,143.56).

### Hormone Levels

The serum $E_2$, FSH, LH, progesterone, and testosterone concentrations are shown in Figure 2. The serum $E_2$ level was lower in the control group, but the FSH and LH levels were significantly higher in the control group, revealing successful disordering of sex hormones in the adult mice. Compared to the control group, all treated groups showed significantly enhanced serum $E_2$ ($P < .05$) and decreased FSH and LH levels ($P < .05$); however, there were no significant difference in progesterone and testosterone among the different groups.

### Histologic Analysis

H&E staining was used to observe the histologic changes of vaginal tissue. The effect of SI on vaginal and ovarian tissues of mice was investigated, and the results are shown in Figure 3. The number of mature follicles was remarkably increased after treatment with SI, indicating that SI promoted the maturation of ovarian follicles. Masson's trichrome staining, widely used in qualitative histology, is a common method for distinguishing cells from connective tissue and estimating the volume of connective tissue fibers.[18] Generally, it results in dark brown to black stained nuclei, red cytoplasm, and blue collagen and muscle fibers. After Masson's trichrome staining, a network of vaginal tissue with blue collagen and red muscle fibers became clearly visible in the

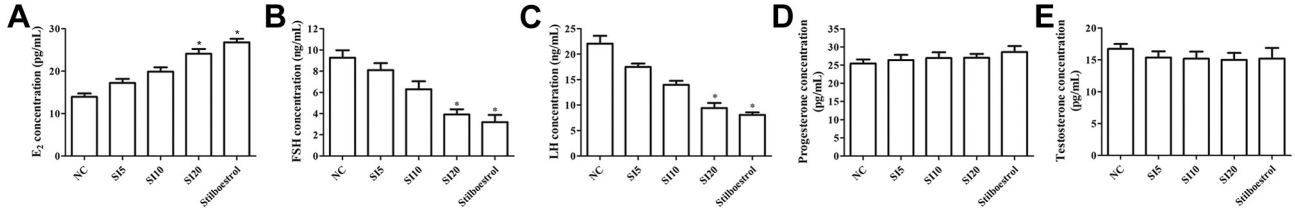


**Figure 2.** Levels of hormones in (A) serum estradiol ($E_2$), (B) follicle-stimulating hormone (FSH), (C) luteinizing hormone (LH), (D) progesterone, and (E) testosterone were examined by enzyme-linked immunosorbent assay. NC = negative control. $^{*}P < .05$, $^{**}P < .01$

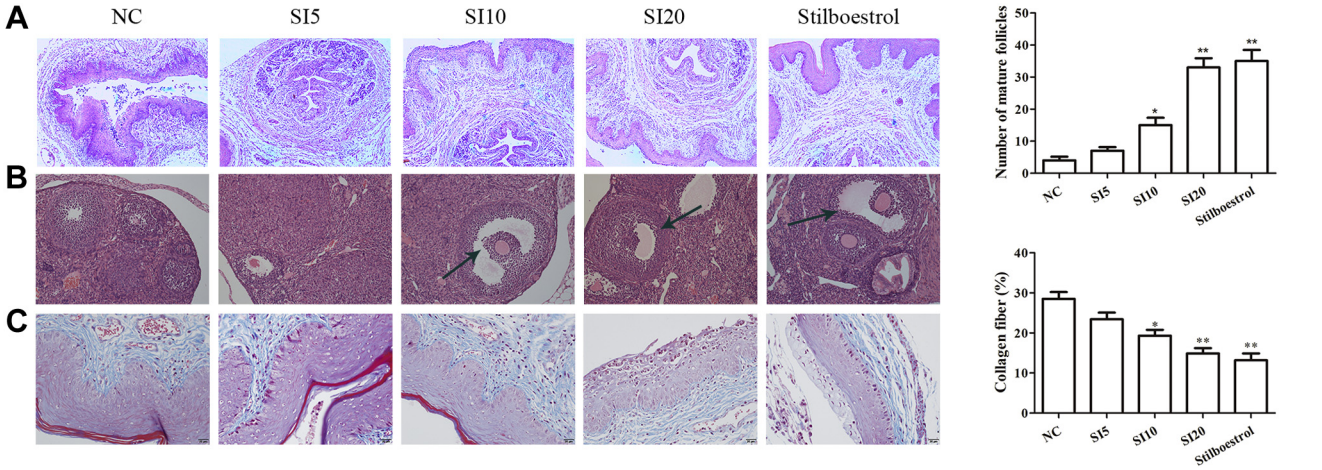


**Figure 3.** (A) Hematoxylin and eosin (H&E) staining was used to assess histologic changes of vaginal and ovarian tissue (20× magnification). (B) Arrows in the images represent the mature follicle. (C) Masson's trichome stain was used to assess the vaginal tissue histology (400× magnification). NC = negative control.

perimenopausal mice. After treatment with SI, decreased collagen was observed compared with the control group (Figure 3C).

## Soy Isoflavone Activated the eNOS Pathway

To detect the effect of SI on the eNOS pathway, western blot assays were applied to determine the protein levels of eNOS and p-eNOS. As shown in Figure 4, no significant changes in the protein expression of eNOS were observed in the different groups; however, SI supplementation increased the protein level of eNOS in a dose-dependent manner when compared with the control group, indicating that SI improved sexual function through activation of the eNOS pathway.

## Soy Isoflavone Upregulated eNOS Through Estrogen Receptor

Crosstalk with estrogen receptor has been well documented, revealing that destruction of estrogen receptor by ICI182780 (fulvestrant, a nonselective antagonist of estrogen receptor) may be a better strategy than blockade of estrogen biosynthesis or antagonism with tamoxifen.[20] Therefore, we evaluated the combined effect of ICI182780 and SI on sexual dysfunction in vivo. It was shown that administration of SI improved sexual function in perimenopausal mice by increasing vaginal blood flow and regulating hormone levels. However, treatment with 0.1 $\mu M$ ICI182780 reversed the SI-induced improvement in sexual function by reducing vaginal blood flow and hormone levels (Figure 5A and B). Nevertheless, ICI182780 alone had no effect on the SI-induced improvement of sexual function compared with the control group. Importantly, the protein expression levels of p-eNOS in mice treated with SI + ICI182780 were lower than those treated with SI alone (Figure 5C). Additionally, there was no significant difference in the expression levels of p-eNOS between the ICI182780 group and the control group. Furthermore, the results demonstrated that ICI182780 suppressed the SI-induced increase in eNOS protein levels. Altogether, we found an inverse correlation between estrogen receptor and eNOS. These results strongly confirmed that SI mitigated sexual dysfunction in perimenopausal mice through the estrogen receptor/eNOS pathway.

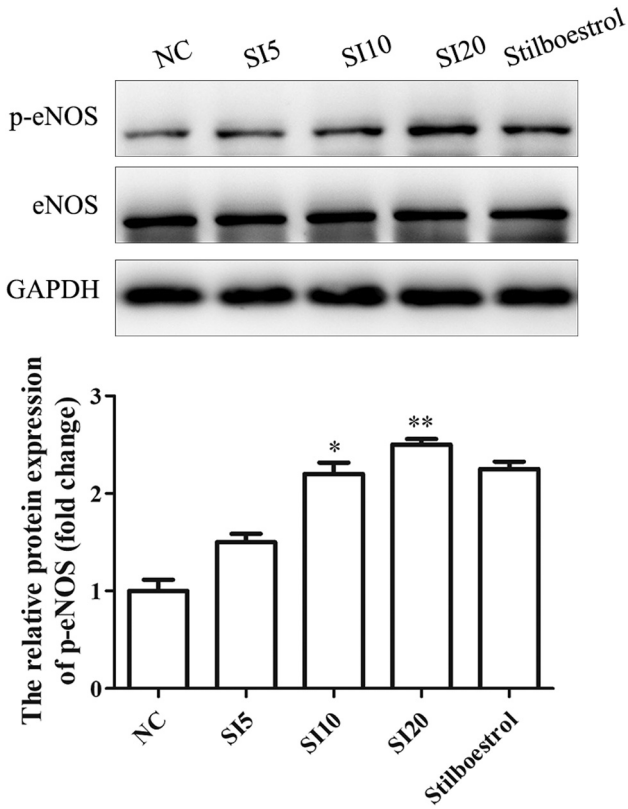


**Figure 4.** Western blot was used to assess the effect of soy isoflavone on protein levels of endothelial nitric oxide synthase (eNOS) in mice vagina in each group. NC = negative control. $^{*}P < .05$, $^{**}P < .01$

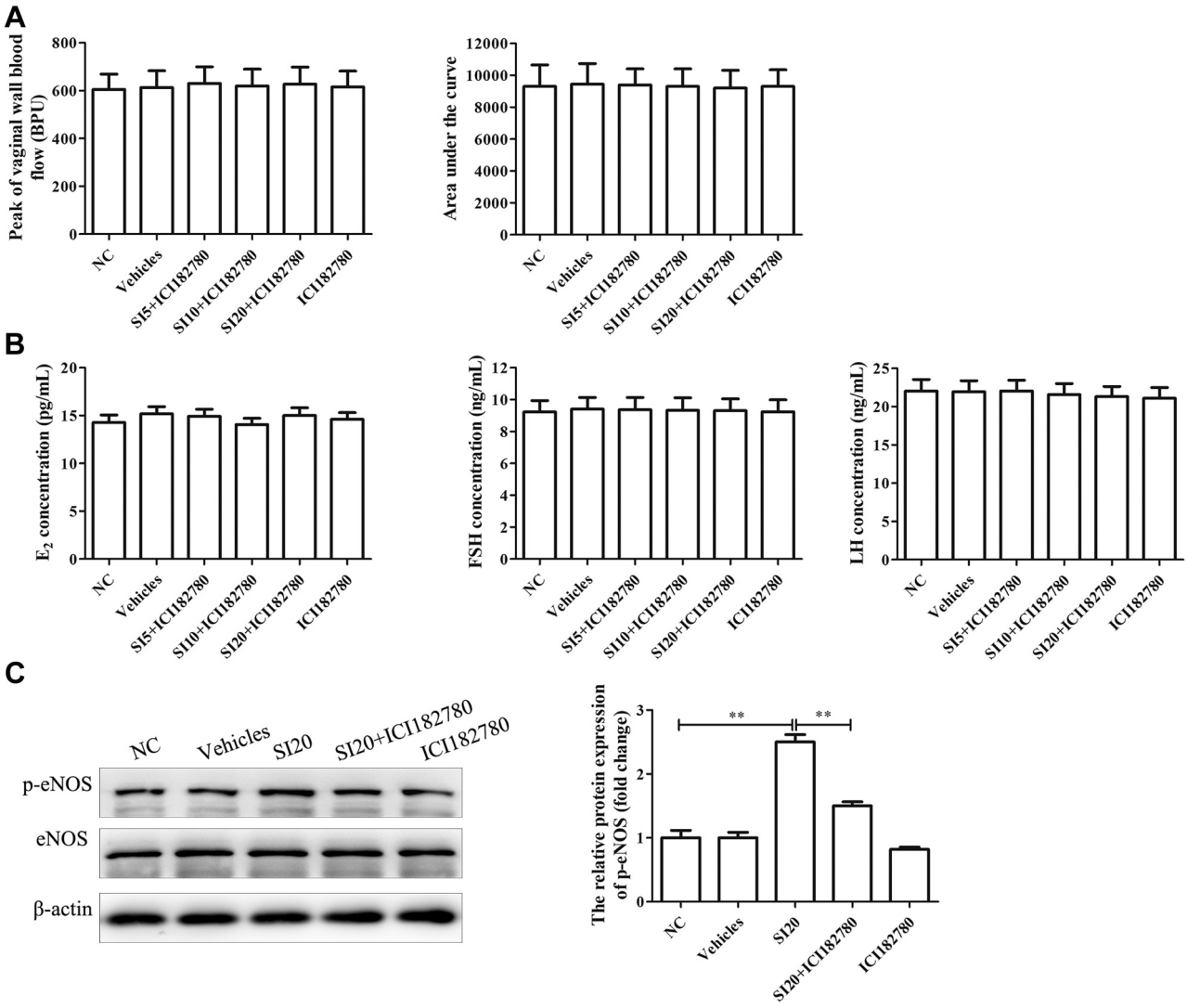


**Figure 5.** Estrogen receptor antagonist ICI182780 reversed soy isoflavone (SI)-induced improvement of sexual function. (A) Vaginal blood flow was measured by laser Doppler flowmetry. (B) Serum hormone levels were detected by enzyme-linked immunosorbent assay. (C) The protein levels of endothelial nitric oxide synthase (eNOS) were analyzed by western blot. $E_2$ = estradiol; NC = negative control. $^{*}P < .05$, $^{**}P < .01$

## DISCUSSION

Although FSD affects most perimenopausal, even reproductive-age, women worldwide and thus is a common medical issue,[21] few studies of FSD have been done. The existing therapy strategies still have many disadvantages; for example, HRT can help maintain sexual function, but it is associated with increasing risks of pulmonary embolism, coronary heart disease, and stroke.[22] Hormone therapy, such as exogenous testosterone replacement, can have a positive impact on sexual function, but it is not effective for all patients. For this reason, it is necessary to explore new pharmacologic treatments that are effective and safe.

As the natural phytoestrogen component of soy, SI has been demonstrated to have a positive impact on many diseases, such as osteoporosis, breast cancer, and ovarian cancer.[23] In the present study, we explored the possible effects of SI on FSD by employing 12-month-old mice as the perimenopausal model, as mice this age have been shown to be similar to perimenopausal women with regard to hormone changes and sexual dysfunction.[24]

An increase in genital blood flow is an important physiological process in female sexual response.[25] In the present study, we observed that mice in the control group had lower vaginal blood flow, but when treated with SI they showed increased vaginal blood flow in response to pelvic nerve stimulation. The results confirmed that after SI treatment sexual function in perimenopausal mice was improved.

Sexual function is also associated with appropriate levels of gonadotropins (FSH and LH) and the ovarian hormones (estrogen and progesterone) responsible for the development and function of estrogen-sensitive organs such as the uterus, vagina, and ovary. Our results indicate that SI elevated the level of $E_2$ and reduced the levels of FSH and LH, but treatment with SI did

not affect the levels of progesterone and testosterone significantly, a finding that is in line with existing publications suggesting that SI can regulate the release of hormones.[26,27] Ovarian development and function play an important role in female sexual function, especially in the period of perimenopause. Ovarian maturation and growth are mainly attributed to the presence of Graafian follicles.[28] The current study showed that SI increased the number of Graafian follicles and modified histologic changes in the vagina.

This study suggests that the NO signaling system plays a key role in regulating sexual function.[29] For example, the NO/cGMP pathway was found to have an effect on vaginal blood flow in rabbits,[30] and eNOS levels in the vagina were reduced in diabetic rats after a decrease of vaginal blood flow.[31] eNOS is regulated by various factors, with estrogen receptor being the most common.[32] Our study revealed that eNOS levels in the vagina were lower in the FSD group. SI significantly increased the mRNA and protein levels of eNOS in the vagina, suggesting that the protective effects of SI on sexual function might be partially mediated by the eNOS pathway. Furthermore, estrogen receptor inhibitor abolished the improved sexual function induced by SI. These results were consistent with a study reporting that SI could regulate eNOS activity.[33]

The current study had some limitations. We employed 12-month-old mice to serve as the perimenopausal model in this study. Although some published reports have demonstrated hormonal changes and sexual dysfunction in 12-month-old mice, we did not measure the related parameters of young mice, and this might be a major limitation. Blood flow determined with laser Doppler flowmetry may exhibit complex variability caused by even small movements of the animal or the probes. Moreover, our study used a relatively small sample that does not have enough statistical power to evaluate differences in eNOS levels.

## CONCLUSIONS

We found that SI improved sexual function in middle-aged female mice by regulating the eNOS pathway. These findings indicate that SI has the potential to improve sexual function.

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## STATEMENT OF AUTHORSHIP

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**(b) Acquisition of Data**
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**(c) Analysis and Interpretation of Data**
Jiehua Ma; Lianjun Pan; Jing Zhang; Yuan Zhu

### Category 2

**(a) Drafting the Article**
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**(b) Revising It for Intellectual Content**
Jiehua Ma; Aixia Zhang

### Category 3

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