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# Sacubitril/Valsartan Improves Sexual Function and Fibrosis of the Clitoral and Vaginal Tissues in Female Spontaneously Hypertensive Rats

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**Abstract:** Female sexual dysfunction is common in hypertension. The effects of sacubitril/valsartan (SAC/VAL) as a potential therapy for hypertension and heart failure have not been studied in relation to sexual function and genital fibrosis in female spontaneously hypertensive rats (SHRs). Thirty female SHRs were administered VAL, SAC/VAL, or saline. Ten normotensive female Wistar–Kyoto (WKY) rats were included in the control group. We assessed estrous cyclicity and sexual behavior in the female rats. In addition, the morphology of clitoral and vaginal tissues was evaluated by histological analyses. Western blotting and enzyme-linked immunosorbent assays were used to assess the levels of fibrotic markers in vaginal and clitoral tissues. Furthermore, the protein levels of phosphatase and tensin homolog deleted from chromosome 10 (PTEN), phosphoinositide-3-kinase (PI3K), and AKT expression were measured by Western blotting. SAC/VAL treatment improved hypertension-induced sexual dysfunction, exhibited as a prolonged estrus phase, increased receptivity and proceptive events, and decreased aggressive events, compared with those of VAL treatment and control SHRs without treatments. In addition, SAC/VAL-treated SHRs had lower levels of fibrotic markers, estradiol, and estrogen receptor α/β than the levels of VAL-treated SHRs or SHRs without treatment. Moreover, SAC/VAL decreased p-PTEN expression and increased p-PI3K and p-AKT expression at the protein level compared with those in VAL treatment alone. VAL and SAC/VAL treatments have significantly increased sexual receptivity and proceptivity, decreased aggressiveness, and improved the fibrosis of vaginal and clitoral tissues in female SHRs. However, SAC/VAL treatment shows more effective results compared with VAL treatment, which may be related to the PTEN/PI3K/AKT pathway.

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**Key Words:** sacubitril/valsartan, female sexual behavior, estrous cycle, fibrosis, spontaneously hypertensive rats

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## INTRODUCTION

Sexual dysfunction is a common complication of hypertension in both men and women and probably has a great impact on overall health and quality of life.[1] Systemic hypertension may result in various functional and structural disorders, which may consequently lead to sexual dysfunction because of its negative effects on the genitals and other closely related organ systems.[2–4] A previous study reported that ≤42.1% of women with hypertension experience sexual dysfunction.[2] Previous meta-analyses have shown that women with hypertension are at risk of sexual dysfunction, and the evaluation of sexual dysfunction needs to be an integral part of the clinical management guidelines for females with hypertension.[5,6] However, studies of female sexual dysfunction in hypertension are still limited in relation to methodology and small sample sizes.

Antihypertensive drugs are mainly classified into 5 categories: diuretics, β-blockers, calcium channel blockers, angiotensin-converting enzyme inhibitors, and angiotensin II receptor blockers (ARBs).[7] Notably, renin–angiotensin–aldosterone system (RAAS) inhibitors, especially valsartan (VAL), improved sexual dysfunction in males with hypertension.[8–10] VAL reduced vascular resistance and improved blood supply to the genital tract by attenuating endothelial dysfunction and preventing the degradation of nitric oxide (NO).[11] Because the sexual response cycle in women is similar to that of men, blood supply can be increased to the erectile tissues of the penis or to the vagina in preparation for intercourse.[12] In addition, VAL has been shown to improve sexual function in men[8] and in women.[13] A randomized controlled trial by Fogari et al [13] reported that VAL improved sexual desire and activity in females with hypertension. According to previous studies, VAL seemed to improve female sexual dysfunction in hypertension.

Sacubitril/valsartan (SAC/VAL) is a new class of cardiovascular drugs with a 1:1 molar ratio of the neutral endopeptidase (NEP) inhibitor prodrug (AHU 377) and VAL.[14,15] Both SAC/VAL and VAL demonstrated important antioxidant and antifibrotic properties.[16] Notably, SAC/VAL was found to be superior to VAL in the treatment of hypertension and heart failure (HF) because of NEP inhibition. NEP inhibition in concert with an ARB reduced hypertension,

ultimately limiting maladaptive cardiac remodeling.[17–19] The innovative concept underlying NEP inhibition displayed a shift from neurohormonal inhibition to neurohormonal modulation.[20] Previous studies reported that NEP inhibitors enhanced genital blood flow without significantly affecting blood pressure.[21,22] However, whether SAC/VAL is superior to VAL in improving female sexual function or fibrosis of vaginal and clitoral tissue is unclear.

Sacubitril attenuates cardiomyocyte cell death, fibrosis, hypertrophy, and impaired myocyte contractility by inhibiting phosphatase and tensin homolog deleted from chromosome 10 (PTEN).[16] PTEN plays a pivotal role in maintaining stratified squamous epithelium and epithelial cell homeostasis.[23] Cell proliferation and differentiation of stratified squamous epithelia must be tightly regulated and coordinated during homeostasis, and the vaginal epithelium is similar to other stratified squamous epithelia. In addition, the PTEN/phosphoinositide-3-kinase (PI3K)/AKT pathway functions to control a myriad of cellular functions involving cell metabolism, proliferation, and survival.[24,25] This pathway is one of the major nongonadotropic insulin signaling pathways that coordinates the activation, growth, and differentiation of follicles.[26] However, little is known about the effect of PTEN/PI3K/AKT signaling on clitoral and vaginal tissues in hypertension.

The biochemistry, physiology, and pharmacology of male sexual function have led to significant improvements in the clinical management of male sexual dysfunction. By contrast, studies on female sexual function have markedly lagged. This lag is partly attributed to the lack of basic science models of sexual responses in female animals. Female spontaneously hypertensive rats (SHRs) have been used as a hypertensive model for studying the impact of a drug on women's sexuality.[27] In this study, we identified for the first time the effect of SAC/VAL on sexual function and the fibrosis of clitoral and vaginal tissues in female SHRs. We also revealed that the effect of SAC/VAL on sexual function and genital fibrosis may be related to the PTEN/PI3K/AKT pathway.

## MATERIALS AND METHODS

### Animals

All animal experiments were approved by the Ethics Committee of Lanzhou University Second Hospital (license number: D2019-098), and all manipulations were performed in accordance with the *National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals*. When reporting animal data, we followed the Reporting *In Vivo* Experiments (ARRIVE) guidelines.[28] A 30-week-old female SHRs (n = 30) and female Wistar–Kyoto rats (WKY, n = 10) were purchased from Lanzhou University Experimental Animal Center. All rats were randomly housed in separate rooms (W 150 × L 210 × H 170 mm) and maintained on a 12-hour light/dark schedule with food and water available ad libitum.[29]

### Cell Culture

Human vaginal epithelial cell line VK2/E6E7 cells was purchased from the American Type Culture Collection (ATCC, VA) and cultured in keratinocyte serum-free medium (Gibco, MA) with bovine pituitary extract (0.05 mg/mL), human recombinant epidermal growth factor (0.1 ng/mL), calcium chloride (44.1 mg/L), and 100 U/mL penicillin and streptomycin (Invitrogen, CA) at 37°C in a 5% $CO_2$ humidified environment.

### Drug Administration

VAL and SAC/VAL were purchased from Novae Artes Pharmaceutical Co, Ltd (Beijing, China). For the *in vivo* studies, dosing was based on pharmacokinetics and pharmacodynamics studies that demonstrated the optimal dosages required to increase atrial natriuretic peptide immunoreactivity in plasma (readout of NEP inhibition) and the ARB concentration.[14] Therefore, after 1 week of acclimatization, a total of 30 female SHRs were randomly divided into 3 groups (n = 10/group): the SHR group (saline, gavage), the VAL group (31 mg/kg/d, gavage), and the SAC/VAL group (68 mg/kg/d, gavage). For both medications, dose selection was based on the previous literature.[30] Ten normotensive female WKY rats of the same age were included in the control group. For the vitro studies, angiotensin II (Ang II) induced VK2/E6E7 cells, which simulated the microenvironment of hypertension. VK2/E6E7 cells were stimulated with 0.1 μM Ang II (A9525, Sigma, MO) for 24 hours. After stimulation, VK2/E6E7 cells were treated with SAC/VAL or VAL for 24 hours. SAC/VAL and VAL were dissolved in 100% dimethyl sulfoxide as 10 mmol stock solutions and stored at −80°C before use. Then, the compound stock solution was diluted in cell culture to a final concentration of 10 μmol/l.[31]

### Blood Pressure Measurement

Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured using a noninvasive tail-cuff system (Kent Scientific, ME) in conscious rats according to the tail-cuff method before, during, and at the end of the treatment. Blood pressure (BP) in SHRs is known to increase age 6 weeks and is maintained during adulthood.[32] BP was only measured once before the experiments. Animals were acclimated to handling and to the restrainer for a training period of 4 days before the measurements. During the procedure, animals were placed in the plastic restrainer on a heating pad to warmup the tail. Three readings were taken for each rat and averaged.

### Vaginal Smear

Vaginal smears were assessed from weeks 6–8 of treatment. All female rats were vaginally lavaged daily with 10 μL of saline solution (NaCl 0.9%), and cells were immediately viewed under a microscope before behavioral assessment. Images were observed with an inverted fluorescence microscope (TE 3000, Nikon Instruments Inc, New York, NY). Vaginal smears were stained using an adaptation of the Papanicolaou (PAP) stain developed by Dr. George N. Papanicolaou.[33] The original PAP staining used a regressive method, where tissue was overstained followed by the removal of excess stain.

### Monitoring the Estrous Cycle by Observation of Vaginal Cytology

For the study of estrus cyclicity, the vaginal fluid of each rat in the 4 groups was collected carefully through a special

device designed for this purpose every morning (10.00–11.00 AM). Three types of cells could be recognized: round nucleated cells (epithelial cells), irregular cells without nuclei (cornified cells), and small round cells (leukocytes). The proportions of each cell type were used for the determination of the phase of estrous cyclicity.[34] The phase of estrous cyclicity was then determined using the following criteria: (1) proestrus with predominantly nucleated epithelial cells; (2) estrus with predominantly cornified epithelial cells; (3) metestrus with the presence of nucleated cells, but predominantly leukocytes; and (4) diestrus with predominantly leukocytes.

## Behavioral Testing

Behavioral testing was assessed from weeks 6–8 of treatment. Evaluation of sexual behavior was performed from 1:00 to 3:00 PM by sexual activity. The stimulus animals were sexually experienced males with ejaculation latencies shorter than 15 minutes in at least 5 consecutive training sessions. Females in the estrus phase (determined by vaginal smears) were then placed in the arena, and the males were allowed to perform. All tests were videotaped for later analysis (assessed by 3 laboratory assistants). The sexual behavior of the female rats involved 2 components: receptive behavior and proceptive events. Female sexual receptivity was assessed by the lordosis quotient (LQ) and the intensity of lordosis (IL). Lordosis was defined as the act of a female lowering her back while raising her tail to expose her genitals to facilitate insertion of the penis.[35] Proceptive behaviors are considered an index of the willingness of females to copulate.[36–38] Furthermore, the rat response was the female's patterning of approaches toward and withdrawal from the male, which regulated or paced its sexual interactions during mating.

The test ended when the female rat received 10 mounts. The LQ was calculated as the number of lordosis postures that females acquired in response to male mounting (number of lordosis/10 mounts). Moreover, the IL was scored on a 3-point scale proposed by Hardy and Debold,[39,40] where (1) 0 = no lordosis; (2) 1 = marginal lordosis, characterized by slight flexing of the spine and raising of the head and hips; (3) 2 = normal lordosis, which consists of flexion of the spine and head to an angle of approximately 30° from the floor with the front paws placed slightly forward and hind legs stiffly straightened; and (4) 3 = exaggerated lordosis, characterized by pronounced spinal flexion with the head at an angle ≥45° from the floor. Preceptive behavior in rats included ear wiggling (rapid alternating movements of the head that provoked vibrations of the ears), hopping (a short leap with the female rats landing on all 4 paws followed by the assumption of a crouching posture), and darting (an abrupt run of several steps).[41,42] In addition, aggressive events in rats comprised the number of attack postures, attacks, lateral postures, on-top postures, kicks, and bites.[41,42]

## Sample Preparation

After behavioral experiments, all animals were euthanized with chloral hydrate (350 mg/kg) during the estrus phase. To measure histology analysis, we performed hematoxylin and eosin (H&E) and Masson's trichrome staining and the clitoral and vaginal tissues were either fixed in 10% buffered formalin or snap frozen in liquid nitrogen. Blood was collected by cardiac puncture. Serum was extracted from the blood samples. To perform enzyme-linked immunosorbent assay (ELISA), tissue homogenates were prepared in ice-cold buffer containing 50 mM Tris and 150 mM NaCl (pH 8.0) and supplemented with 1 mM EDTA, 0.1 mM phenylmethanesulfonyl fluoride, and 1 μg/mL each of aprotinin, leupeptin, and pepstatin (Beyotime Biotech, Shanghai, China) using a homogenizer (OSE-Y50; TIANGEN, Beijing, China), followed by centrifugation at 11,000 g for 15 minutes at 4°C.

## Histological Analysis

The preparations of vaginal and clitoral tissues were cut into the 3-μm cross-sectional sections followed by H&E or Masson's trichrome staining.[43,44] The thickness of the clitoral and vaginal epithelia plus the wall/lumen ratios in the clitoral and vaginal arterioles were evaluated by H&E staining. Collagen deposition was assessed by Masson's trichrome staining. The images were detected using an inverted fluorescence microscope, and the analysis was performed using ImageJ software (National Institutes of Health, MD).

## Enzyme-Linked Immunosorbent Assay

ELISA was performed to determine the levels of collagen (Col)-I, Col-III, and α-smooth muscle actin (SMA) production in vaginal and clitoral tissues (Jianglai, Shanghai, China). The levels of estradiol, estrogen receptor (ER) α, ER β, and androgen were evaluated in the serum (Jianglai, Shanghai, China). Absorbance was measured at 450 nm and 570 nm using an ELISA reader (Infinite M200; Tecan, Männedorf, Switzerland), and the readings at 570 nm were subtracted from the reading at 450 nm.

## Western Blotting

The samples were lysed using RIPA buffer (Beyotime Biotech, Shanghai, China). Protein extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Immobilon P, Millipore, Burlington, MA). Five percent skim milk with Tris-buffered saline containing 0.1% Tween was used to block the blots for 1 hour at room temperature. The membranes were incubated with primary antibodies at 4°C overnight, followed by incubation with secondary antibodies at 37°C for 1 hour. The antibodies included anti-Col-I (ab260043, Abcam, 1:1000), anti-Col-III (ab184993, Abcam, 1:1000), anti-α-SMA (9272, Cell Signaling Technology, 1:1000), anti-p-PI3K (17,366, Cell Signaling Technology, 1:1000), anti-p-PTEN (9551, Cell Signaling Technology, 1:1000), anti-p-AKT (4058, Cell Signaling Technology, 1:1000), anti-PI3K (4257, Cell Signaling Technology, 1:1000), anti-PTEN (ab170941, Abcam, 1:1000), anti-AKT (9272, Cell Signaling Technology, 1:1000), anti-ER α (ab32063, Abcam, 1:1000), anti-ER β (ab196787, Abcam, 1:1000), antiandrogen receptor (ab108341, Abcam, 1:1000), and β-actin (4970, Cell Signaling Technology, 1:1000) proteins for both clitoral and vaginal tissues from all the rats in this study. The protein level of β-actin was used as a loading control. Finally, membranes were washed again and incubated in chemiluminescent ECL substrate (Fisher Scientific,

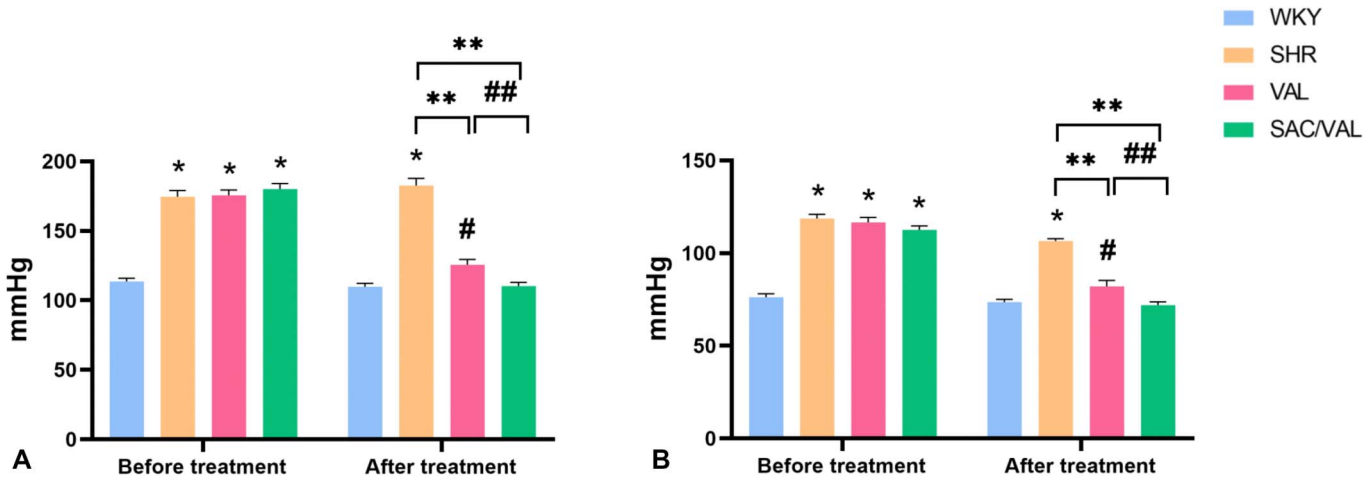


**FIGURE 1.** Effect of SAC/VAL on blood pressure. A, The level of systolic blood pressure (n = 10) and (B) the level of diastolic blood pressure (n = 10). WKY–Wistar rats, SHR-SHRs without treatments, VAL-SHR with valsartan treatment, and SAC/VAL-SHR with sacubitril/valsartan treatment. SHR-spontaneous hypertensive rats. Mean ± SEM, #$P < 0.05$, *$P < 0.01$ versus the WKY group, **$P < 0.01$, ##$P < 0.05$.

Waltham, MA). Images were analyzed by ImageJ software and normalized to β-actin.

## Statistical Analysis

For *in vivo* experiment, data obtained from the experiments are expressed as the mean ± standard error of the mean (SEM). The sexual behavior parameter data were abnormally distributed according to Gaussian distribution analysis, which was performed by Kruskal–Wallis analysis of variance followed by the Mann–Whitney *U* test.[45] Other data were normally distributed and analyzed using 1-way analysis of variance and Tukey's post hoc test. All measurements were performed in triplicate. For in vitro experiment, data obtained from the experiments are expressed as the mean ± standard deviation (SD). Distributed *P* values less than 0.05 or 0.01 were considered significant. All analyses were performed with GraphPad Prism 6.0.

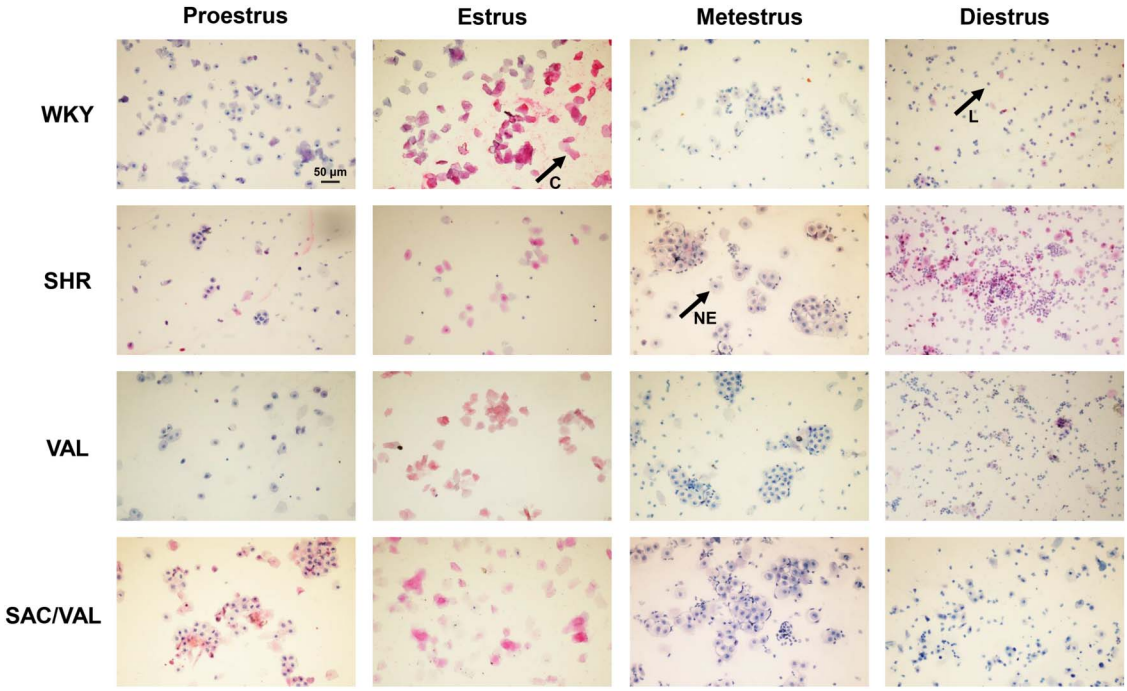


**FIGURE 2.** Photomicrographs showing the changes in cytological properties of proestrus, estrus, metestrus, and diestrus phases of a representative estrus cycle in 4 groups (n = 10): WKY–Wistar rats, SHR-SHRs without treatments,VAL-SHR with valsartan treatment, and SAC/VAL-SHR with sacubitril/valsartan treatment. SHR-spontaneous hypertensive rats, NE-nucleated cell, C-cornified cell, L-leucocytes.

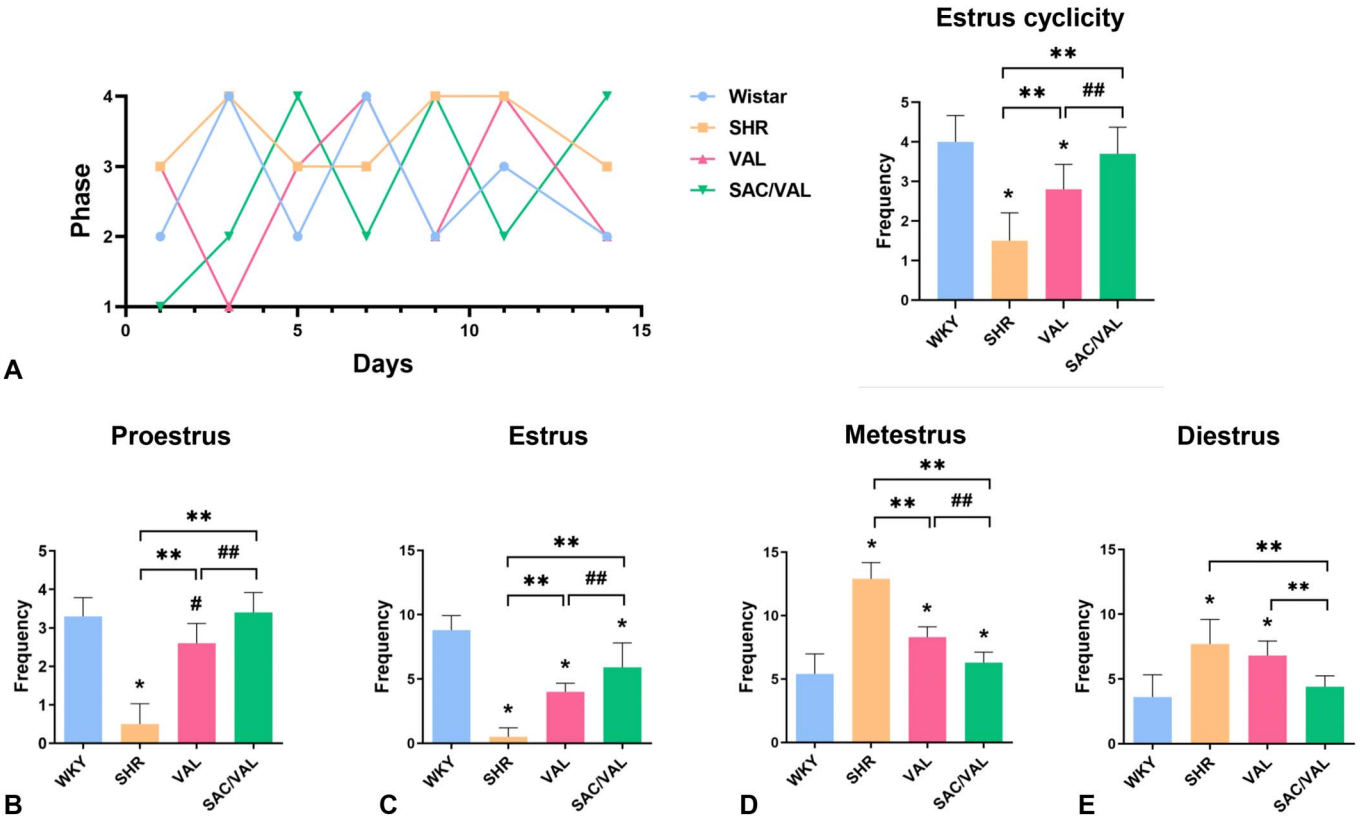


**FIGURE 3.** Effect of SAC/VAL on estrous cycle. A, The cyclicity of estrous cycle (n = 10), (B) the phase of proestrus (n = 10), (C) the phase of estrus (n = 10), and (D) the phase of diestrus (n = 10). WKY–Wistar rats, SHR-SHRs without treatments, VAL-SHRs with valsartan treatment, and SAC/VAL-SHRs with sacubitril/valsartan treatment. Mean ± SEM, #$P < 0.05$, *$P < 0.01$ versus the WKY group, **$P < 0.01$, ##$P < 0.05$.

## RESULTS

### Effects of SAC/VAL on BP

To evaluate the effect of SAC/VAL on BP, we measured BP by using a noninvasive tail-cuff system. Before treatments, increased BP was noted in the untreated SHR controls, VAL-treated SHRs, and SAC/VAL-treated groups, in contrast to the WKY group. There was no difference among the 3 SHR groups. After 8 weeks of treatment, both SAC/VAL and VAL administered alone significantly lowered the SBP and DBP in the SHRs compared with SHRs without treatment (Figs. 1A, B). In addition, SAC/VAL achieved significantly greater SBP and

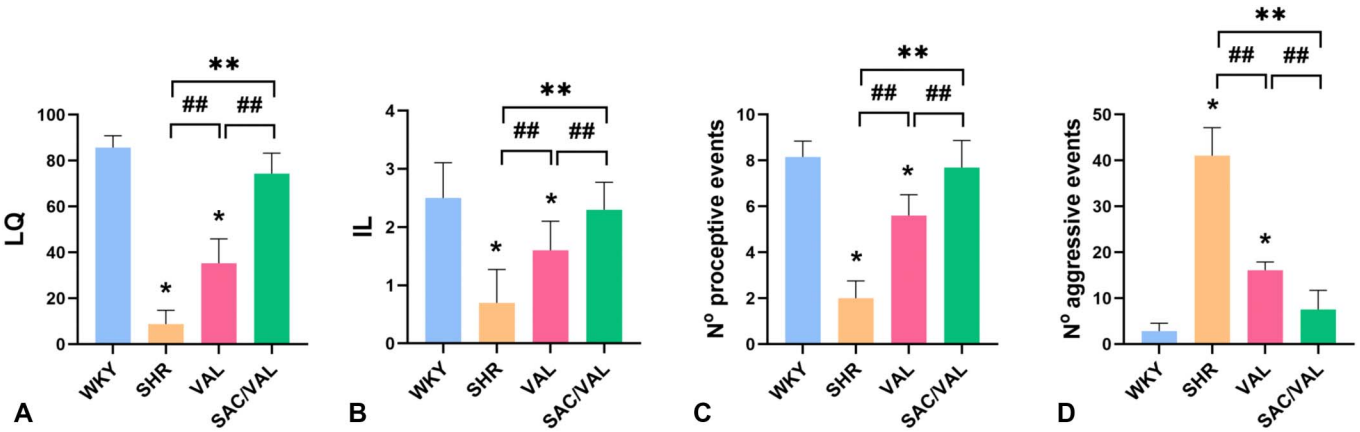


**FIGURE 4.** Effect of SAC/VAL on female sexual behavior. A, LQ (n = 10), (B) IL (n = 10), (C) the number of proceptive events (n = 10), and (D) the number of aggressive events (n = 10). WKY–Wistar rats, SHR-SHRs without treatments, VAL-SHRs with valsartan treatment, and SAC/VAL-SHRs with sacubitril/valsartan treatment. Mean ± SEM, *$P < 0.01$ versus the WKY group, **$P < 0.01$, ##$P < 0.05$.

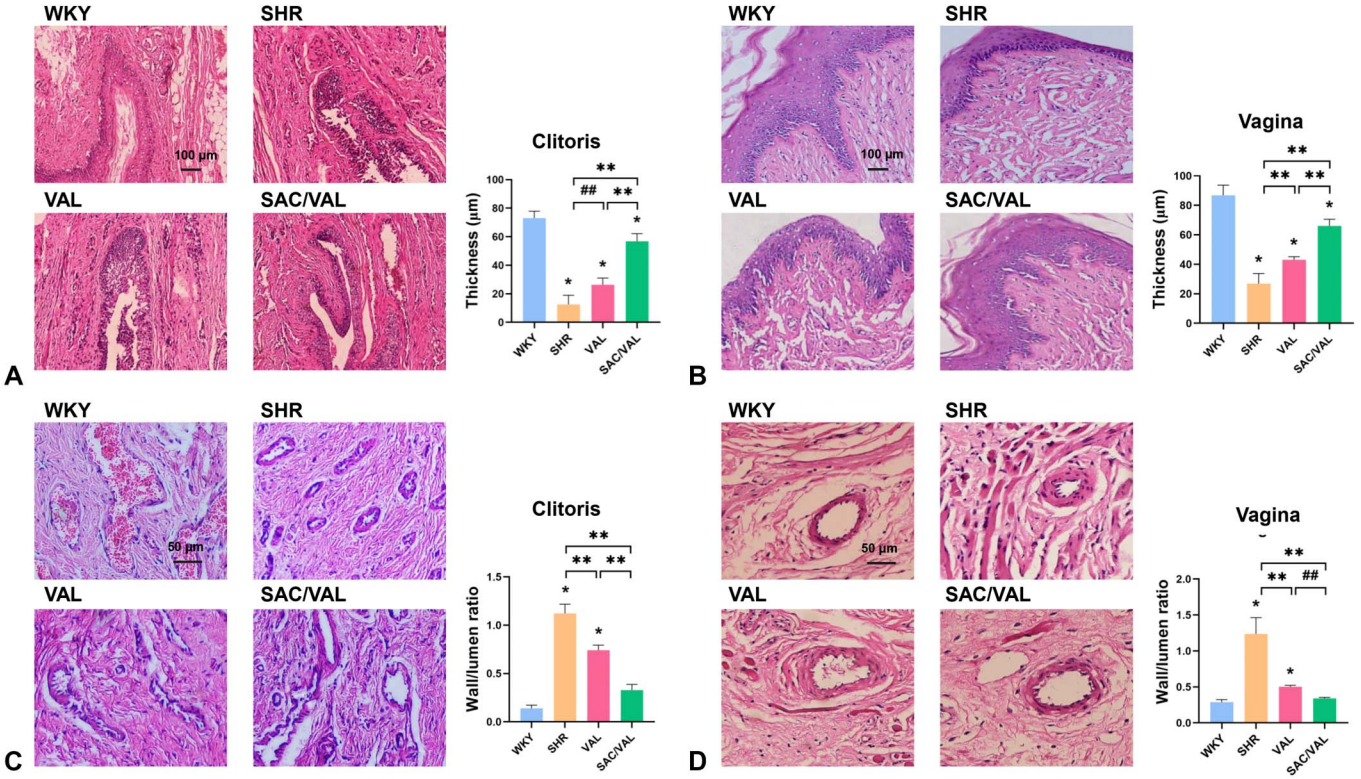


**FIGURE 5.** Effects of SAC/VAL on histology of clitoral and vaginal tissues. A, The thickness of clitoral epithelium (n = 5), (B) the thickness of vaginal epithelium (n = 5), (C) the wall/lumen ratio in clitoral arterioles (n = 5), and (D) the wall/lumen ratio in vaginal arterioles (n = 5). WKY–Wistar rats, SHR-SHRs without treatments, VAL-SHRs with valsartan treatment, and SAC/VAL-SHRs with sacubitril/valsartan treatment. Mean ± SEM, *$P < 0.01$ versus the WKY group, **$P < 0.01$, ##$P < 0.05$.

DBP reductions than VAL after 8 weeks of treatment (Figs. 1A, B). There was no significant difference in BP between the WKY and SAC/VAL-treated groups after a 8-week treatment. The results showed that SAC/VAL administration had a significantly greater BP reduction than VAL administration.

## Effects of SAC/VAL on Vaginal Cytology and Estrous Cyclicity

To evaluate the effect of SAC/VAL on estrous cyclicity, we performed vaginal smears. The estrous cyclicity had 4 phases: proestrus, estrus, metestrus, and diestrus (Fig. 2). In the proestrus phase, the nucleated cells with pink cytoplasm showed dark blue to purple–stained granulated nuclei. The estrus stage showed cornified cells with sheets and clumps and was stained pale orange and pink. Some nucleated cells in the estrus phase had pink, blue, or orange cytoplasm and dark blue–stained nuclei. During the metestrus phase, the leukocytes were stained blue and the nucleated cells had a distinct pale blue border surrounding the cell membranes and dark blue–stained nuclei. The diestrus stage represented leukocytes that were stained dark blue to blue–purple, and the nucleated cells had large opaque nuclei with pink or blue cytoplasm.

During the estrus phase, the intensity of cornified cells was decreased in SHRs without treatments compared with the WKY group, indicating that hypertension had a negative effect on the cornified layer. Moreover, SHRs without treatment had lower estrus cyclicity ($P < 0.01$) and estrus phase ($P < 0.01$) than those in the WKY group during the 21-day period (Figs. 3A, C). However, SAC/VAL-treated SHRs exhibited an increased intensity of cornified cells at the estrus phase compared with SHRs with VAL treatment or without treatment (Figs. 3A, C). In addition, increased estrous cyclicity ($P < 0.05$), proestrus stage ($P < 0.05$), and estrus phase ($P < 0.05$) were observed in SAC/VAL-treated SHRs compared with VAL-treated SHRs (Figs. 3A, C). By contrast, SAC/VAL-treated SHRs had shorter phases of metestrus ($P < 0.05$) and diestrus ($P < 0.01$) than those of SHRs with VAL treatment or without any treatment (Figs. 3D, E). These data suggested that hypertension aggravated estrous cyclicity and the estrus stage. However, SAC/VAL extended (improved) the above indices.

Taken together, these data suggest that hypertension decreased estrus cyclicity and the estrus phase, but increased the diestrus phase. However, SAC/VAL administration extended the estrus stage and increased the intensity of cornified cells at the estrus stage, which is considered a positive effect.

## Effects of SAC/VAL on Female Sexual Behavior

To examine the effect of SAC/VAL on female sexual behavior, rats were observed during the estrus stage. The

receptivity, LQ, and IL were considered as the rhythm of sexual interaction by female rats. The levels of LQ ($P < 0.01$) and IL ($P < 0.01$) were significantly lower in SHRs without treatment than in normotensive WKY rats, which showed that hypertension decreased receptive behavior (Figs. 4A, B). However, the 2 treatment groups exhibited increased (improved) receptive behavior compared with SHRs without treatment. SAC/VAL-treated SHRs had higher levels of LQ ($P < 0.05$) and IL ($P < 0.05$) than those of VAL-treated SHRs (Figs. 4A, B). Similar results were found for the proceptive events (Fig. 4C). SHRs without treatment had decreased proceptive events, including decreased wiggling, hoping, and darting. SHRs treated with SAC/VAL had a higher level of proceptivity ($P < 0.05$) than that of SHRs treated with VAL. By contrast, a higher level of aggressiveness ($P < 0.01$) was observed in SHRs without treatment compared with the WKY group (Fig. 4D). Notably, SAC/VAL administration decreased aggressive events ($P < 0.05$) compared with events in SHRs with VAL treatment or without treatment. These data demonstrate that hypertension decreases female sexual behavior; however, SAC/VAL treatment improves sexual behavior by increasing receptivity and proceptive events while also decreasing aggressive events.

## Effects of SAC/VAL on the Vaginal and Clitoral Tissues Histology

### Histology of Clitoral and Vaginal Tissues

The clitoris complex consists of a surface mucosa, middle erectile, and proximal connective tissue compartments. The glans is the only external portion of the clitoris and is associated with sexual arousal in a murine model.[46] The epithelium of the glans of the clitoris has an arousal property,[44,45] similar to the epithelium of vaginal tissues.[47] To evaluate the effect of SAC/VAL on the morphology of clitoral and vaginal tissues, we first assessed the thickness of the clitoral and vaginal epithelia. H&E staining showed that the thickness of the clitoral and vaginal epithelia was decreased in SHRs without treatment compared with normotensive WKY rats (Figs. 5A, B). The reduced thickness of the clitoral and vaginal epithelia in SHRs without treatment represents a feature of atrophy, which impairs sexual arousal. VAL and SAC/VAL administrations resulted in an increased thickness of the clitoral and vaginal epithelia compared with SHRs without treatment, indicating a positive effect. Notably, the thickness of the clitoral and vaginal epithelia was enhanced (improved) ($P < 0.01$) in SAC/VAL-treated SHRs than in VAL-treated SHRs (Figs. 5A, B). Therefore, the results show that SAC/VAL improved the thickness of the clitoral and vaginal epithelia.

The middle erectile portion is essentially a vascular tissue with a corpus cavernosum structure. Engorgement ensuing by an inflow of blood results in clitoral stimulation, which is most often paramount in sexual arousal.[48] The wall/lumen ratio represents an early step, possibly the earliest step, in hypertension-associated vascular damage and target organ disease.[49] The blood flow is negatively related to the wall/lumen ratio.[50] To evaluate the clitoral and vaginal vessels, we assessed the wall/lumen ratio. The wall/lumen ratio of untreated SHRs was markedly increased not only in the clitoral vessels but also in the vascular structures from the vagina in comparison with those of WKY rats ($P < 0.01$;

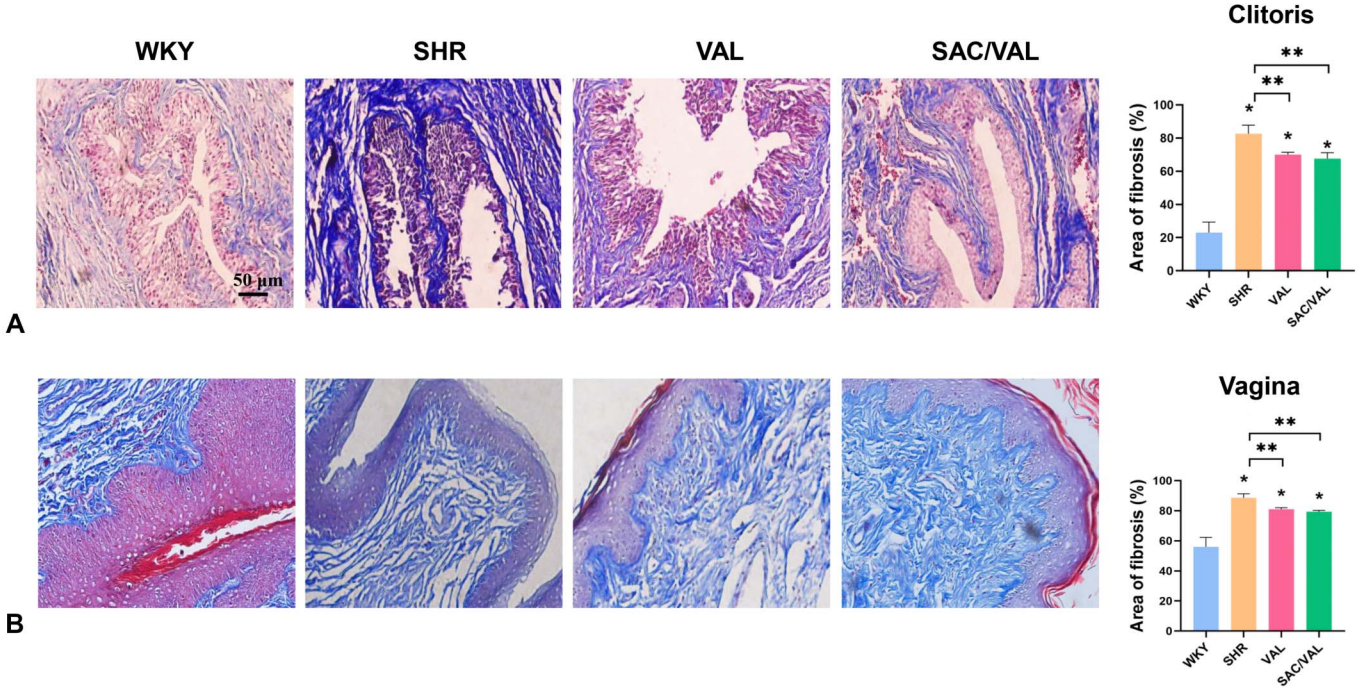


**FIGURE 6.** Masson trichome staining on clitoral and vaginal tissue. A, Fibrosis area on clitoral tissue (n = 5) and (B) fibrosis area on vaginal tissue (n = 5). WKY–Wistar rats, SHR-SHRs without treatments, VAL-SHRs with valsartan treatment, and SAC/VAL-SHRs with sacubitril/valsartan treatment. Mean ± SEM, *$P < 0.01$ versus the WKY group, **$P < 0.01$, ##$P < 0.05$.

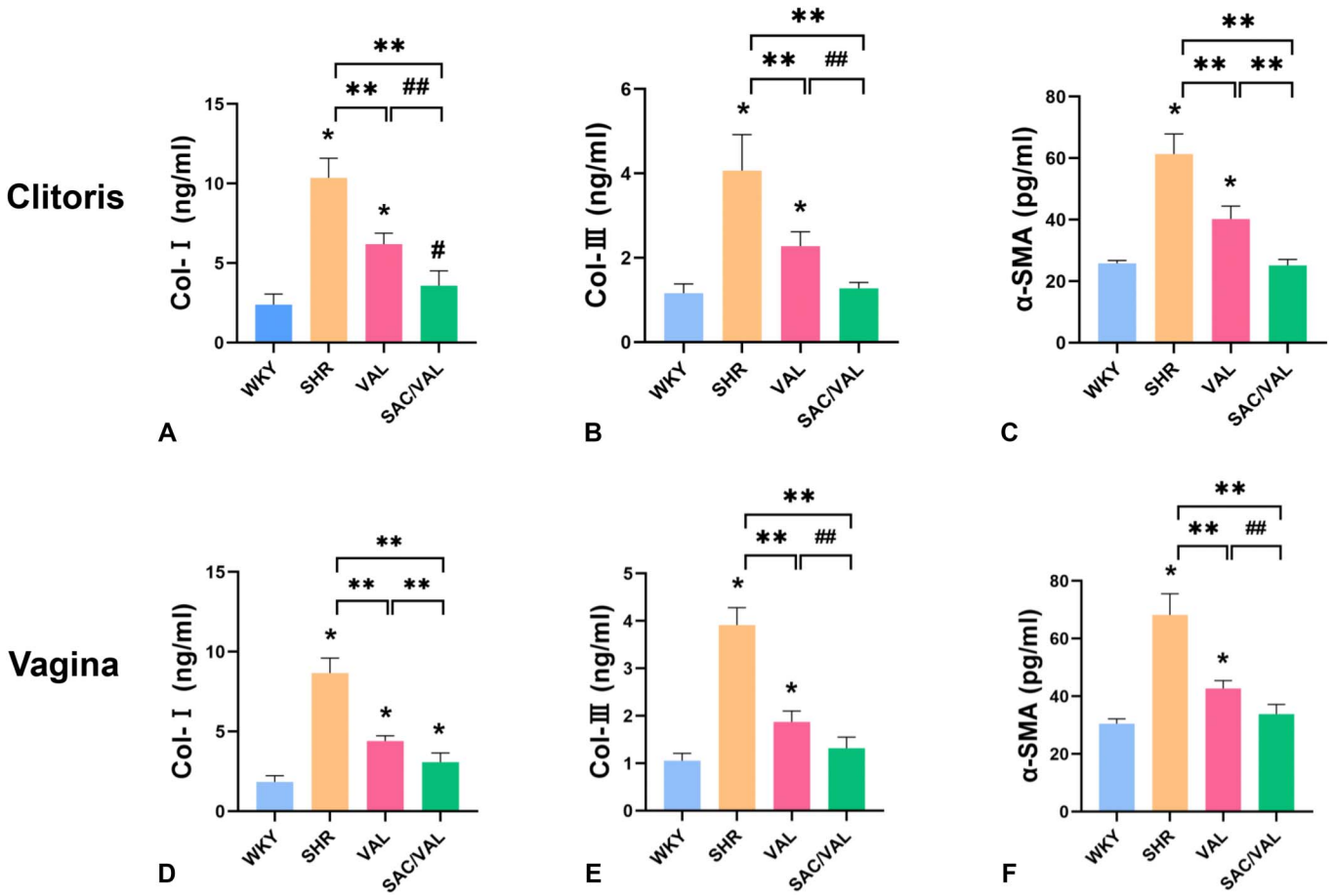


**FIGURE 7.** Effect of SAC/VAL on fibrotic markers (Col-I, Col-III, and α-SMA) by ELISA assay. A–C, The levels of Col-I, Col-III, and α-SMA in clitoral tissue, respectively (n = 5). D–E, The levels of Col-I, Col-III, and α-SMA in vaginal tissue, respectively (n = 5). WKY–Wistar rats, SHR-SHRs without treatments, VAL-SHRs with valsartan treatment, SAC/VAL-SHRs with sacubitril/valsartan treatment, Col-I-collagen I, Col-III-collagen III, and α-SMA-α-smooth muscle actin. Mean ± SEM, #$P < 0.05$, *$P < 0.01$ versus the WKY group, **$P < 0.01$, ##$P < 0.05$.

Figs. 5C, D), suggesting that the lumens were narrowed. Notably, VAL-treated and SAC/VAL-treated rats had lower wall/lumen ratios in clitoral and vaginal tissues than those of SHRs without treatment. In addition, the wall/lumen ratio was decreased (improved) with SAC/VAL treatment relative to VAL treatment ($P < 0.05$), which was displayed in the clitoral and vaginal vessels (Figs. 5C, D). The data suggest that SAC/VAL treatment improved the wall/lumen ratio of the clitoral and vaginal vessels.

### Masson's Trichrome Staining

Masson's trichrome staining is a common method for estimating the volume of connective tissue fibers.[51] After staining, a network of blue collagen and red muscle fibers was observed in vaginal tissue. The ratio of interstitial fibrosis in both clitoral and vaginal tissues was significantly higher in SHRs without treatment ($P < 0.01$) than in the WKY group. VAL and SAC/VAL treatments decreased collagen levels in clitoral and vaginal tissues, but there was no significant difference between these 2 treatment groups (Figs. 6A, B).

Taken together, hypertension decreased the thickness of the vaginal and clitoral epithelium and increased the wall/lumen ratio of clitoral and vaginal vessels; however, SAC/VAL administration significantly improved the above indices (Fig. 7).

## SAC/VAL Improves Fibrotic Markers in Clitoral and Vaginal Tissues

Fibrosis in hypertension results in endothelial dysfunction, increased vasomotor tone, and altered tissue perfusion, which are factors associated with sexual dysfunction.[52] Fibrotic markers include Col-I, Col-III, and α-SMA. To evaluate the effect of SAC/VAL on the fibrosis of vaginal and clitoral tissues, we performed ELISA and Western blotting. SHRs without treatment had higher protein levels of Col-I ($P < 0.01$), Col-III ($P < 0.01$), and α-SMA ($P < 0.01$) in both clitoral and vaginal tissues compared with the levels in the WKY group (Figs. 8, 9). In addition, VAL and SAC/VAL treatments decreased the levels of fibrotic markers in clitoral and vaginal tissues compared with those in SHRs without treatment. Notably, the protein levels of Col-I, Col-III, and

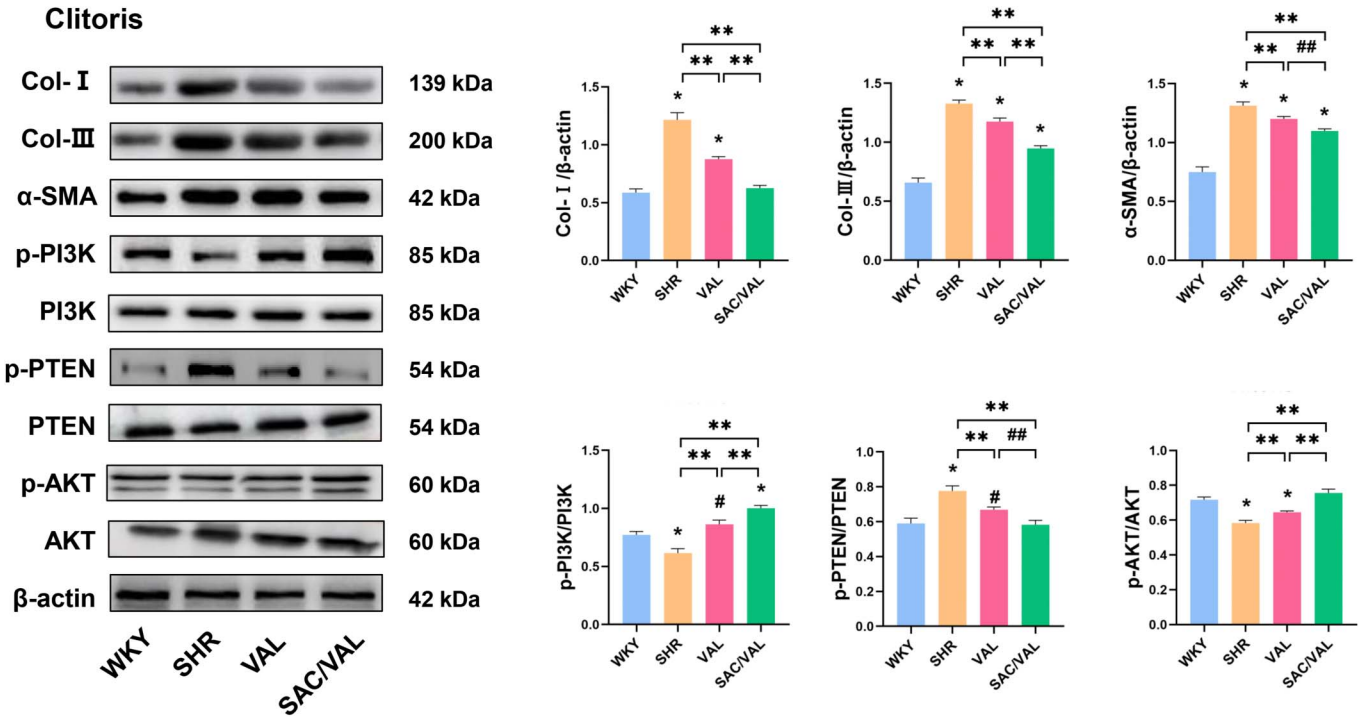


**FIGURE 8.** Effect of SAC/VAL on fibrotic markers and PTEN/PI3K/AKT pathway in clitoral tissues (n = 5). WKY–Wistar rats, SHR-SHRs without treatments, VAL-SHRs with valsartan treatment, and SAC/VAL-SHRs with sacubitril/valsartan treatment. Mean ± SEM, #$P < 0.05$, *$P < 0.01$ versus the WKY group, **$P < 0.01$, ##$P < 0.05$.

α-SMA in vaginal and clitoral tissues were lower in SAC/VAL-treated SHRs than in the VAL-treated SHRs (Figs. 8, 9). However, the protein levels of Col-Ⅰ and Col-Ⅲ in vaginal tissues were not different between VAL-treated SHRs and SHRs without treatment. Similar results were seen for the ELISA. The results showed that the levels of Col-Ⅰ, Col-Ⅲ, and α-SMA secretion from clitoral and vaginal tissues were significantly increased in SHRs without treatments compared with the WKY group (Fig. 7). However, SAL/VAL-treated SHRs had a lower production of fibrotic markers than of VAL-treated SHRs (Fig. 7). These data suggest that SAC/VAL decreased fibrosis of vaginal and clitoral tissues in SHRs.

## SAC/VAL Increases Estradiol, Estrogen Receptor α/β, and Androgen Levels

Estradiol plays an important role in sexual function in female SHRs. To evaluate the effect of SAC/VAL on estradiol, ER, and AR, we performed ELISA and Western blotting. SHRs without treatment had lower levels of estradiol ($P < 0.01$), ER α ($P < 0.01$), ER β ($P < 0.01$), and androgen ($P < 0.01$) than the levels in the WKY group. SAC/VAL and VAL treatments both increased the estradiol, ER α, ER β, and androgen levels compared with those in SHRs without treatment. Notably, SAC/VAL-treated SHRs had higher levels of estradiol, ER α ($P < 0.05$), ER β ($P < 0.05$), and androgen ($P < 0.05$) than the levels of VAL-treated SHRs (Fig. 10). The results from Western blotting were similar to the results from ELISA. SHRs without treatment had lower protein levels of ER α, ER β, and AR in both clitoral and vaginal tissues compared with the levels in the WKY group (Fig. 11). In addition, VAL and SAC/VAL treatments increased the levels of ER α, ER β, and AR proteins in clitoral and vaginal tissues compared with those in SHRs without treatment. Notably, the protein levels of the above parameters in vaginal and clitoral tissues were higher in SAC/VAL-treated SHRs than the levels in the VAL-treated SHRs. Thus, SHRs without treatment had low levels of estradiol, ER α/β, and androgen. SAC/VAL increased the above parameters.

## SAC/VAL Regulates the PI3K/PTEN/AKT Signaling Pathway

SAC/VAL has been reported to inhibit PTEN expression and has been identified as an effective activator of the PI3K/AKT pathway in cardiac fibrosis, epithelium, and follicles.[17,25] Therefore, we examined PTEN/PI3K/AKT pathway-related proteins in clitoral and vaginal tissues by Western blotting. For in vivo studies, lower p-PI3K and p-AKT expression and higher p-PTEN expression at the protein level were observed in SHRs without treatment than in the WKY group. However, SHRs with SAC/VAL treatments had higher protein levels of p-PI3K and p-AKT and lower protein levels of p-PTEN than SHRs with VAL treatment or without any treatment (Figs. 8, 9). For in vitro studies, the expression of the PI3K/AKT/PTEN signaling-related proteins was similar to the results from the in vivo experiments (Fig. 12). Lower p-PI3K and p-AKT expression and higher p-PTEN expression at the protein level were seen in Ang Ⅱ-induced VK2/E6E7 cells than in the control group. However, Ang Ⅱ-induced VK2/E6E7 cells with SAC/VAL

treatment had higher p-PI3K and p-AKT expression and lower p-PTEN expression than Ang Ⅱ-induced VK2/E6E7 cells with VAL treatment or without any treatment. Total AKT expression, total PTEN expression, and total PI3K expression were unchanged in all groups. The results indicate that the effect of SAC/VAL may be related to the PI3K/PTEN/AKT pathway.

## DISCUSSION

Hypertension is a well-known factor that increases cardiovascular disease risk by altering the macrovasculature and microvasculature during target organ injury and causes sexual dysfunction.[13,53] However, the effect of antihypertensive drugs on sexual function has always remained a topic of debate. This study presents evidence of the effect of SAC/VAL treatment on sexual behavior and genital fibrosis (including the clitoral and vaginal tissues) in female SHRs. First, we found that SAC/VAL administration markedly reduced BP relative to that of VAL treatment. Then, we evaluated aspects that had not been studied before, including sexual motivation (using female receptivity and proceptivity), aggressiveness, estrous cycle, genital fibrosis genital morphology, and gonadal hormones. The main findings of this work are that female SHRs have sexual dysfunction, increased genital fibrosis, and decreased gonadal hormones. SHRs with SAC/VAL treatment demonstrated increased sexual motivation, estrous cyclicity, and gonadal hormones and decreased aggressive events compared with those of VAL treatment. Furthermore, activation of the PI3K/AKT pathway and downregulated PTEN expression were found in SAC/VAL-treated SHRs.

The female sexual response is a complex, integrating process, which involves hormonal and vasogenic factors. First, receptive behaviors in the female rat correlate strongly with the animal's degree of proceptivity, thus serving as a useful preclinical measure to test compounds designed to improve libido.[54] Female sexual behavior is intimately linked with the estrous cycle by large-scale presynaptic structural plasticity of the neural pathway that is also required for the behavior.[55] Most females become behavioral receptivity in late vaginal proestrus.[56] Notably, estrogen acts in neurons to trigger the structural plasticity of their projections to elicit sexual receptivity and plays an important role in estrous cycle.[57,58] Hypertension is a medical condition that triggers organ damage and dysfunction, which includes genital damage and decreased gonadal hormones.[59–61] Previous studies showed that estradiol and androgen were greatly reduced in SHRs, which might be linked to reduced expression of ovarian aromatase and decreased uterine weight.[62–64] Meanwhile, insufficient estradiol secretion could also explain the defective follicle development with an increased rate of atresia.[62,63] Second, high blood pressure affects the pelvic region by reducing pelvic blood flow and NO, thus leading to fibrosis of the smooth muscle of the clitoris and the vaginal wall, which leads to the decrease of epithelial thickness and morphologic changes of stratified cornified epithelium.[65] This makes the ability of

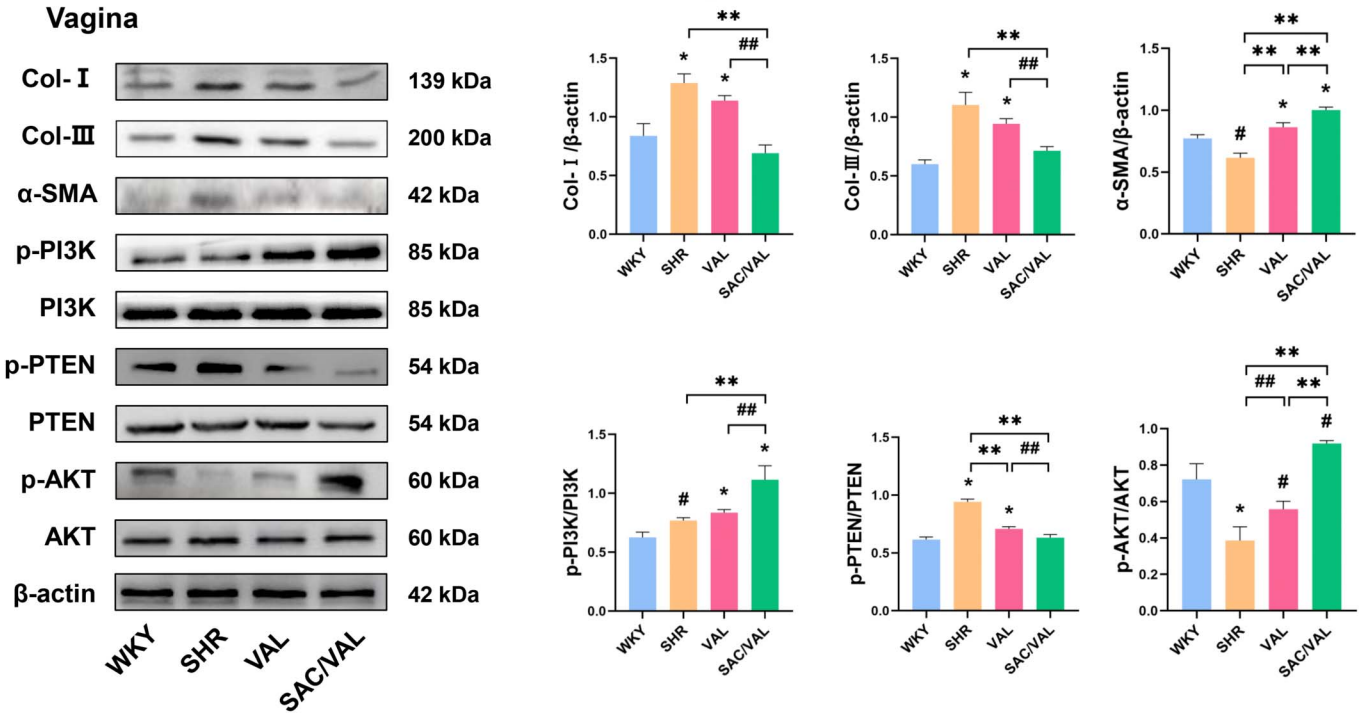


**FIGURE 9.** Effect of SAC/VAL on fibrotic markers and PTEN/PI3K/AKT pathway in vaginal tissues (n = 5). WKY–Wistar rats, SHR-SHRs without treatments, VAL-SHRs with valsartan treatment, and SAC/VAL-SHRs with sacubitril/valsartan treatment. Mean ± SEM, #$P < 0.05$, *$P < 0.01$ versus the WKY group, **$P < 0.01$, ##$P < 0.05$.

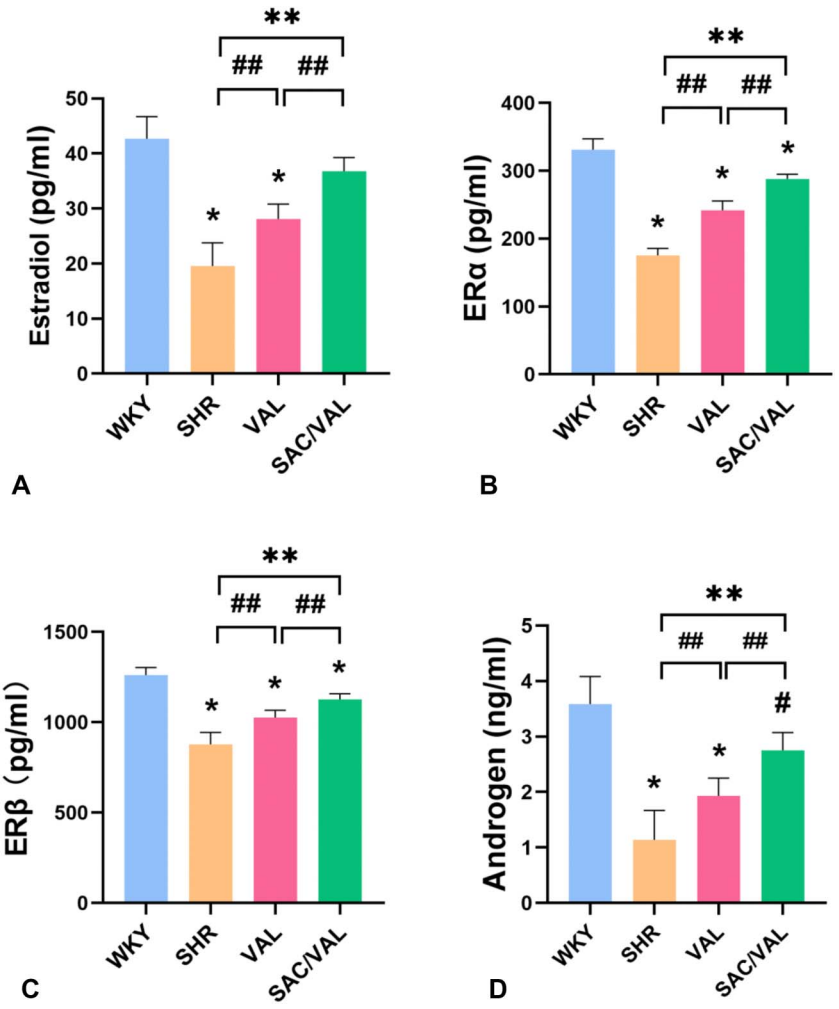


**FIGURE 10.** Effect of SAC/VAL on estradiol, ER α, ER β, and AR on serums by ELISA assay (A–D, respectively) (n = 10). WKY–Wistar rats, SHR-SHRs without treatments, VAL-SHRs with valsartan treatment, and SAC/VAL-SHRs with sacubitril/valsartan treatment; AR, androgen receptor. Mean ± SEM, $*P < 0.01$ versus the WKY group, $**P < 0.01$, $\#\#P < 0.05$.

achieving a response from sexual stimulation extremely difficult.[3] Structural and/or functional abnormalities of the vaginal and clitoral vessels may impair the ability to achieve sexual motivation and represent the underlying cause of female sexual dysfunction. The present results corroborate previous findings. SHRs without treatment had a reduction in estradiol and androgen levels and decreased estrous cycle and estrous stage, which might be linked to decreased sexual receptivity.

Estrogen is important in the maintenance and function of the vaginal epithelium, stromal cells, and smooth muscles of the muscularis and the thickness of the vaginal rugae and vaginal lubrication. ER α and ER β are highly expressed in the basal cells of the stratified squamous epithelium of the vagina which include continuously dividing germinal cells and their offspring because they migrate toward the lumen where they are ultimately shed.[66] High blood pressure decreases the levels of estradiol and ER,[59–61] which affects the epithelial thickness and cornified epithelium. In this regard, this study showed that the alteration of the cornified layer in estrus cycle, which may be related lower levels of estradiol, ER α, and ER β in SHR.

Many factors contribute to sexual dysfunction; thus, its pathological changes are different. α-SMA is an excellent, widely used smooth muscle cell contractile marker.[67] Smooth muscle content reduction in the clitoris has been observed after atherosclerosis or aging in female rabbits.[68] Atherosclerosis-induced arterial insufficiency causes chronic cavernosa ischemia, leading to a decreased smooth muscle/connective tissue ratio.[69] However, Shinde et al[70] reported that α-SMA may be implicated in contraction and remodeling of the extracellular matrix but is not sufficient to induce contraction. α-SMA expression may modulate cellular functions, beyond its effects on contractility. Although data regarding the pathophysiology of sexual dysfunction in hypertensive females are significantly limited compared with those regarding males, there is remarkable evidence supporting functional alterations analogous to those observed in males. Increased blood pressure levels result in significant vascular damage, and thus, hypertension is associated with structural and functional modifications at the level of the endothelium, vascular smooth muscle, and extracellular matrix of blood vessels.[71–73] Previous studies have shown that abundant α-SMA was found in a rat model of hypertension, including in the

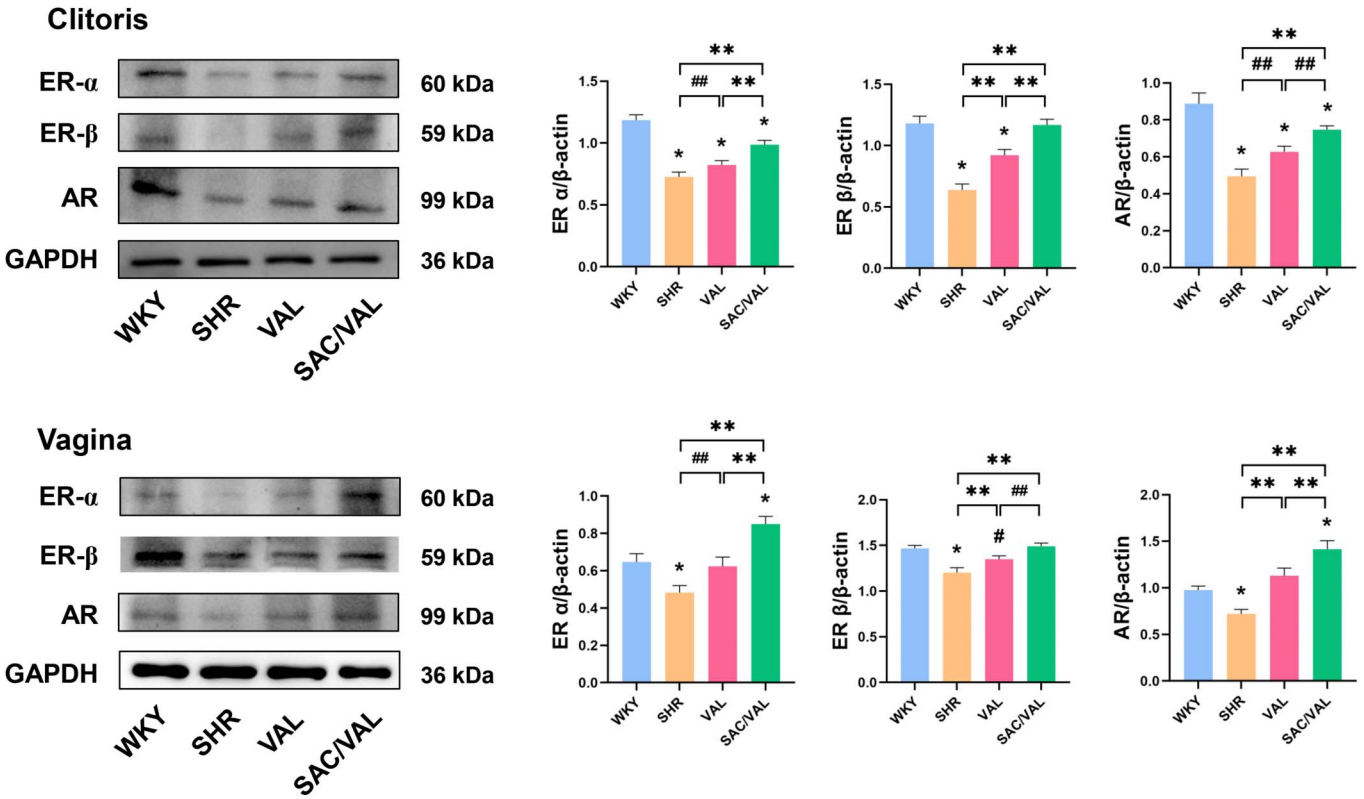


**FIGURE 11.** Effect of SAC/VAL on ER α, ER β, and AR in clitoral and vaginal tissues (n = 5). WKY–Wistar rats, SHR-spontaneous hypertensive rats (SHRs) without treatments, VAL-SHRs with valsartan treatment, and SAC/VAL-SHRs with sacubitril/valsartan treatment; AR, androgen receptor. Mean ± SEM, #$P < 0.05$, *$P < 0.01$ versus the WKY group, **$P < 0.01$, ##$P < 0.05$.

heart,[74,75] kidney,[76] and genitals.[77–80] Consistently, we showed that female SHRs had significantly increased levels of α-SMA in their clitoral and vaginal tissue. Collectively, we proved that hypertension damages female sexual function and increases the levels of α-SMA and collagen deposition (ie, Col-I and Col-III).

Scientific evidence that links antihypertensive drugs to sexual dysfunction is limited, and the question has been raised as to whether the higher rate of sexual dysfunction in hypertensive individuals is due to hypertension or drug treatment of hypertension.[81] However, direct comparison of the effects of major classes of new agents on sexual function

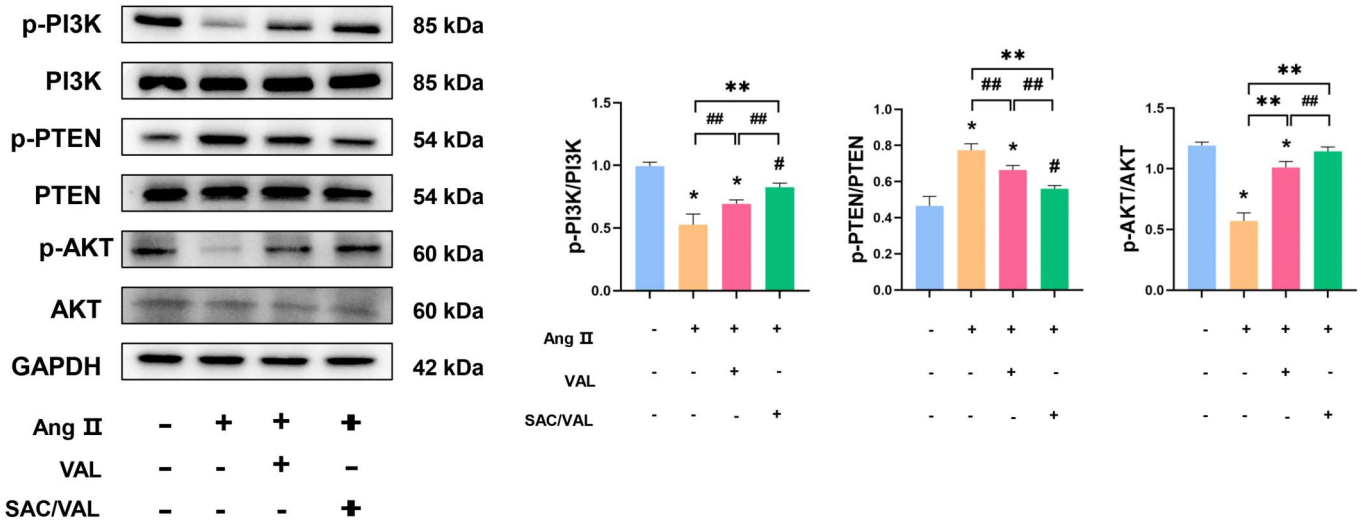


**FIGURE 12.** Effect of SAC/VAL on the PTEN/PI3K/AKT pathway in VK2/E6E7 cells. Ang II-Angiotensin II; VAL-valsartan; SAC/VAL-sacubitril/valsartan. Mean ± SD, #$P < 0.05$, *$P < 0.01$ versus the control group, **$P < 0.01$, ##$P < 0.05$.

has not been made. Previous studies showed that ARBs had the favorable effects on sexual function.[10,13,77,80] The previous clinical study showed that VAL treatment improved sexual function in hypertensive females: the perception of some dimensions of libido, including sexual desire and sexual relations.[13] Some animal experiments also confirmed that RAAS inhibition reversed hypertension-induced alterations in female genital structures.[77,80] Notably, SAC/VAL is a novel combination drug that has proven to be superior to conventional ACE inhibitions or ARBs in hypertension and HF.[30,82–85] The upregulation of RAAS inhibition has been identified as a key pathological pathway resulting in fibrosis, inflammation, and endothelial dysfunction, which is inhibited by the valsartan component. Aside from the important inhibition of valsartan, the peculiarity of SAC/VAL is its capability to enhance many vasoactive peptides. Natriuretic peptides, such as most important vasoactive peptides, are degraded into several potent vasoactive peptides, including adrenomedullin, bradykinin, calcitonin gene-related peptide, natriuretic peptides, substance P, and endothelin-1, and have antiproliferative and antihypertrophic effects.[82,83] In addition, NEP inhibitors regulate female genital arousal and participate in the local control of blood flow, which may be a potential pharmacological strategy for managing female sexual dysfunction.[21,22] The additional beneficial effect of SAC/VAL has been perceived to be due to its effect on the natriuretic peptide system. However, increasing the already elevated natriuretic peptide hormones in HF even further will be of little benefit. Indeed, trials testing other methods of modulating the natriuretic peptide system in HF, such as by supplementing exogenous natriuretic peptides, direct NEP inhibition or dual inhibition of NEP, and angiotensin-converting enzyme, have been disappointing.[86] Therefore, sacubitril monotherapy lacks clinical significance. We designed a VAL group alone without a SAC group. In this regard, both VAL and SAC/VAL treatments improved female sexual function and fibrosis of vaginal and clitoral tissues compared with SHRs without treatment. However, the positive effects of SAC/VAL on sexual function and genital fibrosis were superior to those of VAL alone.

PTEN inhibition mediated by sacubitril's effect on neprilysin seems to be the initiator of a series of cascades that participate in fibrosis, hypertrophy, and inflammation.[16] This study of Miyagawa et al [87] demonstrated that decreased PTEN expression was accompanied by the activation of PI3K/AKT signaling, and AKT was described as a functional mediator of estrogen-induced cell proliferation and differentiation in the mouse vagina and clitoris. Furthermore, the PTEN/PI3K/AKT pathway is one of the major nongonadotropic insulin signaling pathways that coordinates activation, growth, and survival.[88] Activation of AKT expression may result in cell proliferation and squamous differentiation in the vaginal epithelium. In this study, SHRs without treatment displayed lower p-AKT and p-PI3K expression and higher PTEN expression at the protein level than those in the WKY group. SAC/VAL-treated SHRs exhibited increased protein levels of p-AKT and p-PI3K accompanied by decreased protein levels of p-PTEN compared with SHRs without treatments or with VAL treatment. The results show that the effect of SAC/VAL on sexual function and genital fibrosis may be related to the PTEN/PI3K/AKT signaling pathway.

## Limitations and Further Directions

The hormonal regulation of the estrous cycle and sexual behavior is under the neurological control of hypothalamic–pituitary–gonadal axis and ovarian function. Although the limitation of this study is that ovarian function and hypothalamic–pituitary–gonadal axis were not evaluated, this study represented promising results regarding the protective effect of SAC/VAL on sexual function and genital fibrosis. In addition, preclinical and clinical studies have implicated a number of potential neurotransmitter candidates that could be involved in the control of the female sexual response.[89] The melanocortin and oxytocinergic neuronal systems are considered to play pivotal roles in the regulation of female sexual desire and arousal.[90] Further studies should focus on these areas.

Taken together, this study supports the hypothesis that SAC/VAL improved estrous cyclicity, sexual behavior, and fibrosis of vaginal and clitoral tissues in female SHRs, which may be related to the PTEN/PI3K/AKT signaling pathway. The clinical utility and effectiveness of SAC/VAL will need to be confirmed in randomized clinical trials in females with hypertension with sexual dysfunction.

# CONCLUSIONS

This study shows that female SHRs lead to sexual dysfunction, which appears as decreased sexual motivation, increased aggressiveness, shortened estrous stage, and low levels of estradiol and androgen. In addition, female SHRs induce significant morphologic alterations and fibrosis increase in clitoral and vaginal tissues. Both SAC/VAL and VAL treatments improve the above parameters. However, the effect of SAC/VAL is superior to VAL, which is related with the PTEN/PI3K/AKT signaling pathway. These findings showed promising guidance in future clinical trials.

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