Cell J
Cell Journal (Yakhteh)
Homepage: https://www.celljournal.org/
Original Article
Vol 25, No 8, August 2023, Pages: 554-563

β-Sitosterol Inhibits The Proliferation of Endometrial Cells via Regulating Smad7-Mediated TGF-β/Smads Signaling Pathway

Yi Wen, M.D.*, Lili Pang, M.M., Lingxiu Fan, M.M., Yihan Zhou, M.M., Ruonan Li, M.M., Tingting Zhao, M.M., Manli Zhang, M.M.

Departments of Gynecology, Hospital of Chengdu University of Traditional Chinese Medicine, Chengdu, Sichuan, China

Abstract

Objective: To investigate the effect of β-sitosterol on endometrial cells to understand the underlying mechanism.

Materials and Methods: This is a laboratory-based experimental study conducted on animals and cells. Histological assays were performed to determine the effect of β-sitosterol on endometrial cells. The CCK-8 assay was used to assess the inhibitory effect of β-sitosterol on the proliferation of ectopic endometrial stromal cells (hEM15A). Flow cytometry was performed to evaluate the induction of apoptosis by β-sitosterol in hEM15A cells. The transwell invasion assay was conducted to measure the suppression of hEM15A cell migration by β-sitosterol. Western blot analyses were performed to analyze the effect of β-sitosterol on the expression of Smad family member 7 (Smad7) and the activity of transforming growth factor-β (TGF-β1), as well as the phosphorylation of Smad2 and Smad3.

Results: Histological assays showed that β-sitosterol regulates histopathology and induces apoptosis of endometrial cells in vivo. The CCK-8 assay revealed that β-sitosterol could inhibit the proliferation of hEM15A in human endometriosis patients. Flow cytometry showed that apoptosis was triggered by β-sitosterol in hEM15A. The transwell invasion assay indicated that the hEM15A migration under the β-sitosterol treatment group was suppressed. Western blot analyses suggested that β-sitosterol increased the expression of Smad7, decreased the activity of TGF-β1, and reduced the phosphorylation of Smad2 and Smad3. The effect of β-sitosterol was weakened by the silence of Smad7.

Conclusion: The results suggest that β-sitosterol can inhibit the proliferation of endometrial cells and relieve endometriosis by inhibiting TGF-β-induced phosphorylation of Smads through regulation of Smad7.

Keywords: β-Sitosterol, Endometriosis, Smad7, Transforming Growth Factor-β

Citation: Wen Y, Pang L, Fan L, Zhou Y, Li R, Zhao T, Zhang M. β-Sitosterol inhibits the proliferation of endometrial cells via regulating Smad7-mediated TGF-β/smads signaling pathway. Cell J. 2023; 25(8): 554-563. doi: 10.22074/CELLJ.2023.1989631.1230

Introduction

Endometriosis is a gynecological disease caused by women's endometrial tissue metastasis, invasion, and growth outside the uterine cavity. It can lead to severe clinical symptoms such as lower abdomen pain, infertility, and dysmenorrhea, which threaten women's reproductive health, and affect their quality of life and work efficiency (1, 2). According to studies, the proliferation and apoptosis of normal endometrial stromal cells (ESC) in patients with endometriosis are important reasons for decreased endometrial receptivity, difficult embryo implantation, and infertility (3, 4). At present, hormone treatment of endometriosis is inefficient, and the annual recurrence rate after surgical treatment is high (5). Therefore, exploring alternative therapies for endometriosis is expected to alleviate the burden of patients with endometriosis.

There are many causative factors for endometriosis, such as miRNAs which may promote the proliferation, invasion, and metastasis of cancer cells by regulating the expression of upstream target genes, thereby affecting the prognosis of endometrial cancer patients (6). Previous studies have reported that the activity of transforming growth factor-β (TGF-β) in the intimal tissue of endometriosis significantly increased, confirming that the abnormal expression of TGF-β is related to the abnormal growth of ectopic intima and enhanced aggressiveness (7, 8). TGF-β1 is one of the vital cytokines, which has a regulation effect on cell division and proliferation (9). Smad2/3 is the first signal molecule transmitted by the TGF-β1 signal, and they play pivotal roles in the biological effect of TGF-β1 (10). Smad3 is the main signal transmission protein of the TGF-β signaling system to promote adhesion, while smad7 can inhibit the phosphorylation of Smad3 and block the TGF-β signaling system (11). Furthermore, smad7 can inhibit the promoting effect of TGF-β on fibrosis in renal tubular epithelial cells by impairing Smad2 activation (12).

Phytosterols are a class of steroid compounds with multiple biological activities and high clinical application value

Received: 14/February/2023, Revised: 14/May/2023, Accepted: 21/May/2023
*Corresponding Address: Departments of Gynecology, Hospital of Chengdu University of Traditional Chinese Medicine, Chengdu, Sichuan, China
Email: yiwen781108@163.com

Royan Institute
Cell Journal (Yakhteh)

(13). Traditional Chinese herbs rich in β-sitosterol, include *Trifolium repens*, *Houttuynia cordata*, and *Lasia spinosa* (14). The chemical formula of β-sitosterol is $C_{30}H_{52}O$ and its molecular weight is 414.71. It is a white amorphous powder at room temperature and is insoluble in water. Studies have shown that β-sitosterol has various biological activities such as anti-inflammatory, antiproliferative, and anticancer effects (15, 16). In human alveolar epithelial cells, β-sitosterol can inhibit TGF-β1-induced epithelial-mesenchymal transition (EMT) by inhibiting the TGF-1/Smad pathway (17). In addition, the pathogenesis of endometriosis involves EMT, which is a complex process of epithelial cells transforming into mesenchymal cells (18). Overexpression of the *BAMBI* gene encoding the type I receptor of TGF-beta and application of β-sitosterol can inhibit autophagy in non-small cell lung cancer (NSCLC) cells, induce G0/G1 cell cycle arrest, and then inhibit cell proliferation by inactivating the TGF-β/Smad2/3/c-Myc pathway (19). However, the effect of β-sitosterol on the growth of endometrial grafts and the proliferation of hEM15A cells derived from Endometriosis through the Smad7-mediated TGF-β/Smads signaling pathway has received little attention.

The proliferation and apoptosis of ESC play an important role in the pathogenesis of endometriosis. Considering the relationship between Smad7 and cell proliferation, Smad7 could also be a key target for β-sitosterol to inhibit the development of endometriosis. The purpose of this research is to investigate the protective mechanism of β-sitosterol on endometriosis *in vivo* and *in vitro* through the Smad7-mediated TGF-β/Smads signaling pathway.

Material and Methods

Animals and grouping

This is a laboratory-based experimental study conducted on animals and cells. 10-week-old sexually mature C57BL/6 female mice (20 ± 2 g) were all purchased from Chengdu Dashuo Biological Technology Co., Ltd., (Chengdu, China). In this research, all animal experiment operations followed the Animal Experimental Committee and the Ethics Committee of the Hospital of Chengdu University of Traditional Chinese Medicine requirements (No. is 2021DL-02). The animals were housed in an SPF-grade laboratory, fed, and watered ad libitum. Allogeneic endometrial transplantation was used to establish endometriosis models (20). Briefly, mice were given subcutaneous injections of estradiol benzoate (E2, 0.1 mg, Solarbio, China) and anesthetized by intraperitoneal injection of 1% sodium pentobarbital (40 mg/kg). After anesthesia, the donor uterus was removed and isolated under aseptic conditions. The membrane was cut into less than 1 mm^3 pieces and put into serum-free DMEM/F-12 medium. The experimental mice were separated into 3 groups, a control group (endometriosis), β-sitosterol (Meilun, China) high-dose, and low-dose treatment groups, 6 mice per group. In the control group, the harvested uterine tissue was rinsed twice with sterile saline and then cut into pieces, ensuring that the largest diameter fragments were less than 1 mm^3. The uterine fragments were injected intraperitoneally from one donor mouse into recipient mice, and the mice were gavaged with the same amount of normal saline containing β-sitosterol as the treatment groups. The β-sitosterol high-dose (350 μg/kg) and low-dose (35 μg/kg) treatment groups received intragastric administration once a day for 21 consecutive days. After finishing the experiment, the implant was taken out for the next experiment.

H&E assay and TUNEL assay

The tissues of normal endometrium and ectopic endometrium were fixed in 4% paraformaldehyde, and ethanol was used for gradient dehydration. Then, paraffin sections (4 μm) of endometrial tissue were made for H&E staining (Solarbio, Beijing, China). An optical microscope was used to observe the pathomorphological characteristics of the normal uterus endometrium and ectopic endometrium. For Tunel assay, the sections were permeabilized with proteinase-K (Non-specific serine protease) for 20 min and blocked with 5% goat serum for 30 minutes, respectively. Sections were stained with Alexa Fluor 488 (Elabscience, China) for 30 minutes at 37°C and protected from light. Binding Alexa Fluor 488-dUTP to nicked DNA by TdT transferase is one of the common methods to detect apoptosis. Then, the tissues were washed with phosphate buffer saline (PBS), and 1×Equilibration Buffer to immerse the sample. Last, the sections were fixed on slides with a DAPI sealer (Yeasen, China) and were observed through an Olympus BX51 fluorescence microscope (Olympus, Japan).

Immunohistochemical assay

After creating 4 μm paraffin slices, incubating them with primary antibodies for Smad7 (ab216428, Abcam, Cambridge, MA, USA), TGF-β1 (ab215715, Abcam, USA), p-smad2 (ab280888, Abcam, USA), and p-smad3 (ab52903, Abcam, Cambridge, MA, USA) was performed overnight at 4°C. Then, the slices were incubated with an appropriate dosage of biotinylated goat anti-rabbit IgG secondary antibody (D110065, BBILIFE, China) for 30 minutes at 37°C. Finally, the sections were stained with DAB (AR1025, BOSTER, China), and re-stained with hematoxylin (Solarbio, China). The positive cells that appeared under an optical microscope for Smad7/TGF-1/Smad2/3 protein expression were identified and colored yellow or brown. Positive cells were counted using image-Pro+60 image analysis software. The percentage of positive cells=number of positive cells/total number of cells×100.

Cell culture

The ectopic endometrial stromal cell line (hEM15A) from human endometriosis patients was purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). hEM15A cells were cultured in DMEM/high glucose (4.5 g/L D-Glucose) medium (Hyclone, South Logan, UT, USA). Cells were passaged in the logarithmic growth phase. DMEM was supplemented with 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA) and 1% glutamine (Sigma, St. Louis, MO, USA) and incubated at 37°C.

CCK-8 assay

Digestion of the logarithmic growth phase hEM15A cells with 0.25% trypsin, hEM15A cells were collected to adjust the cell concentration to 5×10^4 cells/mL^{-1}. Then, the cells were seeded into a 96-well culture plate with a volume of 200 μL per well at 37°C and 5% CO_2. After 24 hours of cell adhesion, the culture media was swapped out for the wells that contained various concentrations of β-sitosterol (30, 60, 90 μmol/L), and then the plate was incubated at 37°C in 5% CO_2 for 48 hours. 20 μL of CCK-8 (GLPBIO, USA) solution (5 mg/mL) was added to each well, and incubation was continued for 1 hour at 37°C. A microplate reader assessed each well's absorbance (D) value at 450 nm (Thermo Fisher Scientific, Waltham, MA, USA).

Annexin-V/propidium iodide double-staining apoptosis assay

hEM15A cells were collected following treatment with β-sitosterol for 48 hours, and then washed twice with phosphate buffer. 100 μL of 1x binding buffer was taken to resuspend the cells. 1×10^5 cells were used to stain for loss of apoptosis assay. Sequential additions of 5 μL of Annexin V-FITC (Yuanxin, China) and 5 μL of PI (Yuanxin, China) were made, and the mixture was stained after 15 minutes of dark incubation. Within an hour, flow cytometry was utilized to find the cells that had undergone apoptosis.

Transwell invasion assay

A 24-well Transwell chamber (Corning, NY, USA) with 8.0-μm pore membranes covered with Matrigel (BD Biosciences, San Jose, CA, USA) was used to conduct a cell invasion experiment. Briefly, after being suspended in serum-free DMEM, hEM15A cells (1×10^5) were plated in the upper chamber of the Transwell (200 μl). 600 μl of a full medium comprising 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA) was then added to the lower chamber. Transwells were collected 24 hours after incubation, preserved for 30 minutes in -20°C cold methanol, and then stained with 0.5% crystal violet at room temperature for 20 minutes (Solarbio, China). Under an optical microscope (Olympus, Japan), invasive cells were seen.

Western blot assay

RIPA buffer was used to lyse cells and tissues in order to extract the total protein. After the BCA protein detection kit (Abcam, USA) had measured the protein concentration, sodium dodecyl sulfate polyacrylamide gel electrophoresis (10% SDS-PAGE) and electrotransfer were used to estimate the total protein. The protein was transferred to the PVDF membrane, and the membrane was blocked by skim milk solution. The primary antibodies for Smad7 (1:1000, Abcam, UK), TGF-β1 (1:1000, Abcam, UK), p-Smad2 (1:500, Abcam, UK), Smad2 (1:1000, Abcam, UK), p-Smad3 (1:1000, Abcam, UK), Smad3 (1:500, Abcam, UK) were then added, the membrane was sealed with 5% skimmed milk solution, and incubated at 4°C for 12 hours. The membrane was then rinsed with PBST (every 15 minutes) four times before being incubated with the diluted secondary antibody (1:100000, abcam, UK) for 2 hours at room temperature to strengthen the immunological signal detected by the chemiluminescence detection equipment. Optical density analysis was carefully performed using Image J software using β-actin as the reference.

Real-time quantitative reverse transcription polymeras chain reaction

Total RNA was extracted from hEM15A cells using the TRIzol kit (TaKaRa, Japan), and its concentration and purity were assessed using UV spectrophotometry. The sample is deemed eligible when the A260/A280 ratio is between 1.8 and 2.0. Next, total RNA was reversed into cDNA by Real-time quantitative reverse transcription polymeras chain reaction (RT-qPCR) kit steps (TaKaRa, Japan). The primer sequence is shown in Table 1. With *GAPDH* as a reference, the $2^{-\Delta\Delta Ct}$ method was used to calculate *Smad7* relative expression.

Table 1: Primers used in this study

Primer	Prime sequence (5′-3′)
GADPH (Homo)	F: CATCACTGCCACCCAGAAGACTG
	R: ATGCCAGTGAGCTTCCCGTTCAG
SMAD7 (Homo)	F: TTCCTCCGCTGAAACAGGG
	R: CCTCCCAGTATGCCACCAC

Transfection

The siRNA transfection was carried out according to the instructions of lipofectamine 2000™ (Carlsbad, CA, USA). 50 μL of serum-free media was used to dilute one microliter of the transfection reagent before it was incubated at room temperature for 5 min. Then, the cationic vesicles were combined with the diluted siRNA (50 ng) for 20 minutes at room temperature with the diluted transfection reagent. A hundred microliters of RNA cationic vesicles were added to the wells of the cell culture plate, and the plate was then incubated at 37°C with 5% CO_2 for 24 to 48 hours. The siRNAs were synthesized from GenePharma (Shanghai, China), and the sequences are as follows:

si-*SMAD7*(1) sense:
5′-AAGGAAAAAGCCUCUUUCCCC-3′

antisense:
5′-GGAAAGAGGCUUUUUCCUUCU-3′

si-*SMAD7*(2) sense:
5′-AA AUCCAUCGGGUAUCUGGAG-3′

antisense:
5′-CCAGAUACCCGAUGGAUUUUC-3′

Statistical analysis

The research data were statistically analyzed using GraphPad Prism8 software (La Jolla, CA, USA). Measurement data are expressed as mean ± standard deviation ($\bar{x}$ ± SD). Multiple groups underwent one-way analysis, and $P<0.05$ was regarded as statistically significant.

Results

Structure and properties of β-sitosterol

β-sitosterol is a tetracyclic triterpenoid compound with cyclopentane perhydrophenanthrene as the basic skeleton.

Effects of β-sitosterol on the histopathology of the uterus in endometriosis mice

The endometrial tissue of each group of mice was stained by H&E, and the endometrial mucosa injury, proliferation and inflammation were observed under a microscope. In addition, as shown in Figure 1A, in the control group, the cyst-like structure of ectopic endometrial tissue had a structure similar to that of the endometrium under microscopic observation. Pathological changes were degeneration, necrosis, and hyperplasia of endometrioid epithelial cells, neonatal capillary formation, and neutrophil infiltration in the interstitial layer. In summary, pathological changes such as endometrial epithelial cell hyperplasia and neonatal capillary formation in the control group indicated that the cyst-like structure in this group was in the growth stage. Compared with the control group, the β-sitosterol low-dose (35 μg/kg) group and the β-sitosterol high-dose (350 μg/kg) group had more endometrialoid epithelial cell degeneration and necrosis. And the β-sitosterol high-dose (350 μg/kg) group thinned the propria layer and significantly reduced cells. These results indicate that the cyst-like structure growth was inhibited in the β-sitosterol low- (35 μg/kg) and high-dose (350 μg/kg) groups, among which the cyst-like structure growth of β-sitosterol high-dose (350 μg/kg) group was relatively the weakest, and of the β-sitosterol low-dose (35 μg/kg) group was relatively poor. TUNEL staining was used to detect the effect of β-sitosterol on endometrial cells. As shown in Figure 1B and C, the control group have no obvious green fluorescence, the cells are not stained by Alexa Fluor 488, and there is no apoptosis. But when β-sitosterol was used in both low (35 μg/kg) and high (350 μg/kg) levels, the green fluorescence is obvious, and the endometrial cells appear to have undergone apoptosis. Apoptotic cells were increased in the high-dose (350 μg/kg) β-sitosterol compared to the low-dose group.

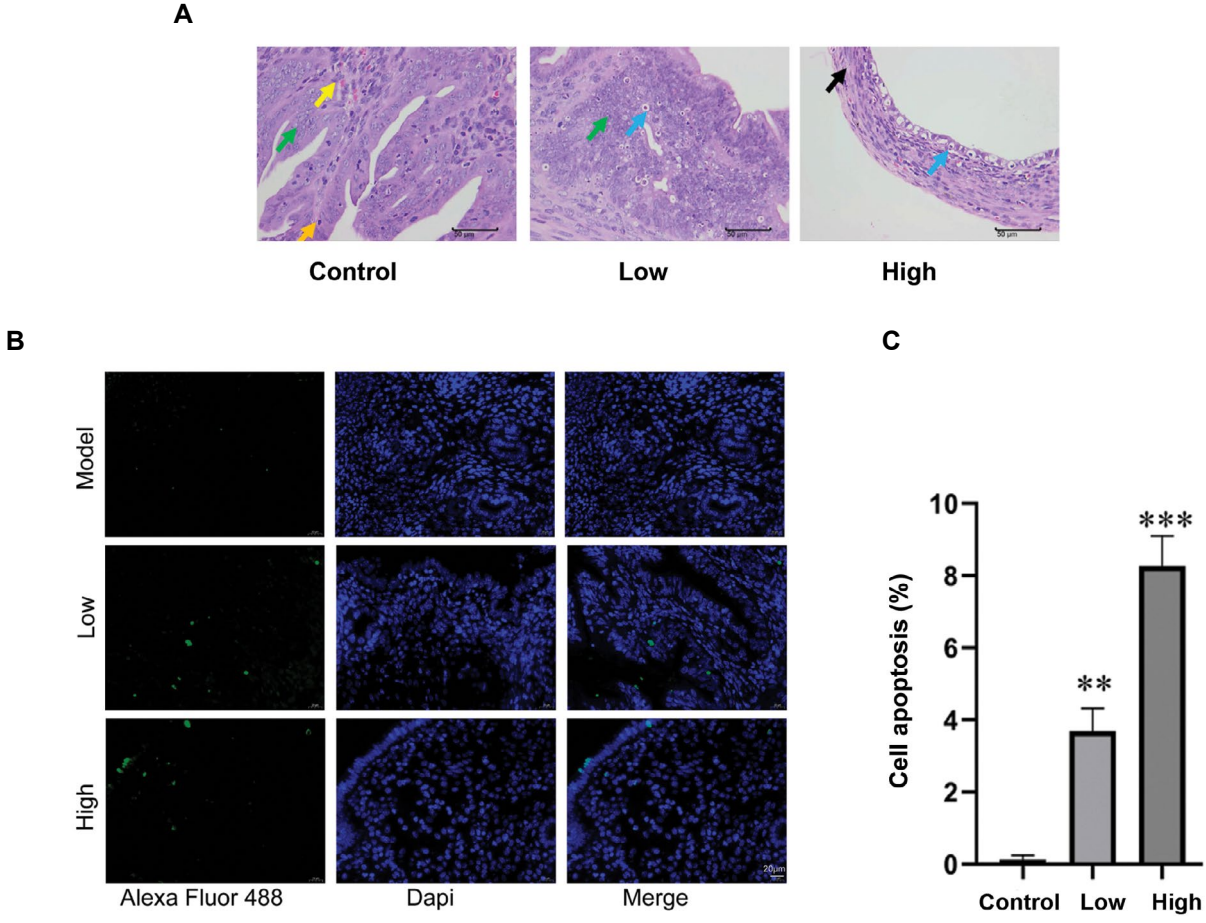

Fig.1: Effects of β-sitosterol on histopathology of uterus in endometriosis mice. **A.** H&E staining images of the endometrium (scale bar: 50 μm). **B.** Images of endometrium tissue following TUNEL staining of Alexa Flour 488 (green fluorescence) (scale bar: 20 μm). **C.** Statistical analysis of apoptosis cells by TUNEL staining. **; P<0.01, ***; P<0.001 compared with the control group.

Effects of β-sitosterol on Smad7-mediated TGF-β1/Smad2/3 in endometriosis mice

Immunohistochemistry was used to detect the changes of Smad7/TGF-β1/Smad2/3 proteins in endometrial tissues. As for smad7, compared with the control group, smad7 was increased in the β-sitosterol low (35 μg/kg) and high-dose (350 μg/kg) treatment groups. However, compared with the control group, the expression of TGF-β1, Smad2, and Smad3 was downregulated in the treatment of high- and low-dose β-sitosterol groups (Fig.2A, B). The above results suggest that changes in Smad7, TGF-β1, and smad2/3 signaling are involved in endometriosis.

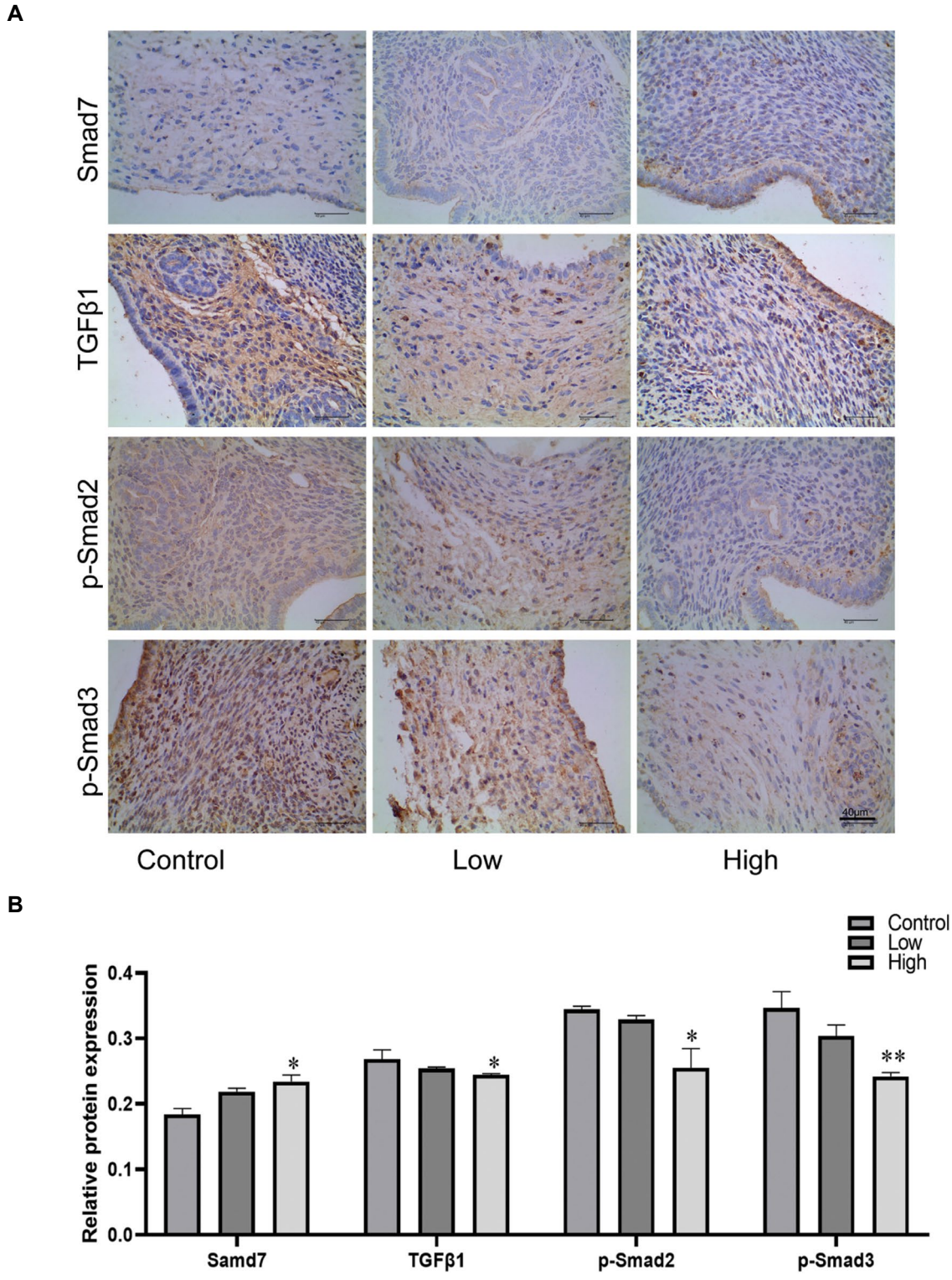

Fig.2: Effects of β-sitosterol on Smad7-mediated TGF-β1/Smad2/3 in endometriosis mice. **A.** Immunohistochemistry of Smad7/TGF-β1/Smad2/3 signaling pathway related proteins (scale bar: 40 μm). **B.** Statistics of Smad7/TGF-β1/Smad2/3 signal pathway related protein. *; P<0.05 and **; P<0.01 compared with the control group.

The effect of β-sitosterol on the biological behavior of hEM15A cells

The biological behavior of β-sitosterol on hEM15A cells was detected by CCK-8, flow cytometry, and transwell test. As shown in Figure 3A, cell viability was decreased in a dose-dependent manner by β-sitosterol, and it was the lowest at 90 μM. As shown in Figure 3B and C, the apoptosis rate was increased by β-sitosterol in a dose-dependent manner, and the highest at 90 μM. As shown in Figure 3D and E, cell migration ability was dose-dependently decreased by β-sitosterol. The migration ability of the blank group was the strongest, and the migration ability was the weakest at 90 μM of β-sitosterol.

To investigate whether the β-sitosterol regulates Smad7/TGF-β1/Smad2/3 expression levels *in vivo*, total protein in hEM15A cells treated with β-sitosterol was detected. As shown in Figure 3F and G, the expression level of Smad7 is the highest at 90 μM of β-sitosterol, and it has a dose-dependent effect. The expression level of TGF-β1/p-Smad2/3 is the highest without treatment of β-sitosterol, and the expression level is the lowest at 90 μM and has a dose-dependent effect.

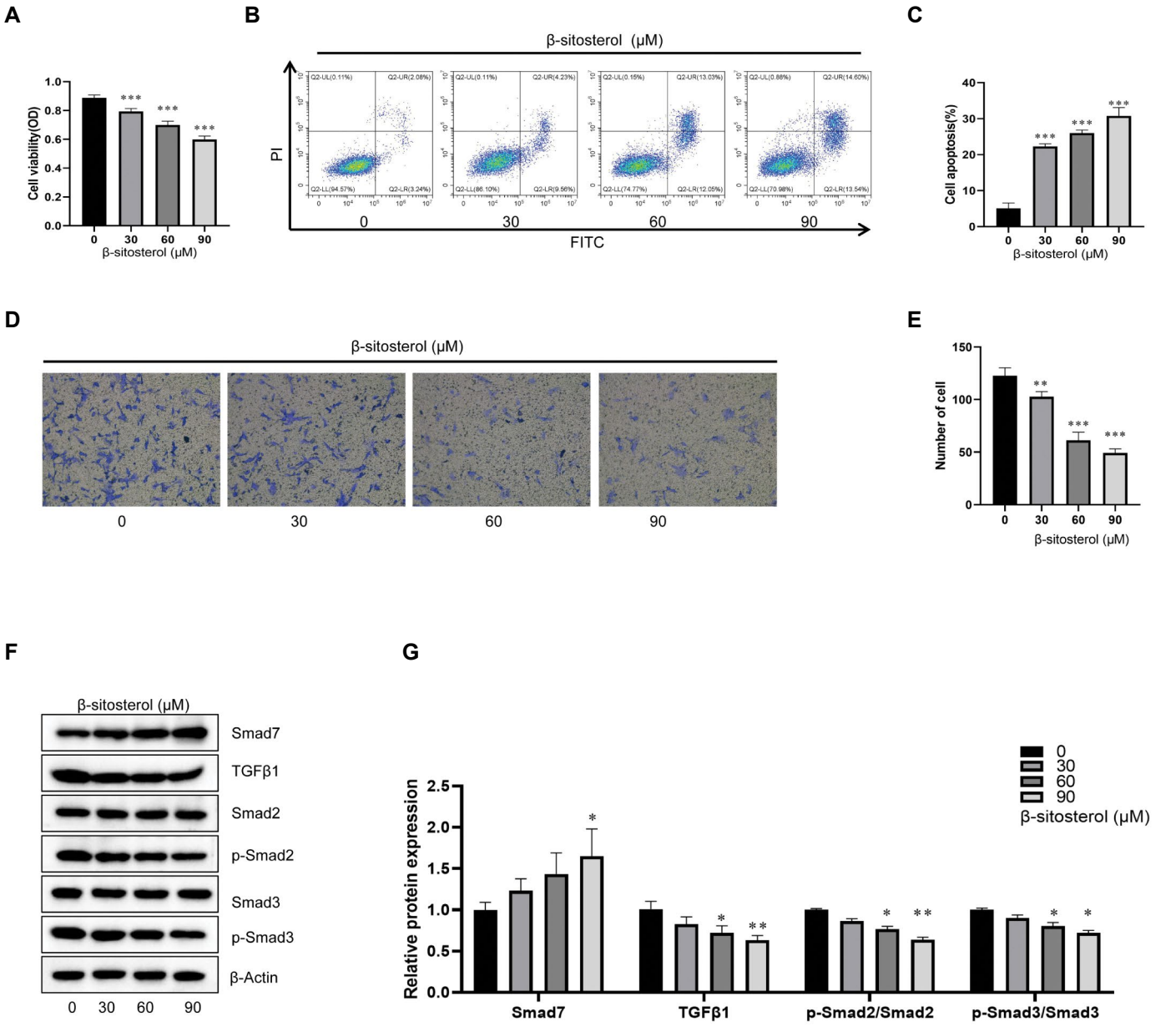

Fig.3: The effect of β-sitosterol on the biological behavior of hEM15A cells. **A.** Inhibition effect of β-sitosterol on hEM15A cells detected by CCK8. **B.** Apoptotic rate of hEM15A cells induced by β-sitosterol. **C.** Statistics of apoptotic cells. **D.** β-sitosterol inhibits hEM15A migration by transwell chamber. **E.** statistics of transwell experimental. **F.** Western blot of Smad7/TGF-β1/Smad2/3 signaling pathway-related proteins. **G.** Statistics of Smad7/TGF-β1/Smad2/3 related protein expression. *; P<0.05, **; P<0.001, and ***; P<0.0001, compared with the 0 group.

The effect of Smad7 on the biological behavior of hEM15A cells caused by β-sitosterol

After hEM15A cells were treated with si-Smad7 or 60 μM β-sitosterol, RNA and protein were extracted to detect the relative expression of Smad7/TGF-β1/Smad2/3. Then, the biological behavior of β-sitosterol and Smad7 on hEM15A cells were detected by CCK-8, flow cytometry, and transwell. As shown in Figure 4A, B, and C, compared with si-Smad7(2), si-Smad7(1) had a better inhibitory effect on smad7 at the protein and mRNA levels. Based on the above results, si-Smad7(1) was chosen for further investigation. As shown in Figure 4D, compared with the blank group, β-sitosterol could cause a significant decrease in cell viability in hEM15A. While compared with the β-sitosterol treatment group, si-Smad7(1) could reverse the decrease in cell viability caused by β-sitosterol. As shown in Figure 4E and F, the β-sitosterol treatment group had the highest apoptotic rate, while the si-Smad7(1) transfection group had the lowest apoptotic rate. Moreover, the apoptosis caused by β-sitosterol could be reduced by transfected si-Smad7(1). As shown in Figure 4G and H, the si-Smad7(1) transfection group had the strongest migration ability while the β-sitosterol treatment group had the weakest migration ability. Moreover, the decline in cell migration caused by β-sitosterol was significantly reversed under the action of si-Smad7(1). As shown in Figure 4I and J, compared with the blank group [si-Smad7(1) and β-sitosterol were both free], the expression level of TGF-β1, p-smad2, and p-smad3 in the β-sitosterol treatment group was low, while the expression level of TGF-β1, p-smad2, and p-smad3 in the si-Smad7(1) transfection group was high. si-Smad7(1) can effectively inhibit increased protein levels caused by β-sitosterol.

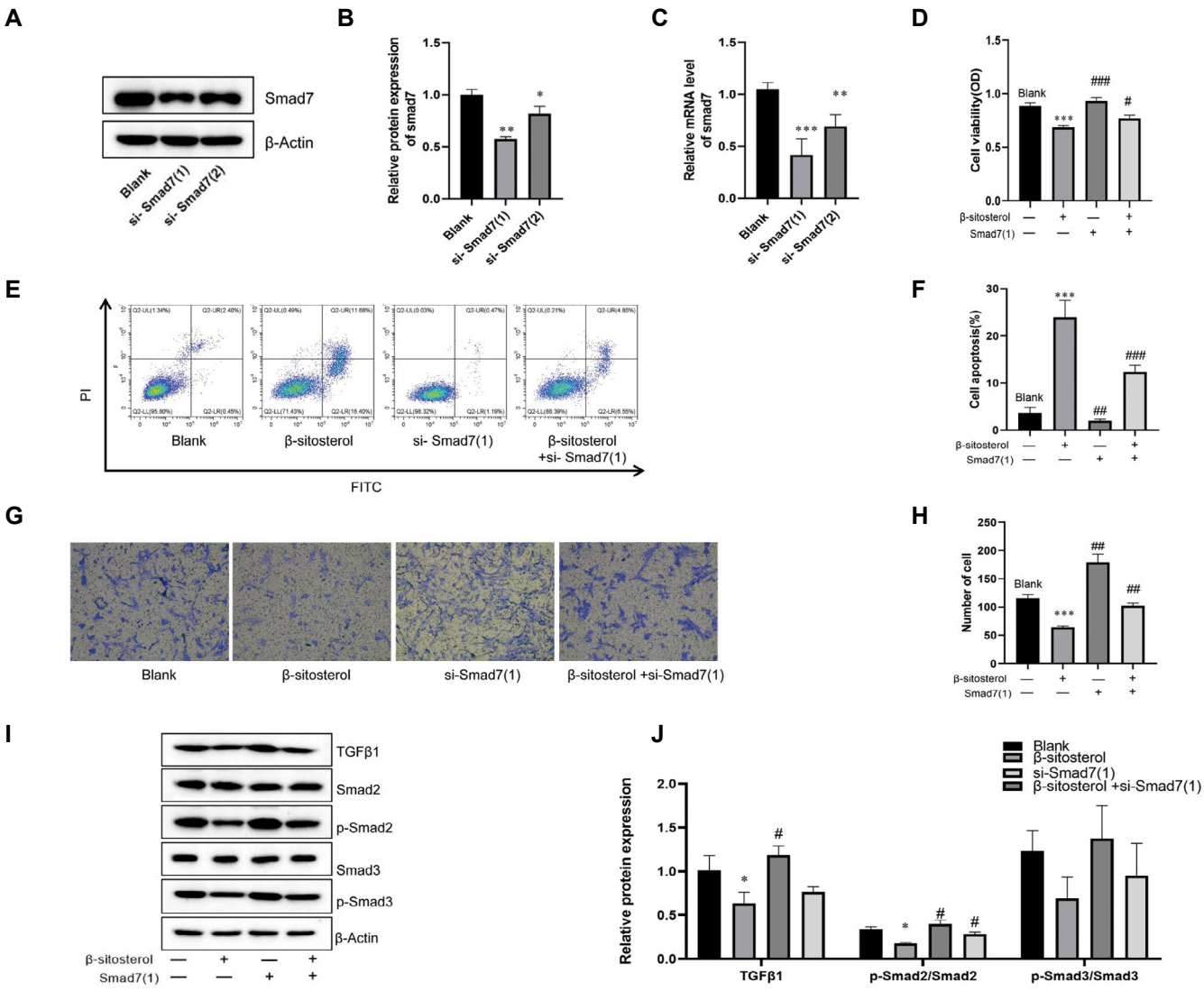

Fig.4: The effect of Smad7 on the biological behavior of hEM15A cells caused by 60μM β-sitosterol. **A.** The protein expression of Smad7. **B.** The statistics of Smad7. **C.** The mRNA level of Smad7. **D.** The effect of β-sitosterol and si-Smad7 on cell survival of hEM15A cells by CCK8. **E.** Effects of β-sitosterol and si-Smad7 on the apoptosis of hEM15A cells. **F.** Statistics of apoptotic cells. **G.** Effects of β-sitosterol and si-Smad7 on the migration of hEM15A cells. **H.** statistics of transwell experimental. **I.** Western blot of TGF-β1/Smad2/3 signaling pathway-related proteins. **J.** Statistics of TGF-β1/Smad2/3 related protein expression. *; Means compared with the blank group, #; Means compared with the β-sitosterol group, *; P<0.05, **; P<0.001, ***; P<0.0001, compared with the blank group, #; P<0.05, ##; P<0.001, and ###; P<0.0001, compared with the β-sitosterol group.

Discussion

Sterols are important physiologically active molecules in various foods and are also important components of cell membranes of all eukaryotes (humans, animals, plants), involved in important life activities, known as the "key" to life, because of its solid state, also known as sterols (21). The most well-known and extensively researched sterol is animal sterol, or cholesterol, which is mostly found in the brain, spinal cord, liver, and blood of both humans and animals (22). β-sitosterol is a natural products, the development and research of which have grown extensilvely, but there are still only a few drugs of natural product origin in the field of gynecological diseases. Therefore, the study of β-sitosterol in endometriosis enriches the study of natural products in gynecological diseases.

There are many reasons for the formation and development of EMS, such as inflammation, abnormal growth, and immune factors (23, 24). The biological characteristics of the ectopic endometrium cells of patients are different from those of normal endometrial cells (25). The eutopic endometrial cells of patients have a stronger ability for migration, invasion, proliferation, and blood vessel formation (26).

Previous research proved that miRNAs might promote the proliferation, invasion, and metastasis of cancer cells by regulating the expression of upstream target genes, thereby affecting the prognosis of patients with endometrial cancer (6). Studies have shown that the expression of TGF-β1 in the eutopic endometrium tissue is higher than that of the normal tissue (7). In the present study, β-sitosterol inhibited the expression level of TGF-β1 and suppressed cell proliferation and migration, thereby inhibiting the formation and progression of endometriosis lesions. Smad7 can inhibit the expression of TGF-β1, which could reveal that Smad7 is involved in the regulation of β-sitosterol on TGF-β1. Therefore, based on the smad7-mediated TGF-β/Smads signaling pathway for the treatment of endometriosis β-sitosterol contributes to the study of clinical treatment.

β-sitosterol is a natural active substance which was widely found in many medicinal plants (27). Previous studies have shown that β-sitosterol plays a helpful role in the prevention and treatment of tumors (28). β-sitosterol was previously reported to inhibit the proliferation and occurrence of tumor cells, inhibit the differentiation and proliferation of tumor or cancer cells, and induce tumor cell apoptosis (39, 30). In addition, after nearly ten years of basic research, it was proved that ectopic endometrial cells were found to be more proliferative than normal endometrial cells (31). Therefore, effectively inhibiting the proliferation of endometrial cells and promoting their apoptosis is a classic method for the improvement of endometriosis.

TGF-β1 was previously reported to be involved in various cell functions such as proliferation, differentiation, adhesion, migration, infiltration, and angiogenesis (32). Previous studies have shown that the level of TGF-β1 is positively correlated with the severity of endometriosis (8). The Smad protein family which is the substrate of the TGF-β receptor, exists in the cytoplasm, and can transmit the signal directly from the cell membrane to the nucleus (32). Smad7 was proven an inhibitor of TGF-β-Smads signal transduction. Smad7 can inhibit TGF-β-Smads signal transduction at the three levels of the TGF-β1 receptor, the Smad2/3 complex (34), and the nucleus. In this study, under-treatment of high-dose β-sitosterol, Smad7 expression level was the highest, while TGF-β1/Smads protein expression level was the lowest. From the above description, TGF-β1 and Smad7 are present in endometriosis, and the two factors exist in the same signal transduction process. It is speculated that TGF-β1 and Smad7 are in normal endometrial tissue. There is a balanced relationship.

hEM15 is an immortalized cell line of eutopic endometrial stromal cells in EMS patients. It retains some of the characteristics of endometrial cell morphology and molecular biology. It has high cell homogeneity and a long survival time, which can be used as research work for endometriosis as *in vitro* models (35). Sulindac has been demonstrated to control the expression of genes and proteins in ESCs from endometriosis-affected women, as well as to reduce nuclear factor-B activation (36). Consistent with the above research, the results showed that β-sitosterol shows a proliferation inhibitory effect, pro-apoptotic effect on hEM15, and cell migration inhibition which was the weakest at 90 μM. β-sitosterol-induced Smad7 inhibited TGF-β1/Smad2/3 signaling, and this effect was positively correlated with the concentration of β-sitosterol.

The Smad7-mediated change in the activity of the TGF-β1/Smad2/3 signaling pathway could be the key to β-sitosterol's influence on the biological behavior of hEM15 cells. In this study, si-SMAD7(1) can significantly silence the expression of Smad7 protein. The ability of cell proliferation and migration was the strongest under the treatment of si-*SMAD7*(1), and apoptotic cells were significantly reduced. Moreover, the signal transduction of TGF-β1/Smad2/3 becomes stronger after transfection with si-*SMAD7*(1). The effects of β-sitosterol and si-*SMAD7*(1) were opposed, indicating that the action of β-sitosterol on hEM15 cells might be caused through Smad7.

In addition, there are already many drugs for the treatment of endometriosis, during which the pain is mostly relieved, but the symptoms usually recur soon after stopping the drug administration (37). Tamoxifen, mifepristone, and aromatase inhibitors are currently in the development stage (38). β-sitosterol could regulate the gut microbiota to treat endometrial disease (39). Young et al. (40) reported that TGF-β1 regulates intraperitoneal VEGF-A expression via the ID1 pathway in patients with endometriosis for the treatment of uterine disease. Our study was in agreement with the Young results that

β-sitosterol has a significant modulatory effect on TGF-β1 signaling and also confirms the therapeutic effect of β-sitosterol on the endometriosis of the animal models.

Conclusion

This study explored the therapeutic effect of β-sitosterol on endometriosis in animal models. Experiments have found that β-sitosterol can effectively promote apoptosis of endometriotic cells and improve endometrial tissue lesions *in vivo*. *In vitro* experiments show that β-sitosterol can inhibit the proliferation of hEM15A cells and promote their apoptosis. In addition, the combined application of si-Smad7 and β-sitosterol counteract the positive effects of β-sitosterol. Smad7-mediated TGF-β/Smads signaling pathway could be the key molecular target for β-sitosterol. These results in this research indicate that β-sitosterol and Smad7 could be used as potential new drugs for the treatment of endometriosis. Admittedly, the limitation is that the species difference between mice and humans is a difficult problem for clinical translation. There are many pathogenic factors of endometriosis, Smad7/TGFβ did not reflect all signaling pathways and future research should be undertaken to explore the more in-depth mechanism.

Acknowledgements

This study did not receive any funding in any form. The authors report no conflicts of interest in this work.

Authors' Contributions

Y.W., L.P.; Designed the study, performed the experiments, and prepared the manuscript. L.F., Y.Z., R.L., T.Z., M.Z.; Collected and analyzed the experiments' data; the data. All authors read and approved the final manuscript.

Reference

1. Amro B, Ramirez Aristondo ME, Alsuwaidi S, Almaamari B, Hakim Z, Tahlak M, et al. New understanding of diagnosis, treatment and prevention of endometriosis. Int J Environ Res Public Health. 2022; 19(11): 6725.
2. Pirtea P, Vulliemoz N, de Ziegler D, Ayoubi JM. Infertility workup: identifying endometriosis. Fertil Steril. 2022; 118(1): 29-33.
3. Song Y, Fu J, Zhou M, Xiao L, Feng X, Chen H, et al. Activated Hippo/Yes-associated protein pathway promotes cell proliferation and anti-apoptosis in endometrial stromal cells of endometriosis. J Clin Endocrinol Metab. 2016; 101(4): 1552-1561.
4. D'Hooghe TM, Debrock S, Hill JA, Meuleman C. Endometriosis and subfertility: is the relationship resolved? Semin Reprod Med. 2003; 21(2): 243-254.
5. Mechsner S. Endometriosis, an ongoing pain-step-by-step treatment. J Clin Med. 2022; 11(2): 467.
6. Ni L, Tang C, Wang Y, Wan J, Charles MG, Zhang Z, et al. Construction of a miRNA-Based nomogram model to predict the prognosis of endometrial cancer. J Pers Med. 2022; 12(7): 1154.
7. Liu Z, Yi L, Du M, Gong G, Zhu Y. Overexpression of TGF-β enhances the migration and invasive ability of ectopic endometrial cells via ERK/MAPK signaling pathway. Exp Ther Med. 2019; 17(6): 4457-4464.
8. Young VJ, Brown JK, Maybin J, Saunders PT, Duncan WC, Horne AW. Transforming growth factor-β induced Warburg-like metabolic reprogramming may underpin the development of peritoneal endometriosis. J Clin Endocrinol Metab. 2014; 99(9): 3450-3459.
9. Hu Z, Li B, Wang Z, Hu X, Zhang M, Chen R, et al. The sympathetic transmitter norepinephrine inhibits VSMC proliferation induced by TGFβ by suppressing the expression of the TGFβ receptor ALK5 in aorta remodeling. Mol Med Rep. 2020; 22(1): 387-397.
10. Li H, Rong P, Ma X, Nie W, Chen Y, Zhang J, et al. Mouse umbilical cord mesenchymal stem cell paracrine alleviates renal fibrosis in diabetic nephropathy by reducing myofibroblast transdifferentiation and cell proliferation and upregulating MMPs in mesangial cells. J Diabetes Res. 2020; 2020: 3847171.
11. Chung AC, Dong Y, Yang W, Zhong X, Li R, Lan HY. Smad7 suppresses renal fibrosis via altering expression of TGF-β/Smad3-regulated microRNAs. Mol Ther. 2013; 21(2): 388-398.
12. Li JH, Zhu HJ, Huang XR, Lai KN, Johnson RJ, Lan HY. Smad7 inhibits fibrotic effect of TGF-Beta on renal tubular epithelial cells by blocking Smad2 activation. J Am Soc Nephrol. 2002; 13(6): 1464-1472.
13. Kim KS, Yang HJ, Lee JY, Na YC, Kwon SY, Kim YC, et al. Effects of β-sitosterol derived from Artemisia capillaris on the activated human hepatic stellate cells and dimethylnitrosamine-induced mouse liver fibrosis. BMC Complement Altern Med. 2014; 14: 363.
14. Vijaya, Yadav AK. In vitro anthelmintic assessment of selected phytochemicals against Hymenolepis diminuta, a zoonotic tapeworm. J Parasit Dis. 2016; 40(3): 1082-1086.
15. Cheng D, Guo Z, Zhang S. Effect of β-sitosterol on the expression of HPV E6 and p53 in cervical carcinoma cells. Contemp Oncol (Pozn). 2015; 19(1): 36-42.
16. Sharmila R, Sindhu G. Modulation of angiogenesis, proliferative response and apoptosis by β-Sitosterol in rat model of renal carcinogenesis. Indian J Clin Biochem. 2017; 32(2): 142-152.
17. Park YJ, Bang IJ, Jeong MH, Kim HR, Lee DE, Kwak JH, et al. Effects of β-sitosterol from corn silk on TGf-β1-induced epithelial-mesenchymal transition in lung alveolar epithelial cells. J Agric Food Chem. 2019; 67(35): 9789-9795.
18. Zondervan KT, Becker CM, Koga K, Missmer SA, Taylor RN, Viganò P. Endometriosis. Nat Rev Dis Primers. 2018; 4(1): 9.
19. Wang X, Li M, Hu M, Wei P, Zhu W. BAMBI overexpression together with β-sitosterol ameliorates NSCLC via inhibiting autophagy and inactivating TGF-β/Smad2/3 pathway. Oncol Rep. 2017; 37(5): 3046-3054.
20. Wen Y, Fan L, Pang L, Zhao T, Li R, Zhang Y, et al. NeiyiKangfu tablets control the progression of endometriosis through inhibiting RAF/MEK/ERK signal pathway by targeting RKIP. Gynecol Endocrinol. 2022; 38(12): 1136-1146.
21. Laitinen K, Gylling H. Dose-dependent LDL-cholesterol lowering effect by plant stanol ester consumption: clinical evidence. Lipids Health Dis. 2012; 11: 140.
22. Lohrey EE, Hughes IR, Gray IK. Effect of dietary lipid oxidation on measurement of protein efficiency ratios. J Assoc Off Anal Chem. 1978; 61(1): 104-110.
23. Guo SW. The pathogenesis of adenomyosis vis-à-vis endometriosis. J Clin Med. 2020; 9(2): 485.
24. Ciarmela P, Critchley H, Christman GM, Reis FM. Pathogenesis of endometriosis and uterine fibroids. Obstet Gynecol Int. 2013; 2013: 656571.
25. Harada T, Kaponis A, Iwabe T, Taniguchi F, Makrydimas G, Sofikitis N, et al. Apoptosis in human endometrium and endometriosis. Hum Reprod Update. 2004; 10(1): 29-38.
26. Wang J, Zhang L, Jiang W, Zhang R, Zhang B, Silayiding A, et al. MicroRNA-135a promotes proliferation, migration, invasion and induces chemoresistance of endometrial cancer cells. Eur J Obstet Gynecol Reprod Biol X. 2019; 5: 100103.
27. Awad AB, Roy R, Fink CS. Beta-sitosterol, a plant sterol, induces apoptosis and activates key caspases in MDA-MB-231 human breast cancer cells. Oncol Rep. 2003; 10(2): 497-500.
28. Sundstrøm T, Prestegarden L, Azuaje F, Aasen SN, Røsland GV, Varughese JK, et al. Inhibition of mitochondrial respiration prevents BRAF-mutant melanoma brain metastasis. Acta Neuropathol Commun. 2019; 7(1): 55.
29. Awad AB, Gan Y, Fink CS. Effect of beta-sitosterol, a plant sterol, on growth, protein phosphatase 2A, and phospholipase D in LNCaP cells. Nutr Cancer. 2000; 36(1): 74-78.
30. Choi YH, Kong KR, Kim YA, Jung KO, Kil JH, Rhee SH, et al. Induction of Bax and activation of caspases during beta-sitosterol-mediated apoptosis in human colon cancer cells. Int J Oncol. 2003; 23(6): 1657-1662.
31. Yin X, Li X, Feng G, Qu Y, Wang H. LINC00565 enhances proliferative ability in endometrial carcinoma by downregulating KLF9. Onco Targets Ther. 2020; 13: 6181-6189.
32. Moreau JM, Velegraki M, Bolyard C, Rosenblum MD, Li Z. Transforming growth factor-β1 in regulatory T cell biology. Sci Immunol.

2022; 7(69): eabi4613.
33. Wang J, Xu Z, Wang Z, Du G, Lun L. TGF-beta signaling in cancer radiotherapy. Cytokine. 2021; 148: 155709.
34. Shu Y, Liu Y, Li X, Cao L, Yuan X, Li W, et al. Aspirin-triggered resolvin D1 inhibits TGF-β1-induced EndMT through increasing the expression of Smad7 and is closely related to oxidative stress. Biomol Ther (Seoul). 2016; 24(2): 132-139.
35. Huang ZX, Wu RF, Mao XM, Huang SM, Liu TT, Chen QH, et al. Establishment of an immortalized stromal cell line derived from human Endometriotic lesion. Reprod Biol Endocrinol. 2020; 18(1): 119.
36. Wieser F, Vigne JL, Ryan I, Hornung D, Djalali S, Taylor RN. Sulindac suppresses nuclear factor-kappaB activation and RANTES gene and protein expression in endometrial stromal cells from women with endometriosis. J Clin Endocrinol Metab. 2005; 90(12): 6441-6447.
37. Becker CM, Gattrell WT, Gude K, Singh SS. Reevaluating response and failure of medical treatment of endometriosis: a systematic review. Fertil Steril. 2017; 108(1): 125-136.
38. Barra F, Scala C, Mais V, Guerriero S, Ferrero S. Investigational drugs for the treatment of endometriosis, an update on recent developments. Expert Opin Investig Drugs. 2018; 27(5): 445-458.
39. Yu Y, Cao Y, Huang W, Liu Y, Lu Y, Zhao J. β-Sitosterol Ameliorates Endometrium Receptivity in PCOS-Like Mice: The Mediation of Gut Microbiota. Front Nutr. 2021; 8: 667130.
40. Young VJ, Ahmad SF, Brown JK, Duncan WC, Horne AW. Peritoneal VEGF-A expression is regulated by TGF-β1 through an ID1 pathway in women with endometriosis. Sci Rep. 2015; 5: 16859.