ORIGINAL RESEARCH

Stigmasterol Depresses the Proliferation and Facilitates the Apoptosis of Fibroblast-Like Synoviocytes via the PI3K/AKT Signaling Pathway in Collagen-Induced Arthritis Rats

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ABSTRACT

Rheumatoid arthritis (RA) is a chronic disease characterized by persistent synovitis and angiogenesis. Its clinical manifestations are synovial hyperplasia and progressive destruction of bone and cartilage, eventually leading to joint deformation and even disability. The healing effect of monomer stigmasterol, the main active ingredient of the Jinwujiangu recipe the Chinese Herbal Compound, on RA has been confirmed in several studies. Fibroblast-like synoviocytes (FLS) are related to the occurrence and development of RA. This study aims to investigate the effects of stigmasterol on FLS cell proliferation and apoptosis, as well as its impact on FLS cell cycle proteins and key genes in the Phosphatidylinositol 3 kinase/protein kinase B (PI3K/AKT) pathway, providing insights into the development of stigmasterol as an alternative therapeutic drug for RA. We administered 20 g/kg stigmasterol to rats continuously for 5 d to obtain stigmasterol-containing serum, and established rat models of osteoarthritis induced by ossein to obtain FLS. To explore the effects of stigmasterol on the viability, migration, proliferation and apoptosis of collagen-induced arthritis (CIA)-FLS cells, we selected 0% (control), 5% (low concentration), 10% (medium concentration) and 20% (high concentration) drug-containing serum to intervene cells and conducted Cell Counting Kit-8 (CCK-8), Transwell, 5-ethynyl-2' -deoxyuridine (EdU) staining and Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) experiments, respectively. The results showed that compared with the control group, low, medium, and high serum significantly inhibited the activity, migration, and proliferation of FLS cells, and promoted their apoptosis, and high serum had the best effect. In addition, we investigated the mechanism of stigmasterol inhibiting FLS proliferation and promoting its apoptosis by qPCR, Western blot, and immunofluorescence assays. The results showed that stigmasterol significantly inhibited the expression of Cyclin D1, cyclin-dependent kinase 4 (CDK4), and Retinoblastoma (Rb), and decreased the expression of key genes kinase insert domain-containing receptor (KDR), PI3K, AKT, phosphorylated PI3K (p-PI3K) and phosphorylated AKT (p-AKT) in the KDR-mediated PI3K/AKT signaling pathway, thus inhibiting the proliferation of FLS and promoting the apoptosis of FLS. It was suggested that stigmasterol may be a potential alternative drug for RA treatment. (*Altern Ther Health Med.* [E-pub ahead of print.])

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INTRODUCTION

RA is an autoimmune disease with synovial inflammation and pannus formation as the basic pathological changes. The prevalence of RA in China is about 0.42%, and the ratio of male to female is about 1:4, which is characterized by a high disability rate and low cure rate.[1] Normal synovial tissues are mainly composed of macrophage-like synoviocytes (MLS) and fibroblast-like synoviocytes (FLS), while the synovial tissues of RA patients are mainly composed of FLS cells, monocytes, and T lymphocytes.[2] In recent years, it has been found that FLS cells in RA patients are similar to abnormal proliferation and functional changes of tumor cells, which is of great significance in the occurrence and development of RA.[3] Studies have shown that inhibition of FLS cell

proliferation can effectively delay the pathological progression of RA and relieve the clinical symptoms of RA patients.[4] Abnormal proliferation of FLS cells in RA patients is associated with changes in the program of contactless inhibition of intracellular death.[5,6]

Traditional Chinese medicine has gradually become a trend in the prevention and treatment of RA due to its advantages of fewer adverse reactions and both samples. The Miao medicine Jinwujiangu recipe (JWJGF) comes from the long-used prescription of miao people in Guizhou to treat bi disease, which is composed of Forrest Silkvine Stem (*Gardneria angustifolia* Wall. in Roxb.), Small-flower Sabia (*Sabia parviflora*), Golden Chicken Fern (*Cibotium barometz*), Homalomenae Rhizoma (*Homalomena occulta*), turmeric (*Curcuma longa* L.), White Peony Root (*Radix Paeoniae Alba*) and other medicines.[7] Researchers have employed mass spectrometry analysis and molecular docking techniques to identify stigmasterol as a primary constituent within Small-flower Sabia (*Sabia parviflora*),[8] Homalomenae Rhizoma (*Homalomena occulta*),[9] turmeric (*Curcuma longa* L.)[10] and White Peony Root (*Radix Paeoniae Alba*),[11] which are main constituents of JWJGF. At present, great progress has been made in basic and clinical research on the treatment of RA with the Jinwujiangu recipe. Researchers mainly cultured RA-FLS in vitro or constructed collagen-induced arthritis (CIA) model rats, and detected inflammatory factors, autophagy-related genes, and the proliferation and apoptosis rates of RA-FLS, to conclude that the Jinwujiangu recipe has good efficacy in preventing and treating RA.[12] In addition, the Jinwujiangu recipe also has the effects of reducing the toxic and side effects of anti-rheumatic drugs (DMARDs) that alleviate the disease, improving the bone metabolism of RA patients, and effectively improving the quality of life of RA patients.[13]

Stigmasterol is a phytosterol which is found in a wide variety of plants. Stigmasterol is the main active component of JWJGF and has many pharmacological activities such as anti-tumor, anti-oxidation, anti-diabetes, and lowering cholesterol.[14-17] Moreover, its anti-inflammatory properties have been substantiated. Stigmasterol is the key component in ginger for treating RA.[18] Research has shown that phytosterols can reduce damage to cartilage tissue by inhibiting various inflammatory mediators, blocking the NF-κB signaling pathway, and decreasing the expression of matrix metalloproteinases (MMPs).[19] Mahmood et al. found that stigmasterol inhibited the expression of pro-inflammatory mediators Tumor Necrosis Factor -α (TNF-α), Interleukin 6 (IL-6), Interleukin 1β (IL-1β), inducible Nitric Oxide Synthase (iNOS) and Cyclooxygenase 2 (COX-2) by blocking the activation of p38 Mitogen-Activated Protein Kinase (p38MAPK) and Nuclear Transcription factor kBp65 (NF-kBp65) signaling pathways, and promoted the expression of anti-inflammatory factor Interleukin 10 (IL-10), suggesting that stigmasterol is a potential drug for the treatment of RA.[20]

FLS is the main cell that affects the pathological development of RA.[21] However, there are few studies on the effect of stigmasterol on FLS. In this study, we used an Ossian-induced rat inflammatory model to obtain CIA-FLS cells and administered stigmasterol to obtain drug-containing serum to study the effects of 0, 5%, 10 and 20% stigmasterol drug-containing serum on the activity, migration, proliferation, and apoptosis of FLS cells. Drug-containing serum refers to the serum obtained from the receptor after feeding animals with traditional Chinese medicine or its compound formula, which belongs to the category of serum pharmacology of Traditional Chinese medicine.[22] At present, the method of using Chinese medicine or its compound drug-containing serum instead of the crude extract of the original drug as the test material for in vitro experiments is more and more favored by many Chinese medicine researchers.[23] This study demonstrated that stigmasterol inhibited the migration and proliferation of FLS and promoted the apoptosis of FLS by blocking the kinase insert domain-containing receptor (KDR)-mediated Phosphatidylinositol 3 kinase/protein kinase B pathway (PI3K/AKT) signaling pathway.

METHODS

Establishment of collagen-induced arthritis (CIA) model rats

Twenty-five-week-old Wistar rats weighing 150±20 g were purchased from Chongqing Tengxin Biotechnology Co., LTD. Then 10mg bovine type II collagen (C7806, Sigma-Aldrich, USA) was dissolved in 0.1 mol/L 5 mL acetic acid solution, and a collagen solution with a concentration of 2 mg/mL was prepared by shaking well, placed in 4°C refrigerator overnight. The next day, the collagen solution (2 mg/mL) was fully emulsified with an equal volume of Freund's complete adjuvant (F5881, Sigma-Aldrich, USA) in an ice bath to prepare a collagen emulsion with a concentration of 1 mg/mL. The prepared mixture was stored in a refrigerator at 4°C for later use. Twenty rats received the following treatment where each was injected with 0.5 mL of collagen emulsion subcutaneously after disinfecting the tail root. On the 14th day, a second injection of 0.3 mL of collagen emulsion was administered using the same method to boost both immunity and inflammation. The rats were monitored for signs of joint inflammation, and significant swelling in the paws and ankles was observed beginning on the seventh day. The animal-related procedures have been approved by the Ethics Committee of the Second Affiliated Hospital of Guizhou University of Traditional Chinese Medicine (approval no. 2017039).

Preparation of stigmasterol-containing serum

The twenty CIA model rats were divided into a stigmasterol-containing serum group and a blank serum group. Ten rats in the drug-containing serum group were given sterol (CAS: 83-48-7, S2424, Sigma-Aldrich, USA) at 20 g/kg, while the other ten rats in the blank control group was given a constant volume of normal saline. The rats were given the drug once a day and were given intragastric administration for 5 d successively. After 1.5 h of intragastric

administration on the 5th day, blood was collected from the rats' posterior orbital vein cluster. The blood samples were centrifuged at 3000 rpm for 10 min to separate the drug-containing serum. The drug-containing serum of the same group was combined, mixed, filtered by 0.2 μm microporous membrane for sterilization, inactivated at 56°C for 30 min, and stored for later use.

Isolation of fibroblast-like synoviocyte (FLS) from rats

Synovial tissues of rats were isolated under aseptic conditions on the 7th day after secondary immunization. The tissues such as blood and fat were washed repeatedly with D-Hanks (H1045, Solarbio, China), and then cut into small pieces. After centrifugation at 3000 rpm for 12 min, tissue fragments were evenly spread in culture bottles. 2 mL Dulbecco's Modification of Eagle's Medium (DMEM) medium containing 20% Fetal Bovine Serum (FBS) was added and cultured in an incubator with 5% CO_2 at 37°C. About 48 hours later, cells were observed under the microscope to crawl out from around the tissue block and fuse into slices. After the tissue blocks were removed, the culture medium was replaced and cells were continued to be cultured. The culture medium was changed once every 48 hours. When the cell density reached about 80%-90%, the cells were digested with 0.25% trypsin and then subcultured, and the third-generation cells were used for subsequent experiments.

Cell grouping

FLS in a healthy and stable growth state were selected and randomly divided into four groups: the Control group, the Low-dose stigmasterol serum group, the Medium-stigmasterol serum group, and the High-stigmasterol serum group. The original culture medium was replaced with DMEM culture medium containing 20% blank serum, 5% drug-containing serum + 15% blank serum, 10% drug-containing serum + 10% blank serum, and 20% drug-containing serum, respectively. Cells were then cultured for an additional 24 hours before being collected for further analysis.

CCK-8

FLSs were inoculated into 96-well plates at the rate of 1×10^5/well, and the medium was discarded after 24 h. 180 μL of the mediums with final concentrations of 0%, 5%, 10%, and 20% drug serum were added to each well, respectively, with 3 triplicate wells in each group. The cells were cultured at 37°C and 5% CO_2. After the supernatant was discarded at 24, 48, and 72 h, 20 μL CCK-8 (C0038, Beyotime, China) solution was added to each well and incubated at 37°C for 2 h. The OD value was measured at 450 nm.

Transwell assay

FLSs were inoculated with 2×10^5 cells/well in a 24-well Transwell chamber (0.2 mL/well), and 600 μL drug-containing serum medium containing 0%, 5%, 10%, and 15% were added into the lower chamber according to the above cells, respectively, and cultured for 24 h. The cells were fixed with 4% formaldehyde for 10 min, stained with 0.1% crystal violet solution for 30 min, washed with phosphate balanced solution (PBS) three times, observed under a fluorescence inverted microscope (IX73, Olympus, Japan), and the purple stained transmembrane cells were counted with Image J software.

TUNEL assay

FLS was inoculated into 6-well plates at a density of 3×10^5 per cell, and supplemented to 2 mL by 0%, 5%, 10%, and 15% drug-containing serum media according to the cells grouped above, and cultured at 37°C in a 5% CO_2 incubator for 48h. The cells were taken out and washed with PBS twice, fixed with 4% paraformaldehyde for 10 min. After being washed with PBS twice, the cells were incubated in an ice bath with immunostaining detergent for 2 min, and washed with PBS twice. The cells were incubated in 0.3% hydrogen peroxide solution (0.3% H_2O_2 in Methanol) at room temperature for 20 min to inactivate the endogenous peroxidase in slices. The cells were washed with PBS 3 times, then 50 μL biotin labeling solution was added to each well, and the cells were incubated at 37°C for 1 hour. The cells were washed once with PBS, and 0.2 mL labeled reaction termination solution was dropped to incubate at room temperature for 10 min. The cells were washed 3 times with PBS, and 50 μL Streptavidin-HRP working solution was added to each well, then cells were incubated with it at room temperature for 30 min. After the cells were washed with PBS 3 times, 0.4 mL Diaminobenzidine (DAB) chromogenic solution was dropped into each well, and cells were incubated with it for 5 min at room temperature. After washing the cells with PBS 3 times, the cells were dehydrated with 95% ethanol for 5 min, and then 100% ethanol for 2 times, 3 min each. Finally, xylene was used to permeate the cells 2 times, 2 min each time, then the cells were sealed, dried, and observed under a microscope.

5-ethynyl-2' -deoxyuridine (EdU) staining

The FLS was inoculated into 24 well plates with 2×10^5 cells/well, and the cells were grouped into the medium containing 0%, 5%, 10%, and 15% drug-containing serum to 2 mL, respectively. After incubating for 48 h, the medium containing 50 μmol EdU was added and incubated for 12 h. The culture medium was discarded and rinsed with PBS buffer 3 times. The cells were fixed with iced methanol at room temperature for 15 min, stained according to the instructions of EdU reagent (ST067, Beyotime, China), and observed under a fluorescence microscope.

qRT-PCR

The cells were inoculated into 6-well plates with 3×10^5 cells/well. According to the above groups, 2 mL drug-containing serum medium containing 0%, 5%, 10%, and 15% were added, respectively. After 48 h culture in 37 ^{0}C, 5% CO_2 incubator, the cells of each group were slowly washed with pre-cooled PBS at 4°C 2 times and collected into 1.5 mL EP tubes. Total RNA was extracted using RNA kits (RC101,

Vazyme, China) according to the instructions of the manufacturer. The obtained RNA samples were reversely transcribed according to the instructions of the Goldenstar™ RT6 cDNA Synthesis Kit Ver.2 (TSK302M, Tsingke Biotechnology Co., Ltd., China) to obtain the corresponding cDNA. qRT-PCR was performed on a fluorescence quantifier (7500, ABI, USA) and the reaction conditions were set as follows: pre-denaturation at 95°C for 10 min, denaturation at 95°C for 10 s, annealing at 60°C for 20 s, and extension at 72°C for 30 s, with a total of 40 cycles.

Western blot

The steps for cell collection are similar to those in qRT-PCR. 1 mL RIPA lysate containing PMSF (P0013C, Beyotime, China) was added to each EP-containing cell, and the cells were lysed on ice for 10 min. The lysed cells were centrifuged at 12 000 R at 4°C for 10 min to obtain the supernatant. The protein concentration of the supernatant was determined by the Bicinchoninic Acid Assay (BCA) method. 5×SDS protein loading buffer was added to the remaining samples and boiled at 100°C for 5 min to obtain protein samples. The corresponding volume of protein was sampled according to the concentration of each pore protein, and the voltage was adjusted to 80 V. After SDS-PAGE electrophoresis, the protein samples were transferred to a Polyvinylidene fluoride (PVDF) membrane. The PVDF membrane was sealed with 5% skim milk for 2 h. Primary antibodies of Cyclin D1 (1:1000, 2922S, Cell Signaling Technology, USA), cyclin-dependent kinase 4 (CDK4) (1:1000, AB68266, Abcam, USA), Retinoblastoma (Rb) (1:1000, A16966, ABclonal, China), KDR (1:1000, AB221679, Abcam, USA), PI3K (1:2000, AB180967, Abcam, USA), p-PI3K (1:1000, AF3241, Affinity, USA), AKT (1:5000, AB182729, Abcam, USA), p-AKT (1:1000, AB38449, Abcam, USA) were incubated with PVDF membrane overnight at 4°C. Tris-buffered saline +Tween (TBST) was used to wash the PVDF membrane for three times, and the PVDF membrane was co-incubated with secondary antibodies at room temperature for 1 h. After the PVDF membrane was cleaned with TBST 3 times, the PVDF membrane was developed and scanned with Enhanced Chemiluminescence (ECL). The obtained protein bands were analyzed by ImageJ software, and the target proteins were quantitatively analyzed with β-actin as an internal reference.

Immunofluorescence

The slides with prepared cells were immersed in PBS for 3 times, 3 min each. The cell slides were fixed with 4% paraformaldehyde for 15 min, and the slides were immersed in PBS 3 times, 3 min each. At room temperature, the cell slides were permeated with 0.5%Triton X-100 (PREPARED by PBS) for 20 min, then the slides were immersed with PBS 3 times, 3 min each, and the PBS was dried with absorbent paper. Normal goat serum (C0265, Beyotime, China) was added to the slides. The samples were incubated for 30 min at room temperature. The sealing fluid on the cell slides was sucked away with absorbent paper, and a primary antibody was added to the cell slipper and placed in a wet box for incubation at 4°C overnight. The cell slides were soaked with PBS+Tween (PBST) 3 times, 3 min each time. The excess liquid on the sliver was dried with absorbent paper, and then a fluorescent secondary antibody was dropped. The cell slides were then incubated at 25°C for 1 h in a wet box, and then the cell slides were soaked with PBST 3 times, 3 min each. 4',6-diamidino-2-phenylindole (DAPI) (C0065, Solarbio, China) was added to the cell slides, and the cell slides were incubated in the dark for 5 min for nuclear staining. Then, the cell slides were immersed with PBST 4 times for 5 min each. The liquid on the cell slides was absorbed with absorbent paper and the cell slides were sealed with an anti-fluorescence quenching sealing solution (P0126, Beyotime, China). The required images were observed and collected under a fluorescence microscope.

Statistical analysis

GraphPad Prism version 9.0 software was used for data analysis and mapping. All results were expressed as mean ± standard deviation. One-way ANOVA was used for comparison between multiple groups, and $P < .05$ was considered a significant difference. The 95% confidence intervals (CIs) for the difference between the means of 2 sets of data were calculated to provide a better understanding of the differences.

RESULTS

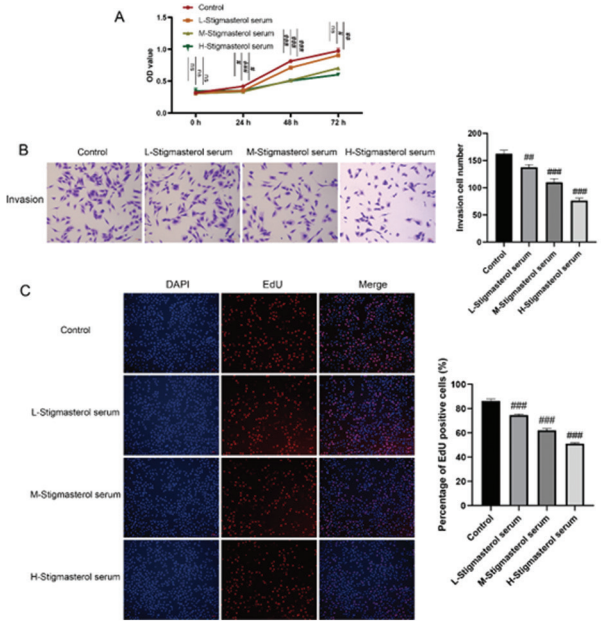
Stigmasterol inhibited the activity, migration, and proliferation of FLS

CCK-8, Transwell and EdU tests were conducted to investigate the effects of stigmasterol on the viability, migration, and invasion of CIA-FLS cells. CCK-8 results showed that after 48 h of culture with drug serum, the activity of FLS cells in medium and high-concentration drug serum groups was significantly lower than that in the control group and low-concentration group ($P< .001$, 95% CI: 0.28 to 0.32; $P < .001$, 95% CI: 0.29 to 0.33). However, after 72 h of culture, FLS in high concentration group was lower than that in the control group and low and medium-concentration groups ($P = .006$, 95% CI: 0.23 to 0.51), indicating that high-concentration serum had the best inhibitory effect on FLS cell viability (Figure 1A). Transwell and EdU staining results showed that the migration and proliferation rate of FLS in low, medium, and high concentration groups were significantly lower than those in the control group after 48h of culture with drug-containing serum, and high concentration group had the best effect ($P < .001$, 95% CI: 72.37 to 99.63; $P<0.001$, 95% CI: 32.20 to 38.11) (Figure 1B-C).

Stigmasterol inhibited the apoptosis of fibro-like synovial cells

In addition, to detect the effect of stigmasterol on the apoptosis of CIA-FLS, the cells were treated with different concentrations of stigmasterol-containing serum for 48h, and the TUNEL test was performed. The results showed that

Figure 1. The CIA model rats were established. Rats were administered 20 g/kg of stigmasterol or an equivalent volume of normal saline by gavage, and drug-containing serum was isolated. After isolating FLS cells from the synovial tissue of CIA rats, the cells were divided into four groups: Control group: 20% blank serum +80% DMEM; L-stigmasterol serum: 5% drug-containing serum +15% blank serum +80% DMEM; M-stigmasterol serum: 10% drug-containing serum +10% blank serum +80% DMEM; H-stigmasterol serum: 20% drug-containing serum +80% DMEM. The cell viability, invasion, and proliferation of FLS cells were examined. A, CCK-8 test was used to assess the FLS cell viability; B, Transwell test was conducted to assess FLS cell invasion; C, EdU staining test was performed to estimate FLS cell proliferation. The results are expressed as the mean of three independent experiments ± SD. With the control group as the comparison, ns: not significant. # $P < .05$. ## $P < .01$. ### $P < .001$.



compared with the control group, medium and high-concentration drugs significantly promoted the apoptosis of FLS (P=0.02, 95% CI: -38.55 to -3.786; P=0.002, 95% CI: -50.50 to -15.74), and the effect of high concentration group was better than that of medium concentration group (Figure 2).

Stigmasterol inhibited the expression of FLS cyclin

To investigate the effect of stigmasterol on FLS proliferation and apoptosis, we detected the expression levels of Cyclin D1, CDK4, and Rb. The results showed that compared with the control group, the expression of Cyclin D1, CDK4, and Rb were significantly inhibited in both medium and high-concentration drug-containing serum, and the inhibitory effect of high-concentration drug-containing serum was better than that of medium concentration drug-containing serum (mRNA: $P < .001$, 95% CI: 0.46 to 0.74; 95% CI: 0.38 to 0.70; 95% CI: 0.35 to 0.75. Protein: $P < .001$, 95% CI: 0.26 to 0.38; 95% CI: 0.088 to 0.15; 95% CI: 0.07 to 0.20) (Figure 3).

Figure 2. TUNEL detection on stigmasterol's effect on FLS apoptosis. The results are expressed as the mean of three independent experiments ± SD. With the control group as the comparison, # $P < .05$, ## $P < .01$. Control group: 20% blank serum +80% DMEM; L-stigmasterol serum: 5% drug-containing serum +15% blank serum +80% DMEM; M-stigmasterol serum: 10% drug-containing serum +10% blank serum +80% DMEM; H-stigmasterol serum: 20% drug-containing serum +80% DMEM.

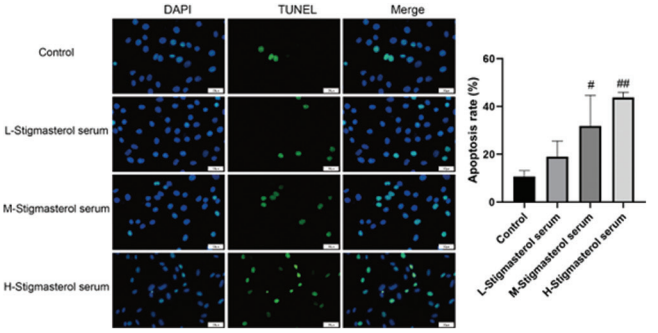
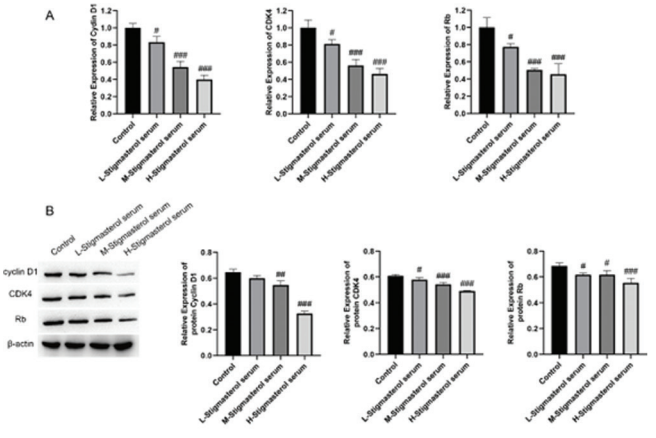


Figure 3. Effect of Stigmasterol on Cyclin D1, CDK4, and Rb expression in FLS cells. A, Cyclin D1, CDK4, and Rb expression detection in FLS cells using qRT-PCR, GAPDH as the internal reference gene; B, The expression of Cyclin D1, CDK4, and Rb proteins in FLS cells was detected with Western blot using β-actin as an internal reference control. The results are expressed as the mean of three independent experiments ± SD. With the control group as the comparison, #$P < .05$, ##$P < .01$, ###$P < .001$. Control group: 20% blank serum +80% DMEM; L-stigmasterol serum: 5% drug-containing serum +15% blank serum +80% DMEM; M-stigmasterol serum: 10% drug-containing serum +10% blank serum +80% DMEM; H-stigmasterol serum: 20% drug-containing serum +80% DMEM.



Stigmasterol inhibited the expression of key genes in the PI3K/AKT signaling pathway

In addition, we further detected the expression levels of stigmasterol on major genes of the PI3K/AKT signaling pathway. qRT-PCR, Western blot, and immunofluorescence results showed that compared with the control group, the

Figure 4. Effects of stigmasterol on the expression of KDR, PI3K, AKT and phosphorylated PI3K, AKT in a KDR-mediated PI3K/AKT signaling pathway. A, KDR, PI3K, AKT mRNA expression in FLS cells was detected using the qRT-PCR method, with GAPDH as the internal reference gene; B) KDR, PI3K, p-PI3K/ PI3K, AKT, and p-AKT/ AKT expression was detected using Western blot, and quantification is conducted using β-actin as an internal reference. The results are expressed as the mean of three independent experiments ± SD. With the control group as the comparison, #P < .05, ##P < .01, ###P < .001. Control group: 20% blank serum +80% DMEM; L-stigmasterol serum: 5% drug-containing serum +15% blank serum +80% DMEM; M-stigmasterol serum: 10% drug-containing serum +10% blank serum +80% DMEM; H-stigmasterol serum: 20% drug-containing serum +80% DMEM.

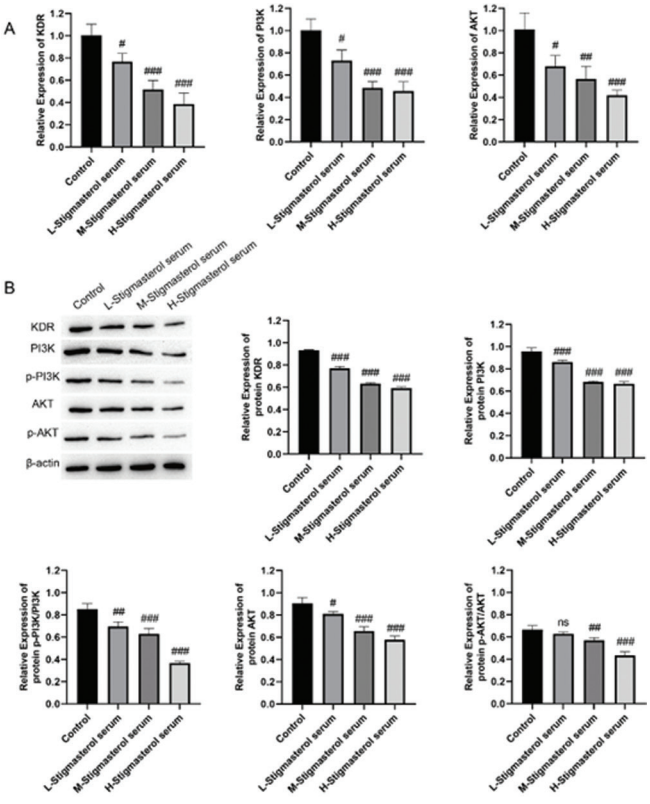
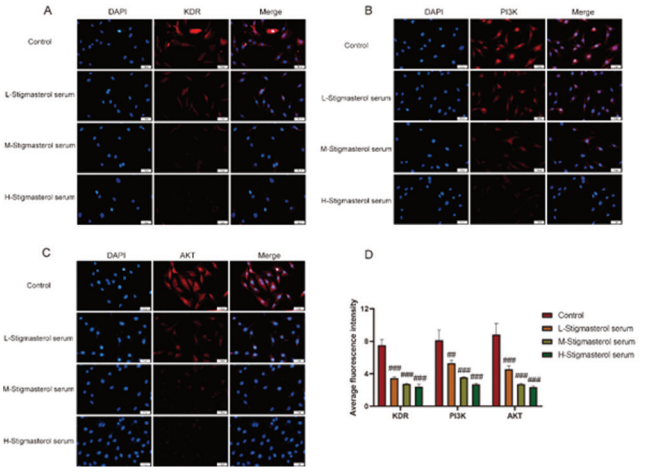


Figure 5. The effects of stigmasterol on KDR, PI3K, and AKT in FLS cells were detected by immunofluorescence. The results are expressed as the mean of three independent experiments ± SD. With the control group as the comparison, ##P < .01, ###P < .001. Control group: 20% blank serum +80% DMEM; L-stigmasterol serum: 5% drug-containing serum +15% blank serum +80% DMEM; M-stigmasterol serum: 10% drug-containing serum +10% blank serum +80% DMEM; H-stigmasterol serum: 20% drug-containing serum +80% DMEM.



expressions of KDR, PI3K, and AKT were significantly decreased in low, medium, and high-concentration drug-containing serum, and the ratio of p-PI3K/PI3K and p-AKT/AKT was also significantly decreased (p-PI3K/PI3K: P=0.004, 95% CI: 0.059 to 0.25; P<0.001, 95% CI: 0.13 to 0.32; P<0.001, 95% CI: 0.39 to 0.58. p-AKT/AKT: P=0.006, 95% CI: -0.1617 to -0.03320; P<0.001, 95% CI: -0.2967 to -0.1683). However, the p-AKT/AKT ratio in the L-stigmasterol serum group showed no significant difference compared to the control group (Figure 4-5). These results indicated that stigmasterol dephosphorylated the proteins PI3K and AKT and prevented the activation of the PI3K/AKT pathway, thus affecting the function of FLS cells, and a high concentration of the drug serum begot the best outcome.

DISCUSSION

RA is characterized by persistent synovitis and angiogenesis, leading to synovial hyperplasia and progressive destruction of bone and cartilage. In the late stage, RA can cause joint deformation or even disability. The pathological examination of RA involved synovial tissue, cartilage, and bone. In the pathological process of RA, the hyperplastic synovial lining contains a large number of activated FLS and macrophages, which extend into the joint space, attach to the cartilage surface (chondrovascular junction), invade and degrade the cartilage matrix, and promote joint destruction. Synovitis is one of the basic pathological changes of RA. During the occurrence of synovitis, there is a significant increase in synovitis cells, which contain a large number of FLS, which continuously express a series of inflammatory mediators and produce inflammatory cytokines, such as Toll-like receptors, IL-6, IL-1β, TNF-α, etc. At the same time, these inflammatory factors can induce and accelerate FLS transformation and metastasis, and promote inflammatory response.[24,25] Pannus synovium is another pathological feature of RA. In the pathological process of RA, FLS produces a large number of Vasoactive Endothelial Growth Factors (VEGF), which can promote vascular formation, while uncontrolled neovascularization can lead to inflammatory cell infiltration, synovial tissue proliferation, and ultimately cartilage and bone destruction.[26] RA is also an inflammatory disease characterized by progressive joint destruction. FLS plays a key role in the pathological process of RA joint injury through invasive migration and stromal invasion. Activated FLS produces active substances such as

matrix metalloproteinases (MMPs), which can worsen symptoms of joint pain, swelling, bone erosion, and cartilage destruction.[27] It is now established that various cytokines, upon binding to their respective receptors, can transmit signals through signaling pathways, regulating gene expression within the cell nucleus, thereby influencing a series of processes in synovial cells, such as growth, differentiation, apoptosis, and more. Among these, the PI3K-AKT signaling pathway is currently a hot topic of research.[28]

Among numerous molecular signaling pathways, the PI3K/AKT signaling pathway is one of the classic signaling pathways, widely existing in a variety of cells, and playing an important role in regulating cell survival, proliferation, differentiation, apoptosis, and metabolism.[29] The PI3K/AKT signaling pathway is intricately linked to abnormal apoptosis in rheumatoid arthritis fibroblast-like synovial cells (RAFLS). Study has revealed the widespread presence and abnormal activation of the PI3K-AKT signaling pathway in RAFLS.[30] PI3K has the functions of both protein kinase and phospholipid kinase and is mainly composed of a regulatory subunit (P85) and catalytic subunit (P110), of which the catalytic subunit can be activated by tyrosine kinase receptor. If the cell membrane is stimulated by a variety of extracellular factors such as cytokines, growth factors, and hormones, intracellular PI3K will be activated and catalyze phosphatidylinositol (PI) phosphorylation at the D3 position, transforming the substrate Phosphatidylinositol 4,5-bisphosphate (PIP2) into Phosphatidylinositol triphosphate (PIP3).[31] PIP3, as a second messenger, binds to the intracellular AKT signaling protein to transmit the signal. AKT, also known as protein kinase B (PKB), is an evolutionarily conserved specific protein kinase that acts during cell survival and apoptosis. AKT is the most studied and universally activated molecule in the downstream of class I PI3K pathway, participating in many downstream activities of PI3K signaling, and also playing an important role in the transmission of messengers in the PI3K/AKT pathway. AKT protects cells from apoptotic genes due to its ability to phosphorylate several downstream targets, including the associated death promoter (Bad), cysteine aspartate protease-9 (Caspase-9), and fork-head transcription factors.[32] KDR is a tyrosine kinase that signals through the PI3K/AKT pathway. PI3K can be activated following KDR activation, and its activation leads to the phosphorylation of AKT. Activated AKT, in turn, further activates various proteins involved in the regulation of RA.[33]

At present, the clinical treatment of RA mainly consists of anti-rheumatism drugs (DMARDs), non-steroidal anti-inflammatory drugs (NSAIDs), analgesics, steroids, and immunosuppressants, but these drugs have many potential adverse reactions, and the high price of immunopreparation limits their long-term application.[34] Therefore, seeking complementary and alternative drugs to treat RA is a current research hotspot. Stigmasterol is one of the active monomers of JWJGF, and its therapeutic effect on RA has been confirmed in many studies. However, as FLS is a key factor in the occurrence and development of RA, studying the effects of stigmasterol on FLS is also a new direction for finding therapeutic targets for RA. The results of this study demonstrated that stigmasterol significantly inhibited the proliferation and migration of CIA-FLS, promoted the apoptosis of FLS, and significantly reduced the expression of Cyclin D1, CDK4, and Rb. These results align with Song's findings, where stigmasterol was shown to inhibit the expression of cyclin D1 and CDK4 in Lung Adenocarcinoma Cells NCI-H1975.[35] Our results suggest that stigmasterol down-regulates Cyclin D1 by inhibiting Rb, thus reducing the binding of Cyclin D1 to CDK4 and inhibiting cell proliferation. Furthermore, a study has indicated that phytosterols can upregulate pro-apoptotic proteins, and activate the PI3K/Akt, Akt/mTOR, and KDR signaling pathways, thereby participating in the regulation of various types of cancers.[36-38] Similarly, our results indicate that stigmasterol reduced the expression of key genes (KDR, PI3K, AKT) in the PI3K/AKT signaling pathway, along with a decrease in the ratios of p-PI3K/PI3K and p-AKT/AKT, suggesting that stigmasterol can induce apoptosis of FLS by blocking KDR-mediated activation of PI3K/AKT signaling pathway, thus playing a role in the treatment of RA.

CONCLUSION

In conclusion, stigmasterol down-regulates the expression of Cyclin D1, CDK4, and Rb in CIA-FLS, and inhibits the migration and proliferation of CIA-FLS. Other than that, stigmasterol promotes FLS apoptosis by blocking KDR-mediated activation of the PI3K/AKT signaling pathway and reducing the expression of key genes KDR, PI3K, AKT, and phosphorylated proteins p-PI3K and p-AKT in this pathway. These biological effects suggest the potential therapeutic effect of stigmasterol on RA.

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AUTHOR CONTRIBUTIONS

Daomin Lu (MM) and Yuzheng Yang (MM) contributed equally to this work.

CONFLICT OF INTEREST STATEMENT

No potential conflict of interest was reported by the authors.

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