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Curcumol Attenuates Endometriosis by Inhibiting the JAK2/STAT3 Signaling Pathway

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Manuscript Preparation E
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Conflict of interest:

None declared

Background:

Curcumol is a hydrogenated austenitic compound with hemiketal. In this study we evaluated the effects of curcumol on local inflammatory response, cell proliferation, and metastasis in endometriosis, and elucidated the underlying mechanisms.

Material/Methods:

Ectopic endometrial stromal cells were treated with increasing doses of curcumol. The MTT assay was used to assess cell viability. FITC-labeled annexin-V/PI double-staining method and flow cytometry were used to determine cell apoptosis. Cell migration was evaluated using a wound healing assay. ELISA kits were used to detect the levels of TNF- α , IL-6, and IL-1 β . Western blot assay was used to examine the phosphorylation degree of JAK2 and STAT3 and the expression of Bax, Bcl2, and caspase-3 proteins. Autologous endometrial transplantation was used to establish a rat model to assess the anti-EMS effect of curcumol in vivo.

Results:

Curcumol can inhibit the proliferation of ectopic endometrial stromal cells, promote cell apoptosis, and weaken cell migration ability. Curcumol can reduce the expression of Bax and caspase-3 protein and increase the expression of Bcl2 protein. Curcumol also can inhibit the secretion of inflammatory cytokines, including tumor necrosis cytokines (TNF)- α , interleukin (IL)-6, and IL-1 β , by ectopic endometrial stromal cells. In addition, curcumol can also inhibit the phosphorylation of JAK2 and STAT3. In vivo experiments also proved that curcumol could inhibit the growth of ectopic lesions in EMS model rats.

Conclusions:

Curcumol can inhibit the JAK2/STAT3 pathway, reduce the inflammatory cytokines secreted by ectopic endometrial stromal cells, inhibit cell proliferation and migration, and reduce the volume of ectopic lesions.

Keywords:

Curcumol • Endometriosis • JAK2 Protein, Human • STAT Transcription Factors

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Background

Endometriosis (EMS) is a common chronic disease characterized by the growth of endometrial glands and stroma outside the uterus, mainly on the pelvic peritoneum and ovaries [1]. This disease affects 10-20% of women of childbearing age, 20-30% of women with infertility, and 40-60% of women with dysmenorrhea [2]. Although EMS is a benign disease, EMS cells are known to have characteristics similar to those of malignant cancer cells, such as continuous proliferation, angiogenesis, migration, and invasion [3]. However, the cellular and molecular mechanisms behind these pathological changes have not been fully defined. Sampson found that endometrial cells are transported through the fallopian tube during menstruation, are implanted into the peritoneal surface, undergo heterotopic implantation, and grow as lesions [4]. However, it is unclear why most women have menstrual blood reflux during menstruation, but only 10% of women develop EMS. It is increasingly recognized that the peritoneal microenvironment plays an important role in the migration, implantation, and invasion of ectopic endometrium, and the severity of the inflammatory microenvironment is related to the development of EMS [5,6]. The disease is characterized by increased production of pro-inflammatory cytokines such as IL-6 and tumor necrosis factor alpha (TNF α), which can promote endometrial cell proliferation and adhesion [7,8].

Janus kinase-signal transducers and activators of transcription (JAK-STAT) is an important pathway of cytokine signal transduction. JAK2/STAT3, as an important signal axis in the JAK-STAT pathway, is an important transduction pathway mediated by a variety of cytokines and growth factors and is involved in cell proliferation, differentiation, apoptosis, migration, and immune cell apoptosis [9,10]. There are many activators of the JAK2/STAT3 signaling pathway in patients with EMS, such as IL-6 and TNF α , and these cytokines can activate the JAK2/STAT3 signaling pathway, phosphorylate STAT3, participate in the expression of target genes, and activate its downstream cytokines such as Bcl2 and caspase-3, which leads to the continuous proliferation of ectopic endometrium and enhances anti-apoptosis ability [8,11].

Curcumol is an active component extracted from a traditional Chinese medicine plant, *Curcuma zedoaria*. Clinical and experimental research have found that curcumol can inhibit proliferation and promote apoptosis of many kinds of solid tumor cells, such as lung cancer, gastric cancer, and ovarian cancer [12-14]. The results also show that curcumol can not only kill tumor cells directly, but it can also achieve anti-tumor effects by improving the body's immune function [15]. These effects may be related to inhibition of the JAK2/STAT3 signal transduction pathway by curcumol [16]. We searched the literature on traditional Chinese medicine in the treatment of EMS, and found that *Curcuma zedoaria* is the most frequently used drug [17].

We also found in previous research that curcumol has a good therapeutic effect on EMS, and its effect is related to the inhibition of local inflammatory response [18,19]. Based on the above results, we speculated that curcumol may affect the EMS process by inhibiting the JAK2/STAT3 signal transduction pathway, but we found no previous research on this topic.

In this research, we conducted cell experiments and animal experiments, from the perspective of anti-inflammatory, promoting apoptosis, and inhibiting proliferation and migration, to explore whether curcumol can block the JAK2/STAT3 signal transduction pathway, reduce local inflammatory response, promote cell apoptosis, inhibit cell proliferation and migration, and inhibit the growth of ectopic lesions.

Material and Methods

Reagents and Antibodies

Curcumol (4871970), purity $\geq 98\%$, was purchased from Shanghai Jizhi Biochemical Technology Co., Ltd. Tumor Necrosis Cytokines (TNF- α) Kit (C06PZB), interleukin-6 (IL-6) Kit (C12PDB), and interleukin 1 β (IL-1 β) Kit (C09PDB) were all purchased from Beijing North Institute of Biotechnology. PCR primer (Shengggong Biological Products Co., Lt. T), Trizol reagent (10296010), and SYBR Green Master Mix (A25742) were purchased from Thermo Fisher Technology (USA). JAK2-specific antagonist α -cyano-(3,4-hydroxy) N-benzylstyrylamine (AG490, 100 mg/piece) was purchased from Santa Cruz Biotechnology (Shanghai) Co. Goat anti-Rabbit IgG TNF- α (ab6671) polyclonal antibody, JAK2 (Rabbit polyclonal antibody, 1: 1000, ab39636), JAK2 (phospho Rabbit polyclonal antibody, 1: 1000, ab32101), STAT3 (Mouse monoclonal antibody, 1: 1000, ab119352), STAT3 phospho Y705 (Rabbit monoclonal antibody, 1: 1000, ab76315), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Mouse monoclonal antibody, 1: 1000, ab8245) were all purchased from Abcam Trading Co.

Acquisition of Ectopic Endometrium

Between January 2020 and December 2020, 5 patients with postoperative pathologically confirmed EMS were enrolled at Hebei Provincial Hospital of Traditional Chinese Medicine (according to rAFS: stage III-IV). The patients ranged in age from 24 to 33 years old, with a mean age of 32.0 ± 5.6 (mean \pm SD) years.

The inclusion criteria were: 1) diagnosis of EMS by pathology; 2) regular menstruation cycle; 3) no any hormonal treatment within 6 months prior to surgery.

The exclusion criteria were: 1) abnormally developed uterus; 2) diagnosis of malignant tumor, hepatic and renal insufficiency, and hematological disease.

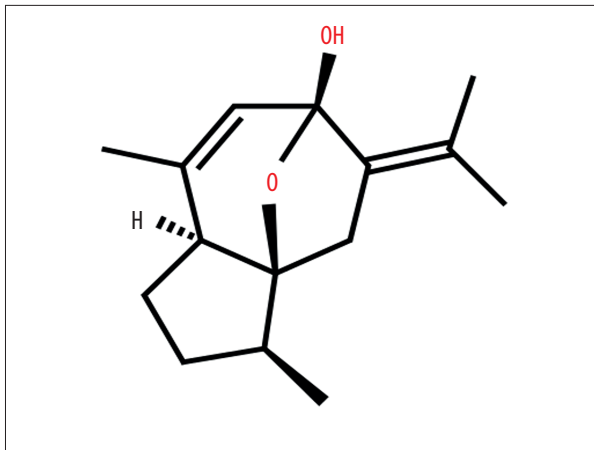


Figure 1. Chemical structure of curcumin.

The samples were collected during the proliferative period of the menstrual cycle. Written informed consent was obtained from all participants and the study was approved by the local ethics committee (No: YXLL2020004).

Cell Culture

The ectopic endometrium of EMS patients was washed with F-12/DMEM (FD) medium, cut into pieces <1 mm³, and digested with 1% collagenase at 37°C for 60-90 min. After filtration with a cell filter, the cells were gradient-precipitated with 3.0% and 1.5%

bovine serum albumin (BSA) solutions, and then the stromal cells were obtained and inoculated in a culture flask. Cell morphology and growth were observed under an inverted microscope.

MTT Cell Viability Assay

Ectopic endometrial stromal cells were seeded in 96-well plates (5×10³ cells/well), cultured in medium for 24 h, and then treated with curcumin at the corresponding concentration or at the specified time. After absorbing the supernatant, medium containing 0.5% MTT was added, the culture was continued for 4 h, then DMSO was added. After repeated dissolution of the crystals, the absorbance of each pore was measured at 490 nm by microplate, and the inhibition rate of cell growth was calculated.

Flow Cytometric Analyses of Apoptosis

FITC-labeled annexin-V/PI double-staining method and flow cytometry were used to determine cell apoptosis. The ectopic endometrial stromal cells were treated with curcumin for 48 h. This experiment was carried out according to the operating procedures of the annexin-V-FITC apoptosis detection kit (Nanjing keygen Biotechnology Co., Nanjing, China). Only the fluorescein-positive cells without PI staining were regarded as apoptotic cells, and the percentage was determined by flow cytometry (FACS Calibur flow cytometry, BD, USA). CellQuest software was used to analyze the data.

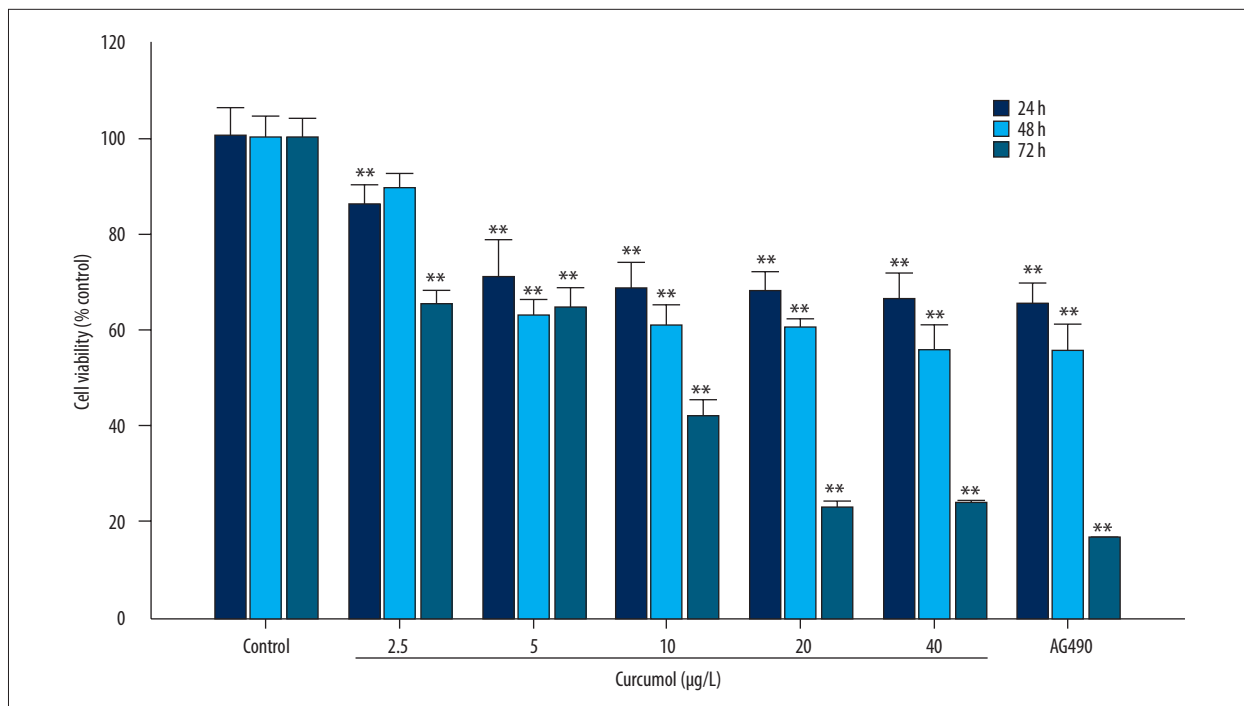


Figure 2. The effects of curcumin on cell viability in vitro. MTT cell viability assay shows the dose-dependent growth-inhibitory effects of curcumin on ectopic endometrial stromal cells. The experiments were performed in triplicate, and the results were expressed as mean±SD. Compared with the control group, ** $P < 0.01$.

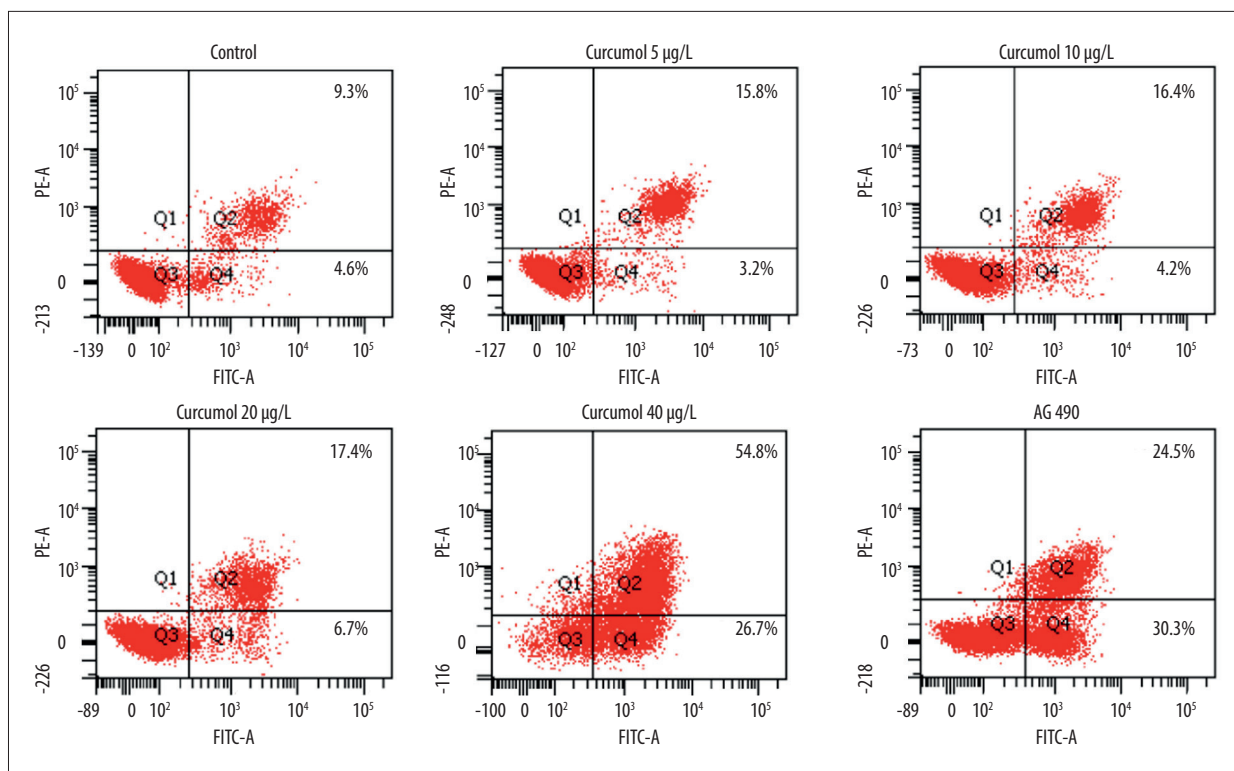


Figure 3. The results of flow cytometry showed that curcumol could induce apoptosis of ectopic endometrial stromal cells. The experiments were performed in triplicate.

Wound Healing Assay

The medium was discarded, the cells were washed with PBS 3 times, digested with 0.25% trypsin, counted, and inoculated on 24-well plates at a density of 1×10^6 cells/ml. After the cells spread to the bottom of the well plate, they were treated with 20 µg/L curcumol. We replaced the supernatant with a serum-free medium, used a pipette tip to make a vertical scratch in the middle of the bottom of the plate, and took pictures under an inverted microscope. Image J software was used to measure the scratch width at 24 h and to assess cell mobility.

Measurements of Cytokines

The cells were incubated with curcumol 2.5-40 µg/L for 48 h, and the supernatant was taken. Different ELISA kits were used to detect the cytokines (TNF- α , IL-6, IL-1 β) in the cells or rat peritoneal fluid according to the operating procedures provided by the manufacturer. According to the instructions, the concentration of cytokines was calculated in linear regression.

Western Blot Analysis

The ectopic endometrial stromal cells were treated with curcumol 2.5-40 µg/L for 48 h. The protein was extracted from ectopic endometrial stromal cells or endometrial tissue and

quantified. Then, SDS loading buffer was added and denatured by heating. The processed samples were electrophoresed, transferred, and sealed. The antibody of the corresponding protein was added to the membrane and kept overnight at 4°C. The next day, samples were incubated with the corresponding secondary antibody at room temperature for 1 h, and then were assessed by chemiluminescence. The immune complex development was carried out using an enhanced chemiluminescence (ECL) detection kit and the bands were analyzed using the Gel Doc 2000 imaging system. The gray value of the target protein band and the gray value of the internal reference GAPDH protein band were assessed, and the relative expression of each target protein was calculated.

Animal Experiments

The rat EMS model was reproduced by transplantation autoplasmic. The protocol was approved by the Animal Ethics Committee of Hebei University of Traditional Chinese Medicine. Sprague-Dawley rats of SPF grade were purchased from the Experimental Animal Center of Hebei Medical University. The rats were fed in the SPF environment, with 23-25°C ambient temperature, and fed standard laboratory rat chow. All possible efforts were made to treat experimental animals humanely and we followed the Guidelines for the Management and Use of Experimental Animals issued by the National Institutes of Health.

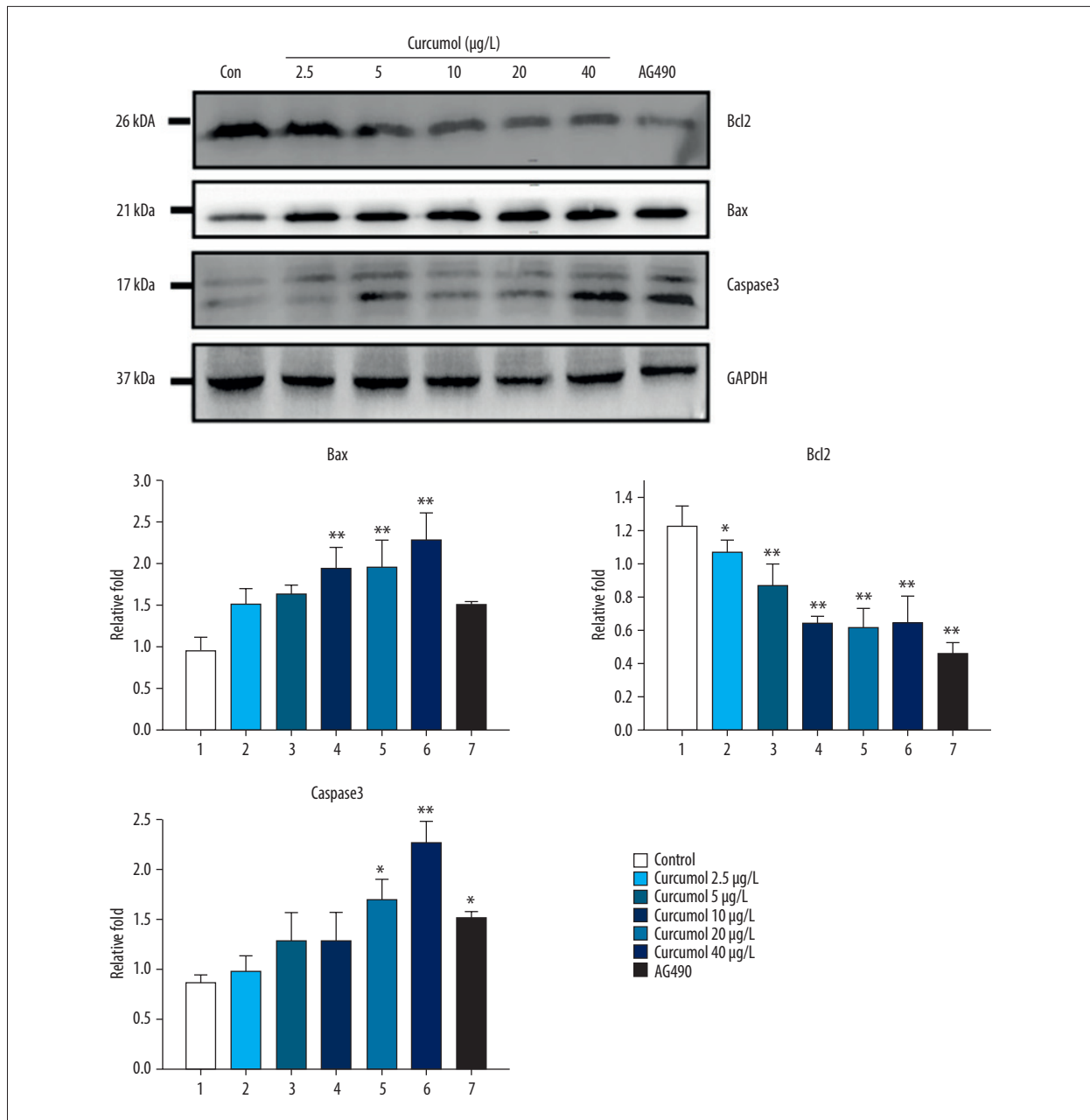


Figure 4. Western blot shows that curcumol resulted in increased expression of Bax and caspase-3, reduced expression of Bcl2. The experiments were performed in triplicate, and the results were expressed as mean±SD. Compared with control group, * $P<0.05$, ** $P<0.01$.

After general anesthesia, rats were laparotomized, and 5×5 mm uterine fragments were cut and sutured into the peritoneum. On day after surgery, rats were given estradiol benzoate at 0.1 mg/kg body weight, once every 5 days for 3 times to promote the growth of ectopic endometrium. Another 10 rats in the sham operation group underwent only a simulated operation. After 30 days, the volume of ectopic lesions was detected by B-ultrasound. The successfully modeled rats were divided into 3 groups according to lesion volume: the model

group, the positive control group (intraperitoneal injection of AG490, 4 mg/kg body weight, twice a week), and the experimental group (intragastric administration of curcumol, 20 mg/kg body weight, once a day), with 10 rats in each group. There were 10 rats in the sham operation group. Curcumol was continuously administered for 30 days. On the 28th day of administration, the ectopic lesions of the rats were assessed by B-ultrasound. After the experiment, the long diameter and short diameter of ectopic endometrium were measured by vernier

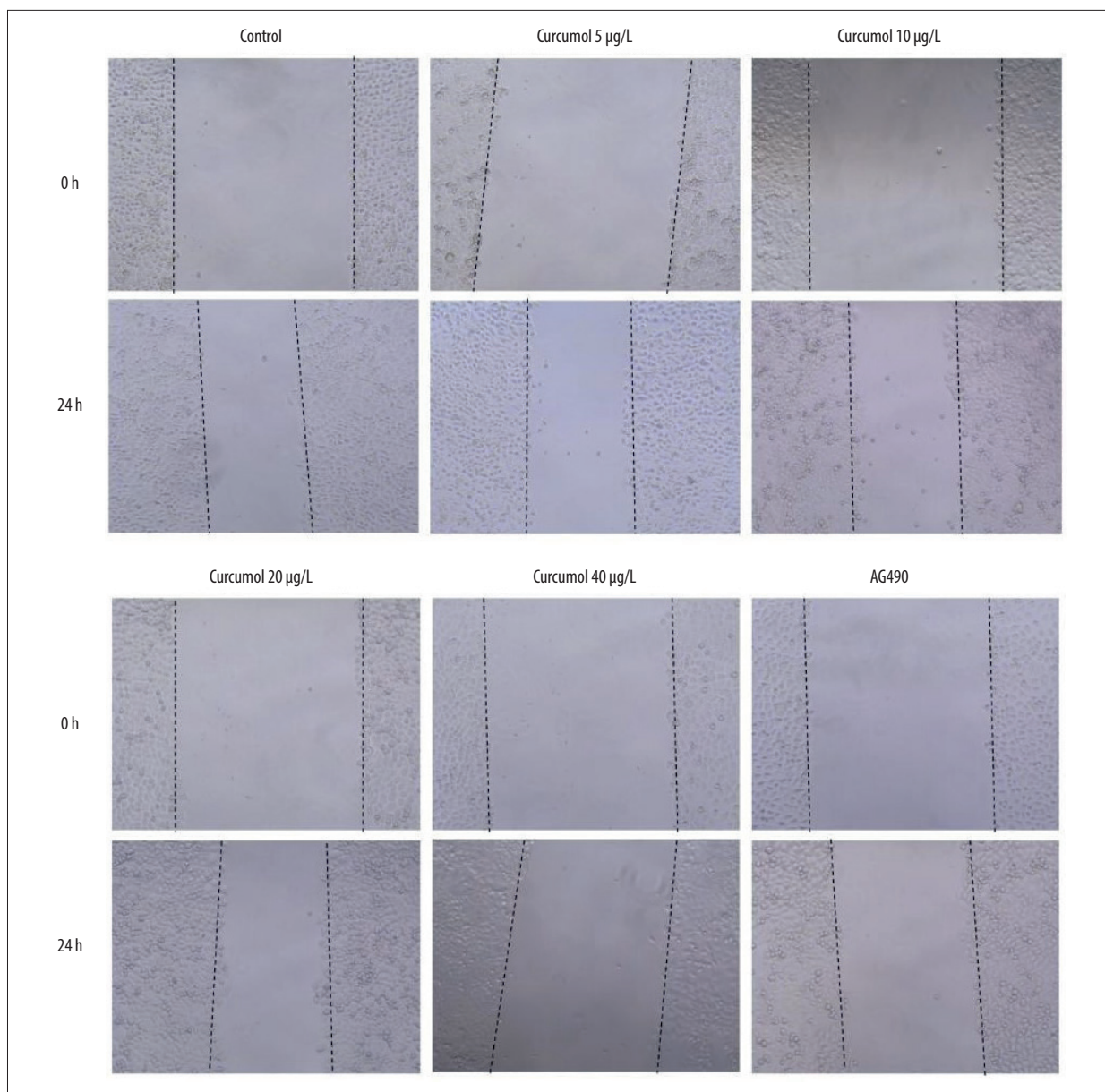


Figure 5. Effect of curcumol on ectopic endometrial stromal cell migration. Cell wound healing assay proved the inhibited effect of curcumol on the migration of ectopic endometrial stromal cells. The experiments were performed in triplicate.

caliper, and we calculated the area. HE staining of ectopic lesions was performed.

Statistical Analysis

For in vitro studies, all experiments were performed in triplicate. The data are expressed as mean values \pm standard deviation (SD). The quantitative data were tested for normality using the Shapiro-Wilk test, and normally distributed data were evaluated using SPSS19.0 software to perform one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

Results

Curcumol Reduced the Viability of Ectopic Endometrial Cells

We preliminarily determined that curcumol (**Figure 1**) has an effect on the activity of ectopic endometrial stromal cells. Cell proliferation was measured by MTT assay. At a concentration of 5-40 $\mu\text{g/L}$, curcumol has a significant inhibitory effect on the viability of ectopic endometrial cells when it acts for 24, 48, and 72 h (24 h: $P = 0.000$, 48 h: $P = 0.013$, 72 h: $P = 0.000$) (**Figure 2**). Especially when treated with curcumol for 72 h, the

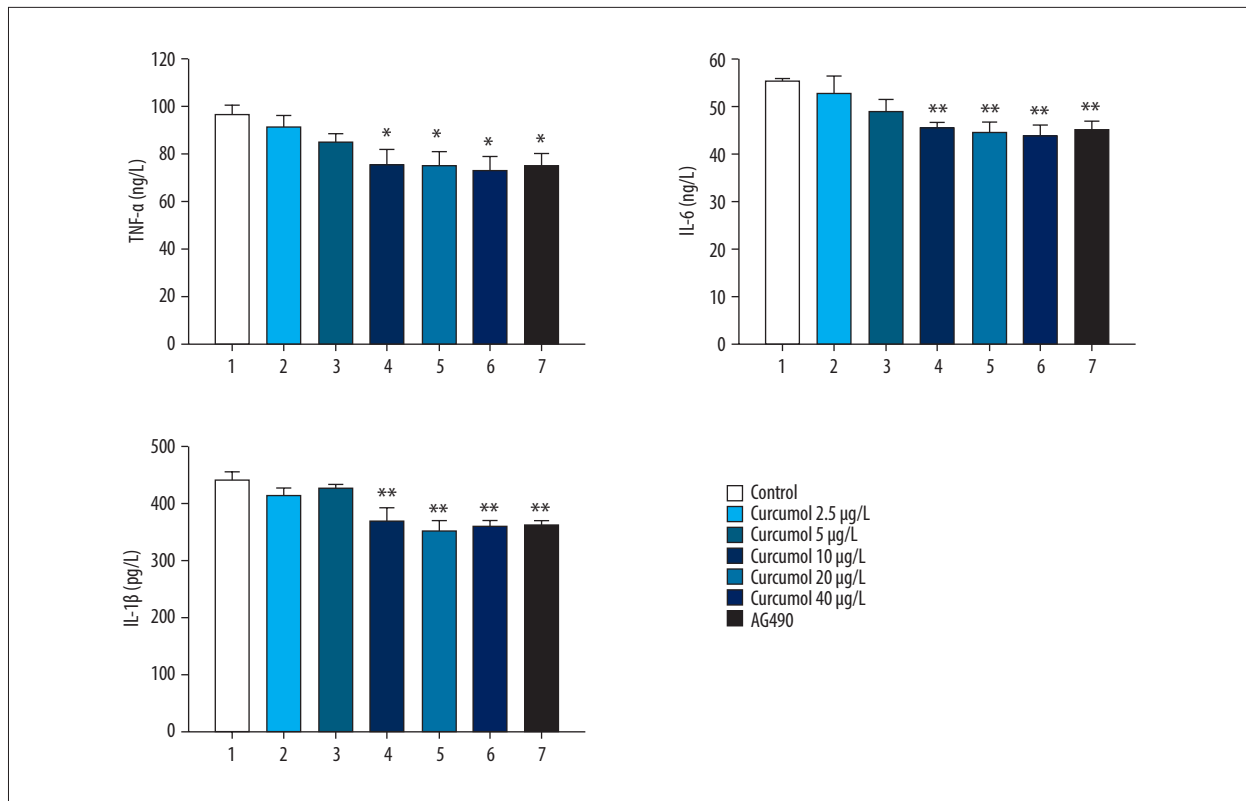


Figure 6. Effects of curcumol on levels of inflammatory cytokines (TNF- α , IL-6, and IL-1 β) in the supernatant of ectopic endometrial stromal cells. The experiments were performed in triplicate, and the results were expressed as mean \pm SD. Compared with control group, * $P<0.05$, ** $P<0.01$.

cell viability was significantly inhibited, and 20 and 40 $\mu\text{g/L}$ curcumol reduced cell activity to 22% of normal.

Curcumol Induced Apoptosis in Ectopic Endometrial Cells

The results of flow cytometry showed that curcumol could induce apoptosis of ectopic endometrial stromal cells. At a concentration of 5-40 $\mu\text{g/L}$, when curcumol acts on ectopic endometrial stromal cells for 48 h, the apoptosis rate is significantly increased in a concentration-dependent manner. The number of apoptotic cells was found to be significantly increased by curcumol treatment with 9.3% in the control group and 54.8% at 40 $\mu\text{g/L}$ (Figure 3). In addition, we also observed that curcumol at the concentrations of 10, 20, and 40 $\mu\text{g/L}$ significantly upregulated the expression of Bax in ectopic endometrial cells ($P=0.026$). The protein expression of Bcl2 was decreased at 5-40 $\mu\text{g/L}$ ($P=0.002$). The expression of caspase-3 was increased at 20 and 40 $\mu\text{g/L}$ ($P=0.004$) (Figure 4).

Curcumol Inhibits Migration of Ectopic Endometrial Cells

Cell wound healing assay proved the effect of curcumol on the migration of ectopic endometrial cells. The results showed that curcumol could significantly inhibit the migration of ectopic

endometrial stromal cells at the concentrations of 5, 10, 20, and 40 $\mu\text{g/L}$ for 24 h, which was measured as the wound width (Figure 5).

Curcumol Inhibits the Secretion of Inflammatory Cytokines in Ectopic Endometrial Cells

The inhibitory effect of curcumol on the secretion of inflammatory cytokines by interstitial cells was evaluated. We measured the levels of inflammatory cytokines (TNF- α , IL-6, and IL-1 β) in the supernatant of ectopic endometrial cells treated with curcumol by ELISA. The levels of TNF- α , IL-6, and IL-1 β in the supernatant of curcumol at 10, 20, and 40 $\mu\text{g/L}$ were significantly decreased (TNF- α : $P=0.047$, IL-6: $P=0.013$, IL-1 β : $P=0.001$). These data indicate that curcumol can inhibit the secretion of inflammatory cytokines in ectopic endometrial cells at relatively low concentrations (Figure 6).

Curcumol Interferes with JAK2/STAT3 Signal in Ectopic Endometrial Stromal Cells

Because the phosphorylation level of JAK2/STAT3 in ectopic lesions of EMS patients is increased, we studied the effect of curcumol on JAK2/STAT3 signaling in ectopic endometrial cells.

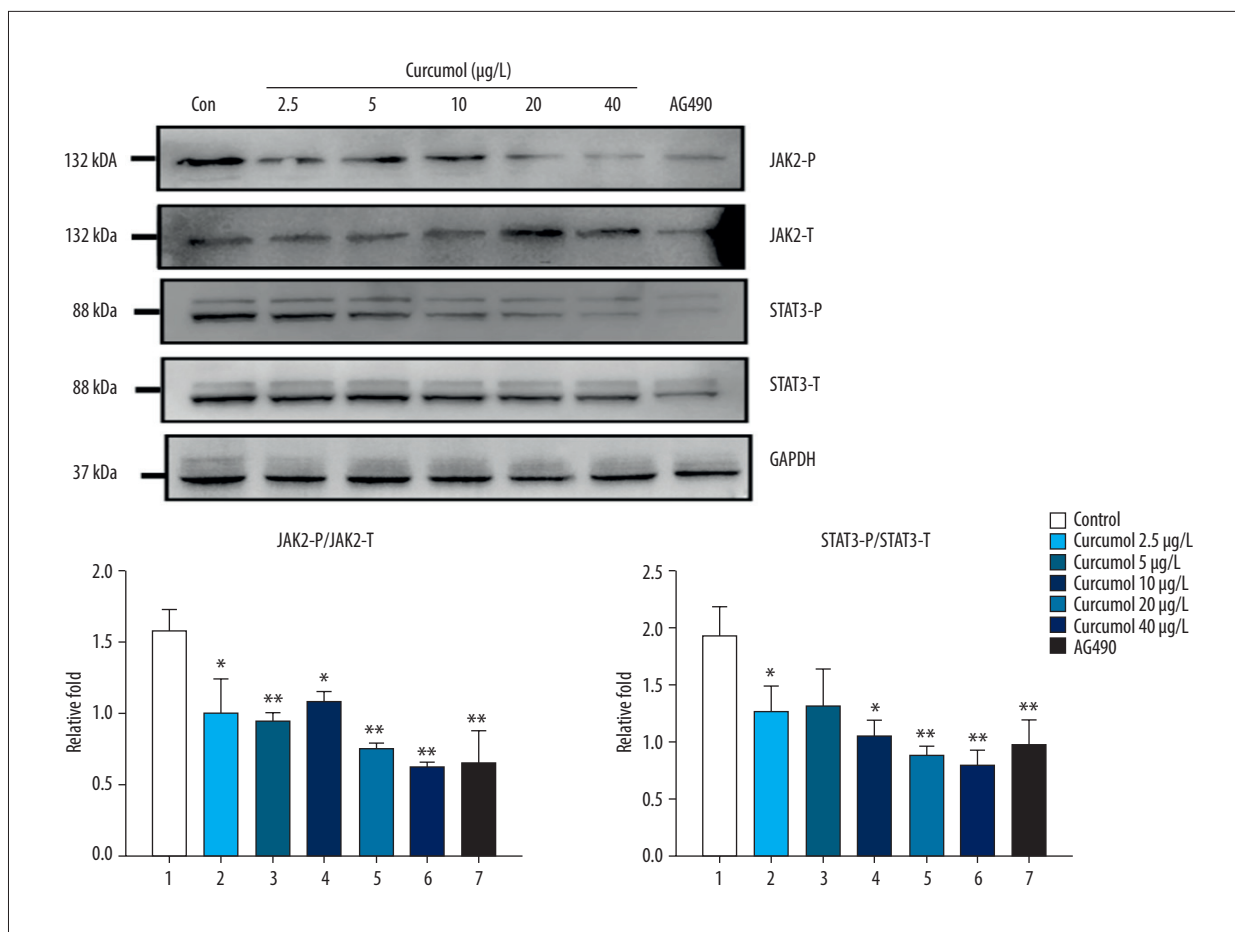


Figure 7. Western blot results show that curcumol is associated with a dose-dependent decrease in the phosphorylation levels of JAK2 and STAT3 in ectopic endometrial stromal cell. The experiments were performed in triplicate, and the results were expressed as mean±SD. Compared with the control group, * $P < 0.05$, ** $P < 0.01$.

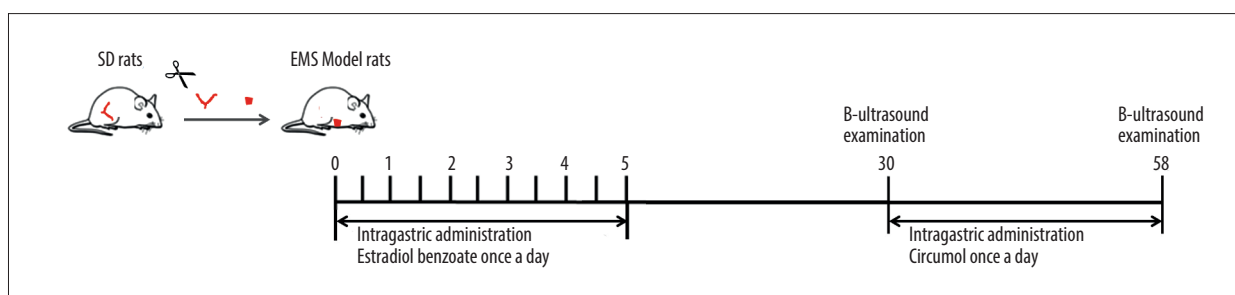


Figure 8. We used the method of autologous endometrial transplantation to copy the rat model of EMS.

As expected, similar to AG490, an inhibitor of JAK2/STAT3 signaling, curcumol can inhibit the phosphorylation of JAK2 and STAT3 (JAK2: $P = 0.006$, STAT3: $P = 0.033$) (Figure 7).

Curcumol Can Inhibit the Growth of Ectopic Lesions in EMS Model Rats

We used the method of autologous endometrial transplantation to construct the rat model of EMS. After the formation

of ectopic lesions, rats were continuously fed with curcumol for 28 days (Figure 8). The results showed that curcumol and AG490 could inhibit the growth of ectopic lesions. By using B-ultrasound detection and caliper measurement, we found that the volume of ectopic lesions in the treatment group was significantly smaller than that in the model group (B-ultrasound detection: $P = 0.000$, caliper measurement: $P = 0.000$) (Figure 9).

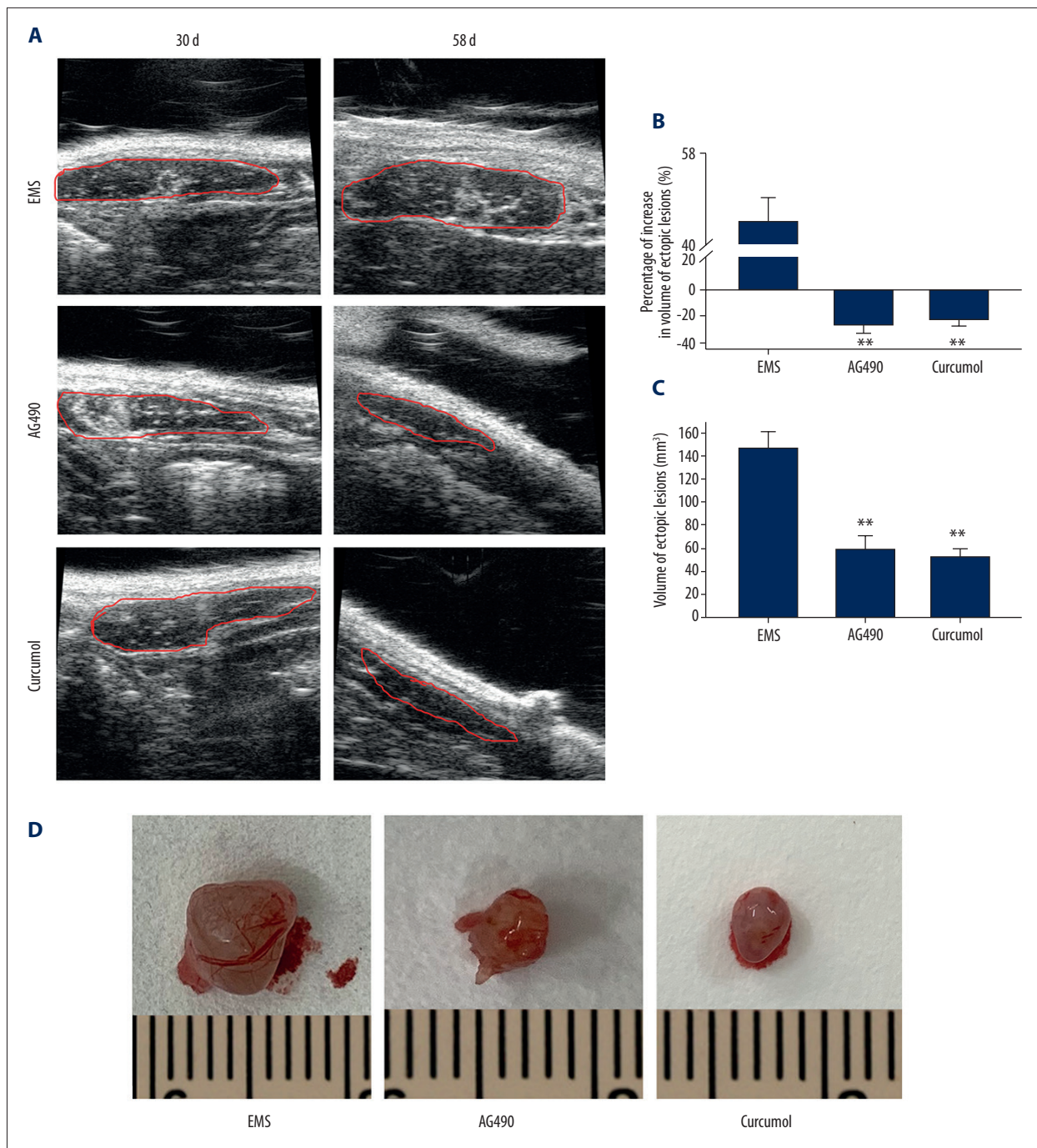


Figure 9. Effect of curcumol on growth of ectopic lesions in EMS model rats. **(A)** The volume of ectopic lesions was detected by B-ultrasound (the lesions are marked by red line). **(B)** Percentage of volume proliferation of ectopic lesions calculated from B-mode ultrasound results. **(C)** The ectopic lesion volume calculated according to caliper measurement. **(D)** The appearance of ectopic lesions. The experiments were performed in duplicate, and the results were expressed as mean±SD. Compared with control group, ** $P<0.01$.

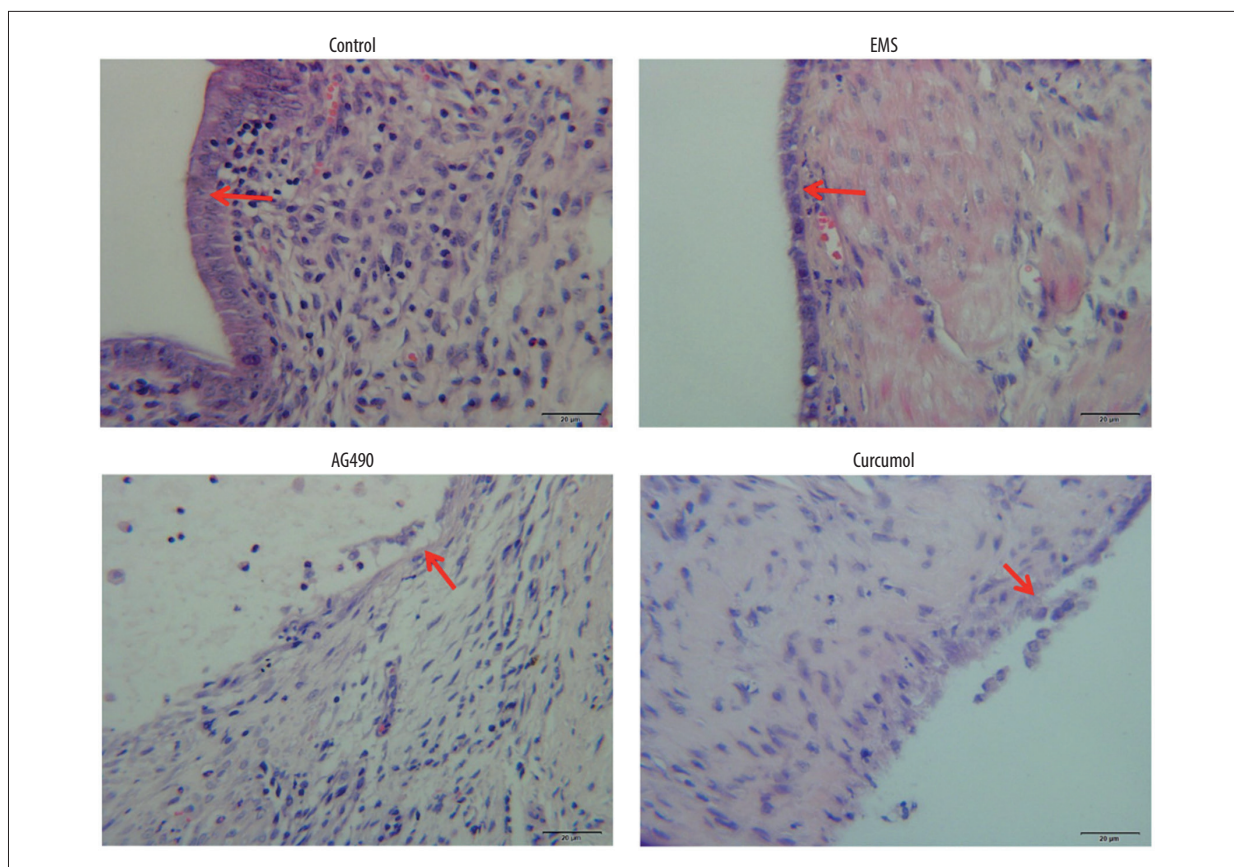


Figure 10. HE staining was used to observe the pathological morphology (epithelial cells are marked by red arrows). The experiments were performed in triplicate.

HE staining (**Figure 10**) showed that the histomorphology of ectopic lesions was similar to that of eutopic uterus, the surface layer was composed of neatly arranged flat columnar epithelial cells, and glands were seen in the interstitium. After treatment, the epithelial cells, which were originally neat and dense, became incomplete.

The results of the western blot experiment show that, similar to the JAK2/STAT3 inhibitor AG490, curcamol can also inhibit the phosphorylation of JAK2 and STAT3 (JAK2: $P=0.000$, STAT3: $P=0.000$). After the signal transduction of JAK2/STAT3 is inhibited, the protein expression levels of Bax and caspase-3 in the ectopic lesion tissue were significantly increased, while the protein expression level of Bcl2 was significantly reduced (Bax: $P=0.005$, caspase-3: $P=0.003$, Bcl2: $P=0.044$) (**Figure 11**). Detecting the levels of inflammatory cytokines in the rat's peritoneal fluid showed that after treatment, the levels of TNF- α , IL-6 and IL-1 β had decreased significantly (TNF- α : $P=0.002$, IL-6: $P=0.036$, IL-1 β : $P=0.001$) (**Figure 12**). In conclusion, curcamol has an obvious therapeutic effect on EMS rats.

Discussion

In recent years, the incidence rate of EMS has increased year by year. It is often accompanied by chronic pelvic pain, adhesions, and infertility, which seriously affect the quality of life and reproductive capacity of patients, thus causing widespread concern in the field of gynecology. At present, the methods for treating EMS are surgery and ovarian inhibitors, including GnRH agonists, progestins, oral contraceptives, and androgen drugs. However, hormone therapy is associated with adverse effects such as delayed conception [20]. More seriously, EMS increases the risk of epithelial ovarian cancer by 50% [21]. Therefore, it is still necessary to find effective drugs for the treatment of EMS. The findings of the present study showed that curcamol, an important component of the herbal medicine compound derived from *Curcuma zedoaria*, exerted growth-inhibitory effects on ectopic lesions in EMS. We also found in previous research that curcamol's inhibition of ectopic lesion growth is associated with anti-inflammatory effects [18]. Therefore, we continue the discussion of the relevant mechanisms in the present article.

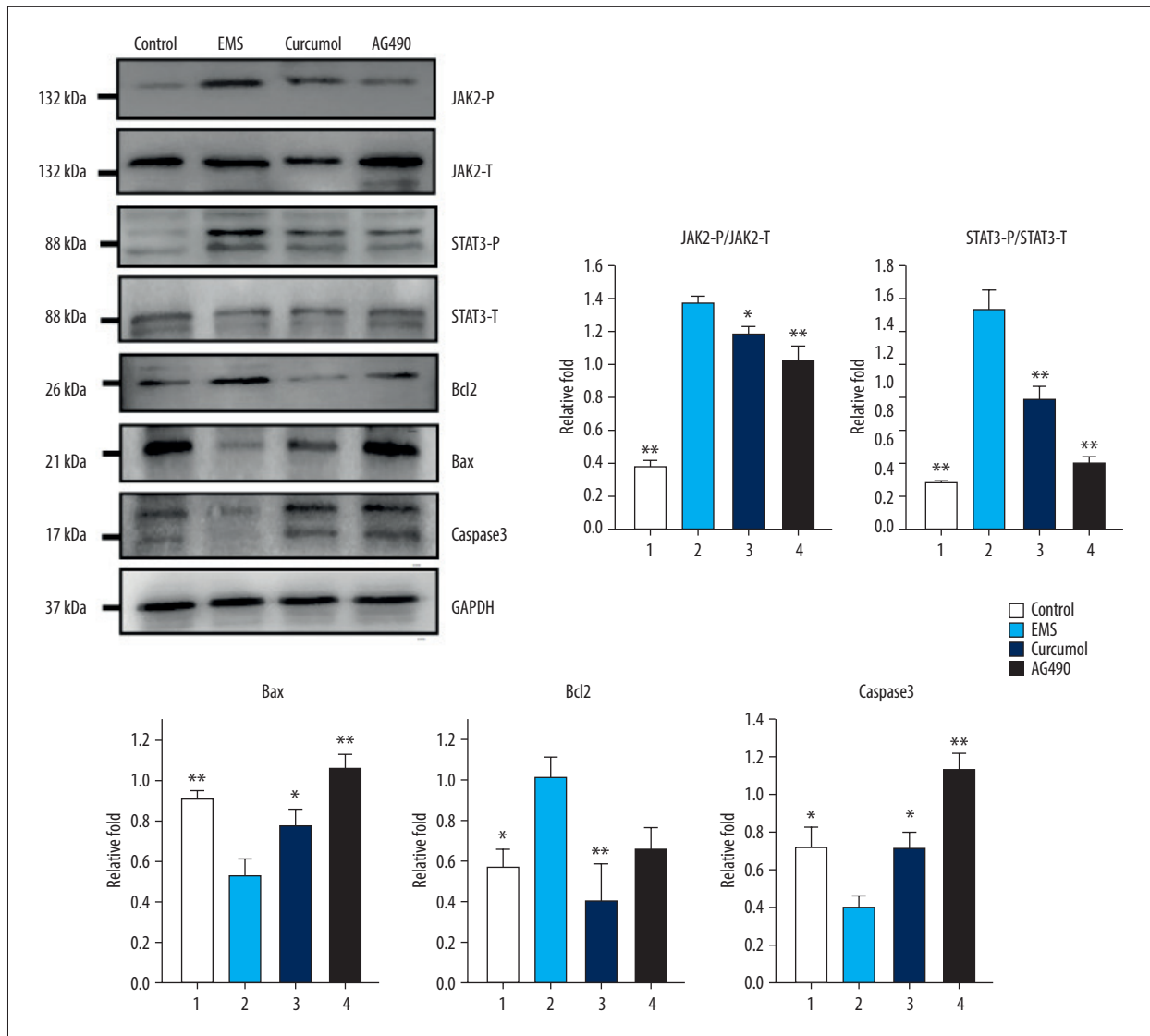


Figure 11. Effects of curcumol on phosphorylation levels of JAK2, STAT3 and expression levels of Bax, Bcl2, caspase-3 in ectopic lesions as shown by western blot analysis. The experiments were performed in triplicate, and the results were expressed as mean±SD. Compared with control group, * $P<0.05$, ** $P<0.01$.

In the present study, we found that curcumol decreased ectopic endometrial stromal cell viability in a dose-dependent manner. Flow cytometry assay showed that curcumol triggered apoptosis in cells. Increased apoptosis was associated with up-regulation of the expression of apoptosis-related proteins Bax and caspase-3 and downregulation of Bcl2 by curcumol. The expression of cleaved caspase-3 was considerably increased after treatment of the ectopic endometrial stromal cells with curcumol, indicating an increase in apoptosis. Bax and Bcl2 are considered to be important biomarkers of apoptosis, and an increase in the Bax/Bcl2 ratio promotes apoptosis [22].

In this research, curcumol treatment was also shown to reduce the secretion of inflammatory cytokines TNF- α , IL-6, and

IL-1 β in ectopic endometrial cells. Research has shown that TNF- α and IL-1 β are necessary for chronic inflammation, and their activation of the JAK2/STAT3 signaling pathway curcumol is closely related to the development of EMS [23,24]. IL-6 can also cooperate with other cytokines, such as TGF- β , to induce epithelial–mesenchymal transition, thus promoting the proliferation, movement, and invasion of endometrial cells [25]. Research has shown that stimulation of the JAK2/STAT3 signal transduction pathway can also promote the proliferation and migration of human endometrial cells, and it can inhibit apoptosis and promote inflammatory reaction in EMS model rats [26,27]. Increasing evidence also shows that blocking JAK2/STAT3 signaling not only inhibits the proliferation of endometrial cells, but also reduces the inflammatory response in

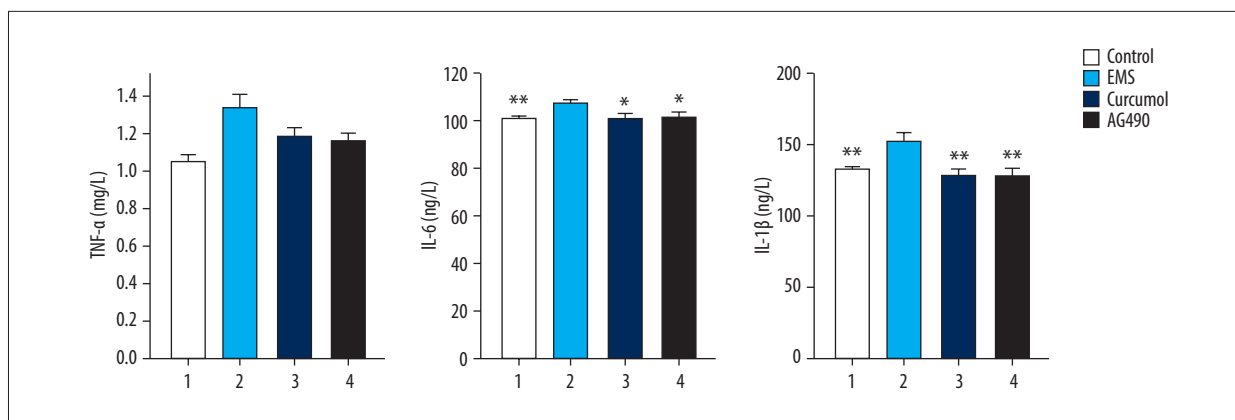


Figure 12. Curcumol can decrease the levels of inflammatory cytokines (TNF α , IL-6, and IL-1 β) in rat peritoneal fluid. The experiments were performed in duplicate, and the results were expressed as mean \pm SD. Compared with the control group, * $P<0.05$, ** $P<0.01$.

the microenvironment [28]. Previous research has confirmed that curcumol can inhibit the JAK2/STAT3 pathway [29]. The findings of this study show that the effect of curcumol is similar to that of the JAK2/STAT3 signal inhibitor AG490 in reducing the phosphorylation of JAK2 and STAT3 in ectopic endometrial stromal cells.

In animal experiments, curcumol showed a therapeutic effect equivalent to that of AG490, and curcumol can reduce the volume of ectopic lesions and atrophy of the endometrium of EMS model rats. During this experiment, we also observed an increase in the phosphorylation of JAK2 and STAT3 in the ectopic lesion tissue of the EMS model rats. The expression of apoptotic protein decreased, while the expression of protective protein Bcl2 increased significantly.

Here, we report that curcumol can inhibit the growth of EMS ectopic lesions by affecting JAK2/STAT3 signaling and reduction of inflammatory response, suggesting that curcumol could prove to be an important component for the treatment of EMS. However, curcumol requires further investigation to determine whether it can be developed into a lead molecule for the treatment of EMS. The lack of observation of the effects of curcumol on normal endometrial mesenchymal cells is the most important limitation of our study. In the future, we will

also continue to assess the role of curcumol in normal endometrial stromal cells overexpressing JAK2 or STAT3 to clarify the therapeutic effect of curcumol on EMS.

Conclusions

Our current research shows that curcumol can inhibit the proliferation and migration of ectopic endometrial stromal cells, reduce the secretion of inflammatory cytokines, and attenuate endometriosis. These effects may be related to inhibition of the JAK2/STAT3 pathway.

Ethics Statement

The human experiment was approved by the Medical Ethics Committee of Hebei University of Chinese Medicine (No: YXLL2020004), and animal research was approved by the Animal Ethics Committee of Hebei University of Chinese Medicine.

Declaration of Figures' Authenticity

All figures submitted have been created by the authors who confirm that the images are original with no duplication and have not been previously published in whole or in part.

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