Inhibitory effect of curcumin in human endometriosis endometrial cells via downregulation of vascular endothelial growth factor

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Abstract. Endometriosis, which affects up to 10% of women of reproductive age, is defined as endometrial-like gland and stroma tissue growths outside the uterine cavity. Despite increasing research efforts, there are no current effective treatment methods for this disease, therefore investigations for therapeutic strategies are of primary concern. In preliminary work, the authors demonstrated that curcumin inhibits endometriosis *in vivo*. The present *in vitro* study aimed to investigate the association between endometriotic stromal cells and curcumin and to clarify the underlying mechanism of action. A total of 14 patients with endometriosis were enrolled in the present study. The purity of endometrial stromal cell cultures was proven by standard immunofluorescent staining of vimentin. The cell proliferation and curcumin effects on endometrial stromal cells were assessed by the MTT assay and Hematoxylin and Eosin staining. For cell cycle analysis, phase distribution was detected by flow cytometry. Vascular endothelial growth factor (VEGF) protein expression was examined using immunohistochemistry staining. Apoptosis was assessed using Annexin V-fluorescein isothiocyanate staining. The results indicated that the treatment of curcumin decreased human ectopic and eutopic stromal cell growth. Following treatment with curcumin, human endometriotic stromal cells demonstrated an increased percentage of G_1-phase cells and decreased percentages of S-phase cells, particularly in the group treated with 50 μmol/l curcumin. Treatment with curcumin additionally decreased expression of VEGF. The data provide evidence that curcumin reduces cell survival in human endometriotic stromal cells, and this may be mediated via downregulation of the VEGF signaling pathway.

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Introduction

Endometriosis, which is a chronic benign gynecological disease, is defined as endometrial-like gland and stroma tissue growths which are present outside the uterine cavity. Endometriosis affects up to 10% of reproductive age women and is a primary contributor to infertility and dysmenorrhea (1,2). Despite previous research efforts, the pathogenesis is still unclear, and treatment remains a controversy, with current approaches including pharmacological therapy and surgical treatment. Pharmacological therapy aims at suppressing the estrogen production to prevent progression of the disease, however this may result in a risk of adverse effects associated with decreased estrogen (3). Surgical treatment allows histological diagnosis, resolves symptoms, and may restore fertility by removing endometriotic lesions (4) and previous studies have demonstrated that laparoscopy has a statistically significant benefit in improving pregnancy rates in endometriosis patients (5,6). However, numerous complications may occur following surgical treatment (7), and a previous systematic review estimated a high recurrence rate (21.5% at 2 years and 40-50% at 5 years) of endometriosis (8). Consequently, the search for effective therapeutic treatment remains of primary concern (9).

A previous study suggested that traditional Chinese medicine may be used to treat reproductive system diseases, including menstruation and non-infectious disorders, which may potentially result from endometriosis (10). Curcumin ($C_{21}H_{20}O_6$; molecular weight 368.4; Fig. 1), is a hydrophobic low molecular weight flavonoid, extracted from dried turmeric rhizomes (11). Various studies have revealed the health benefits of curcumin (12), and the authors previously demonstrated that curcumin inhibits endometriosis (13,14). The present *in vitro* study aimed to investigate the association between endometriotic stromal cells and curcumin and elucidate the underlying mechanism of action.

Materials and methods

Patients. For investigation of endometriotic (ectopic) stromal cells, 14 patients (n=14; aged 24-45 years, mean 31.13±4.90)

with endometriosis were enrolled in the present study. The direct visualization of the lesions by invasive procedure, using a laparoscopy is currently the primary method of diagnosis (15), and the final diagnosis was verified by histopathology.

For endometrial (eutopic) stromal cells, 9 premenopausal patients (n=9; aged 29-43 years, mean 35.06±5.74) who had undergone hysteroscopy for infertility were enrolled; endometrial tissue verified histologically however there was no evidence of endometriosis.

The ages of the patients were statistically similar between the endometriotic (ectopic) group and endometrial (eutopic) group. None of the patients had received any hormonal treatments prior to the laparoscopy or endoscopic operations. In order to maintain a high degree of cell proliferation capacity, all the endometrial tissue was obtained during the early secretory phase (16). The present study was approved by the ethics committee of Hubei University of Medicine (Shiyan, China) and written informed consent was obtained from all enrolled patients.

Materials. Curcumin (molecular weight 368.4, purity 99%) was purchased from Fluka Chemie GmbH (Buchs, Switzerland). Following dissolving in Dulbecco's modified Eagle's medium (DMEM), curcumin was sterilized through 0.22 μm membrane filtration. Prior to use, it was diluted to the desired concentrations as follows: 0, 20 and 50 μmol/l with DMEM supplemented with 20% fetal calf serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Culture system. Following extraction of endometrial tissue, the human endometrium was cut into slices (20-50 mg wet weight each), and dissociated in phosphate buffered saline (PBS, pH 7.4) at room temperature. It was then digested with trypsin, cultured and the medium was replaced every 48 h. Cell passage was carried out when cells reached 80% confluence on the Petri-dish. Isolated cells were cultured in DMEM supplemented with 1% penicillin and streptomycin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), and 10% charcoal-stripped heat-inactivated FBS (Thermo Fisher Scientific, Inc.). Cells were plated in 24-well plates at a density of $3x10^5$, and were incubated in an atmosphere containing 5% CO_2 and 95% air at 37˚C.

Identification of endometrial stromal cells by immunocytochemistry. The human endometrial stromal cell cultures were identified by immunostaining. Cells were fixed in 4% paraformaldehyde in PBS for 15 min at room temperature, rinsed twice in PBS for 5 min at room temperature, and incubated with 0.5% (v/v) Triton X-100 for 15 min. Non-specific reaction was blocked for 24 h using 10% normal goat serum (Invitrogen; Thermo Fisher Scientific, Inc.) at 4˚C. Following this, cells were incubated overnight at 4˚C with an anti-human vimentin antibody (cat no. ab185030; dilution, 1:500; Abcam, Cambridge, UK) and counterstained with hematoxylin at room temperature for 30 min. Stained cells were examined using an IX70 fluorescence microscope (Olympus Corporation, Tokyo, Japan).

Hematoxylin and Eosin staining. Cells were grown in 4-well Lab Tek II chamber slides until they reached 80-90% confluence. Various concentrations (0, 20 and 50 μmol/l) of curcumin were added to the cells and cultures were incubated at 37˚C for 48 h. The slides were stained at room temperature for 30 min as follows: 100% ethanol twice, 95% ethanol twice, 80% ethanol, dH_2O, Hematoxylin (Statlab, Baltimore, MD, USA), dH_2O, 0.25% acid alcohol, dH_2O, 0.3% ammonium water, dH_2O, 70% ethanol, Eosin Y (Statlab), 95% ethanol twice, 100% ethanol twice, and Xylene three times (AAPER Alcohol and Chemical Co., Shelbyville, KY, USA). Following this, they were mounted in Acrymount media (Statlab) and cover slipped using micro-cover glass. Stained cells were visualized under an IX70 inverted microscope (Olympus Corporation).

Cell proliferation assay. Cell proliferation and curcumin effect on human endometrial stromal cells were assessed by the MTT assay. Briefly, cells at a density of $5x10^4$ /ml were incubated overnight in a 10% FBS medium. Following cell adhering to 96-well plates, various concentrations (0, 20 and 50 μmol/l) of curcumin were added to the cultures for 48 h. Then, the cells were incubated with the MTT reagent (0.5 mg/ml) for 4 h and the formazan crystals were dissolved with dimethyl sulfoxide. The relative number of viable cells was measured using spectrophotometry at a wavelength of 570 nm against a reference wavelength of 630 nm.

Flow cytometry analysis. Phase distribution was detected by flow cytometry for the cell cycle analysis. Briefly, the cells were incubated with various concentrations (0, 20 and 50 μmol/l) of curcumin for 48 h and then fixed with ice-cold 70% ethanol overnight at 4˚C. Prior to analysis, cells at 80-90% confluence were washed twice with PBS and then incubated with 50 μg/ml propidium iodide (PI) in a solution containing 100 μg/ml RNase A and 0.2% Triton X-100, for ~30 min in the dark at room temperature. DNA content was measured by flow cytometry (Beckman Coulter, Inc., Brea, CA, USA), using an excitation wavelength of 488 nm, and measuring fluorescence at 610 nm. Results were analyzed using the FCS Express software version 2.0 (De Novo Software, Glendale, CA, USA).

Vascular endothelial growth factor (VEGF) expression detection via immunostaining assay. VEGF protein expression levels in curcumin treated endometrial stromal cells were examined using immunocytochemistry staining. Second-passage endometrial stromal cells were seeded at a density of $5x10^5$/ml onto 6-well plates with α-minimal essential medium supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher, Scientific, Inc.). Cells were incubated at room temperature with various concentrations (0, 20 and 50 μmol/l) of curcumin for 72 h. Cells grew to 80% confluence and were washed three times with PBS prior to fixation in 4% paraformaldehyde at room temperature for 5 min. Immunocytochemistry was performed as follows: Firstly, cells were blocked using StartingBlock (PBS) blocking buffer (Thermo Fisher Scientific, Inc.) for 15 min at room temperature and then incubated with rabbit anti-human VEGF (cat no. sc-152; dilution, 1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) antibody overnight at 4˚C, then incubated with rhodamine-conjugated goat anti-rabbit (cat no. sc-2091; dilution, 1:1,000; Santa Cruz Biotechnology, Inc.) secondary antibodies for 30 min

at 37˚C. Finally, cells were counterstained with hematoxylin at room temperature for 5 min. Of the four wells, one was selected at random and images were obtained using an IX70 fluorescence microscope (Olympus Corporation). The images were processed using ImageJ software version 6.0 (National Institutes of Health, Bethesda, MD, USA) for analysis.

Quantification of apoptosis by flow cytometry. Apoptosis was assessed using Annexin V-fluorescein isothiocyanate (FITC) staining. Human endometriotic stromal cells and endometrial stromal cells were plated in 6-well plates and treated with 50 μmol/l curcumin for 48 h at 37˚C. Subsequently, adherent cells were harvested, washed twice with PBS at 4˚C, resuspended in binding buffer at a density of $5x10^5$ cells/500 μl, and stained with 5 μl Annexin V-FITC (Sigma-Aldrich; Merck KGaA) and 10 μl PI (Sigma-Aldrich; Merck KGaA) for 15 min at room temperature in the dark, prior to flow cytometric analysis. Analysis was performed using a CytoFLEX flow cytometer (Beckman Coulter, Inc., Brea, CA, USA) and data was analyzed using Kaluza analysis software version 1.3 (Beckman Coulter, Inc.).

Statistical analysis. Data are expressed as the mean ± standard deviation of 3 independent experiments. Statistical analysis was performed using SPSS software, version 12.0 (SPSS, Inc., Chicago, IL, USA) and data were analyzed using one-way analysis of variance followed by a post hoc Tukey test to compare differences among the groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Cell morphology. Following digestion of the endometrium with trypsin, stromal cells had attached within the first 24 h. The passage time was as follows: Primary cultures (P0 passage) 3 to 4 days; P1-P10 passage 3 days. The cell morphology between ectopic and eutopic stromal cells was similar. Stromal cells exhibited a fibroblast morphology with a spindle-shape, abundant cytoplasm and oval-shaped nucleus. Ectopic and eutopic stromal cells were easy to passage, following long-term culture, and these cells were arranged in parallel bundles, with dense piles of regional aggregation (Fig. 2).

Identification of stromal cells. The purity of human endometrial stromal cell cultures was proven by detection of the specific biomarker vimentin, and positive vimentin images are presented in Fig. 3.

Hematoxylin and Eosin staining. Following incubation with 20 μmol/l curcumin for 48 h, cells began to exhibit an altered morphology, the permeability reduced and suspension cells increased; with 50 μmol/l curcumin, adherent cells decreased, suspension cells increased significantly and cell debris was noted. These effects were better noted in ectopic cells, however ectopic and eutopic endometrial stromal cell adherence ability decreased following curcumin treatment, and they were more easily digested, as treatment with curcumin appeared to decrease the adherence capabilities of stromal cells (Fig. 4).

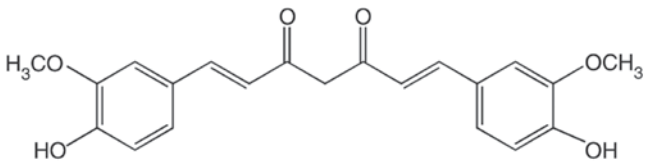

Figure 1. Chemical structural formula of curcumin. $C_{21}H_{20}O_6$, molecular weight 368.4.

Curcumin treatment decreases cell proliferation. To investigate the curcumin potential to induce cell death in human endometriotic and endometrial stromal cells, the present study first examined the effect of curcumin on cell survival using the MTT assay (Fig. 5). The results indicated that treatment of curcumin decreased ectopic and eutopic stromal cell growth.

Curcumin suppresses division in the cell cycle. The present study then investigated the effect of curcumin on human endometriotic and endometrial stromal cell division by analyzing the cell cycle. Following treatment with 20 and 50 μmol/l curcumin, endometriotic and endometrial stromal cells demonstrated increased percentages of G_1-phase cells and decreased percentages of S-phase cells, particularly in the group with concentrations of 50 μmol/l curcumin. The cell cycle phases with various concentrations of curcumin are presented in Fig. 6.

Curcumin effects on ectopic and eutopic stromal cells are mediated through VEGF. Then, the present study investigated if the curcumin effects on endometriotic and endometrial stromal cells were mediated through VEGF. Yellow and brown cells were identified as positive cells for VEGF. Treatment with 20 and 50 μmol/l curcumin decreased the proportion of VEGF positive expression compared with the 0 μmol/l group and accordingly, the fluorescence intensities of VEGF staining were decreased, as presented in Fig. 7.

Quantification of apoptosis by flow cytometry. Finally, the effects of 50 μmol/l curcumin on apoptosis were assessed by using Annexin V-FITC staining. Following treatment with 50 μmol/l curcumin, 4.7% early apoptosis (bottom right) and 28.4% late apoptosis (top right) was observed in endometriotic stromal cells, and 2.8% early apoptosis (bottom right) and 21.4% late apoptosis (top right) was observed in endometrial stromal cells (Fig. 8).

Discussion

Endometriosis is a frequently occurring disease in women during childbearing age (17), and is the primary reason for infertility, significantly impacting patient quality of life (18). The pathogenesis of endometriosis involves several mechanisms, including migration, adhesion and invasion, high oestradiol biosynthesis, progesterone resistance, inflammation and neuroangiogenesis (19). A previous study demonstrated that angiogenesis is important in the pathogenesis of endometriosis, as it establishes a new blood supply that is critical for the development of endometriotic lesions (20). VEGF is a signaling protein produced by cells, which functions to

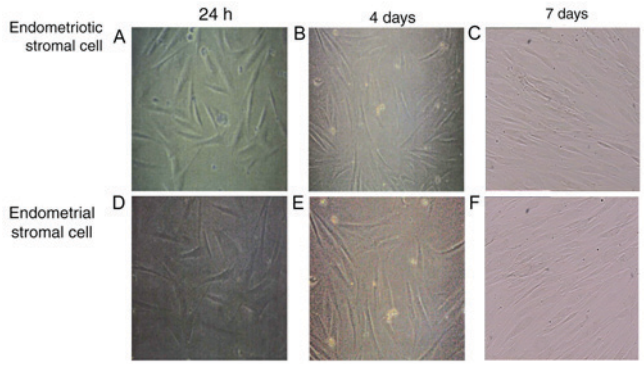

Figure 2. Morphological features of human stromal cells. Endometriotic stromal cells at (A) 24 h, (B) 4 days and (C) 7 days. Human endometrial stromal cells at (D) 24 h, (E) 4 days and (F) 7 days. Magnification, x200.

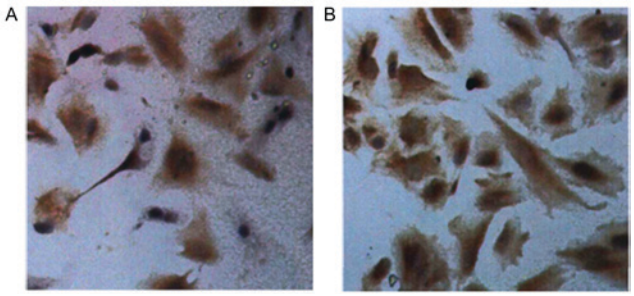

Figure 3. Identification of human stromal cells. Cells were positively immunostained by anti-human vimentin antibody to detect purity. Magnification, x200. (A) Endometrial eutopic stromal cells. (B) Endometriotic ectopic stromal cells.

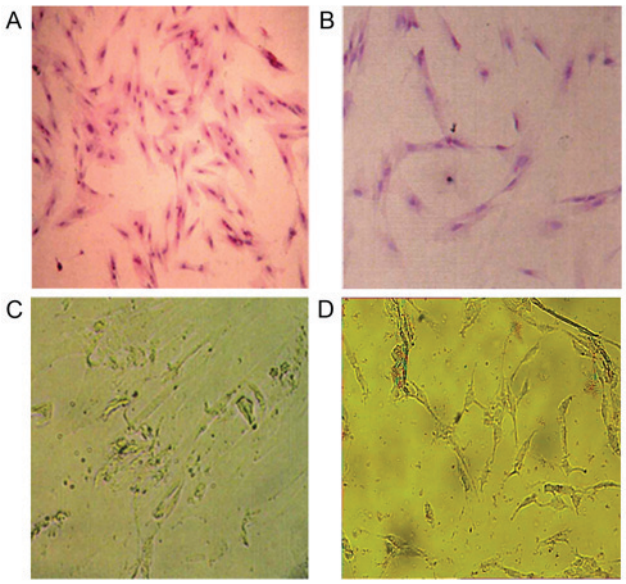

Figure 4. Effects of curcumin treatment of stromal cells. (A) Stromal cells exhibit fibroblast morphology, a spindle-shape, are abundant in cytoplasm and have oval-shaped nuclei. (B) Higher magnification of the image in panel A. (C) Following 20 μmol/l curcumin treatment for 48 h, cells began to alter in morphology, their permeability reduced and suspension cells increased. (D) With 50 μmol/l curcumin, adherent cells decreased, suspension cells increased significantly and cell debris was noted. Images representative of hematoxylin and eosin staining. (A, magnification x100; B-D, magnification, x200).

stimulate angiogenesis, vasculogenesis and vascular permeability (21). A previous study demonstrated that VEGF is involved in endometrial activity; McLaren *et al* (22) demonstrated that the VEGF levels are increased in the peritoneal fluid of patients with endometriosis.

Curcumin, which is a traditional Chinese herb derived from turmeric, has been used for its safety and efficacy, and exhibits anti-oxidant, anti-inflammatory, analgesic and antiseptic properties (11,23). The therapeutic properties of curcumin have been evaluated in clinical trials, and have been used as a supplement in several countries for the treatment of various diseases (24). Angiogenesis is important in the development of endometriosis, and is regulated by a variety of pro-angiogenic genes and signaling molecules, which include VEGF. Curcumin exhibits an anti-angiogenic effect *in vivo* in xenograft models (25,26), and the authors previously demonstrated that curcumin has the ability to inhibit endometriosis via decreasing VEGF protein expression, in a heterotopic endometrium rat model (13).

Cellular proliferation is a highly-regulated event in the endometrial cell (27). Following treatment with 20 and 50 μmol/l curcumin, endometriotic and endometrial stromal cells demonstrated increased percentages of G_1-phase cells and decreased percentages of S-phase cells, particularly in the 50 μmol/l curcumin treatment group. The modified percentages of G1 and S-phase cells suggested that curcumin suppressed DNA synthesis and cell proliferation *in vitro*.

In the present study, apoptosis was assessed by using Annexin V, which is a protein that binds to the cell surface of apoptotic cells (28). Apoptosis is the process of programmed cell death that occurs in multicellular organisms (29,30). It has previously been demonstrated that curcumin may be used in the treatment of cancer due to its ability to induce apoptosis and inhibit angiogenesis (31,32). Phase III and phase II clinical trials indicate that curcumin is safe and may exhibit therapeutic efficacy in patients with cancer (33). However, the role of curcumin in human endometriotic and endometrial stromal cells remain unclear, therefore the present study aimed to determine the apoptotic potential of curcumin in human endometriosis. Treatment with 50 μmol/l curcumin resulted in 4.7% early apoptosis and 28.4% late apoptosis in endometriotic stromal cells, and 2.8% early apoptosis and 21.4% late apoptosis in endometrial stromal cells, respectively. The data provided evidence that curcumin reduced cell survival in human endometriotic and endometrial stromal cells *in vitro*.

In conclusion, the results of the present *in vitro* study demonstrated that treatment with curcumin altered cell morphology in human endometriotic and endometrial stromal cells, decreased cell proliferation, and demoted cell division. In further analyses, it was demonstrated that curcumin induced the apoptosis of human endometriotic and endometrial stromal cell via downregulation of VEGF expression. The present observations suggested that curcumin effects on human ectopic and eutopic stromal cells may be mediated through the VEGF signaling pathway. Further studies are required to fully elucidate the molecular mechanisms underlying the effects of curcumin, and to investigate its potential for the treatment of endometriosis in humans.

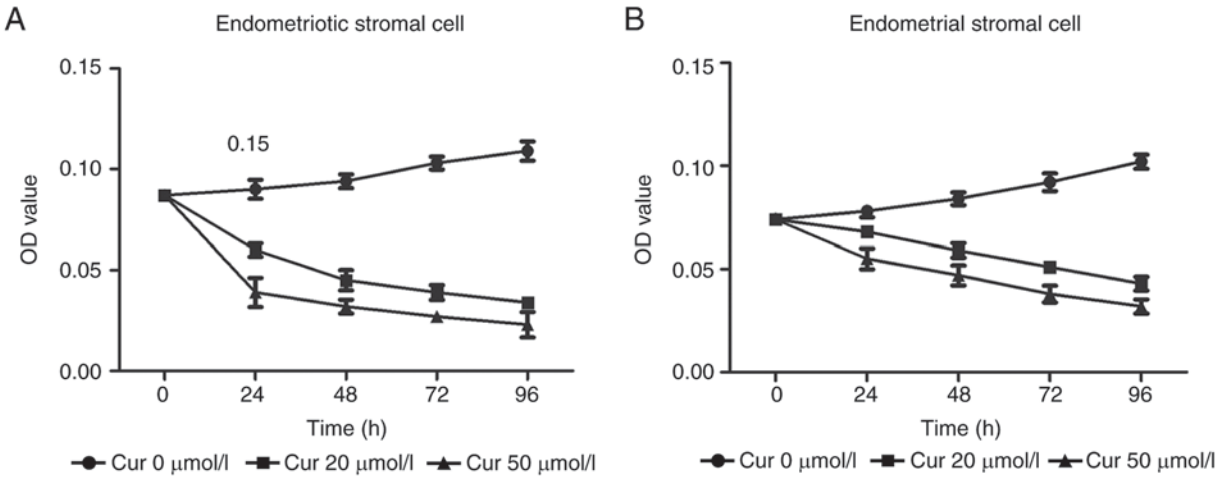

Figure 5. Effect of curcumin treatment of cell proliferation. Cells were exposed to 0, 20 and 50 μmol/l curcumin. (A) Influence of curcumin on human endometriotic stromal cell growth curves. (B) Influence of curcumin on human endometrial stromal cell growth curves. The OD values of the 20 and 50 μmol/l groups were different compared with the 0 μmol/l group. Following treatment with curcumin, the optical density curves were decreased, suggesting that curcumin may influence proliferation. OD, optical density; Cur, curcumin.

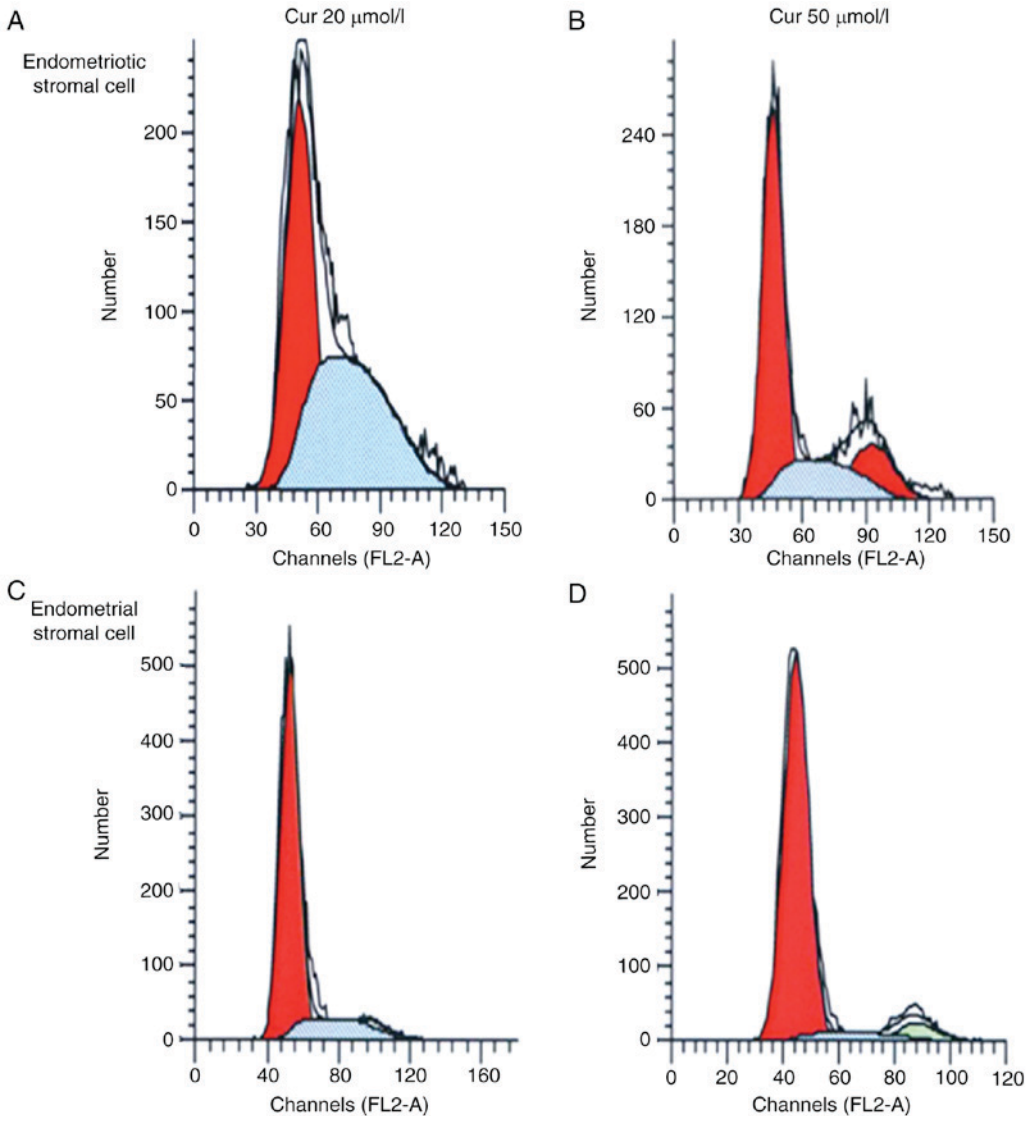

Figure 6. Flow cytometry analysis of human endometriotic stromal cells. Human endometriotic stromal cells were treated with (A) 20 μmol/l and (B) 50μmol/l curcumin for 48 h. Endometrial stromal cells were treated with (C) 20 μmol/l and (D) 50 μmol/l curcumin for 48 h. Following treatment, cells demonstrated increased percentages of G_1-phase cells and decreased percentages of S-phase cells, particularly in the group with concentrations of 50 μmol/l curcumin. Cur, curcumin.

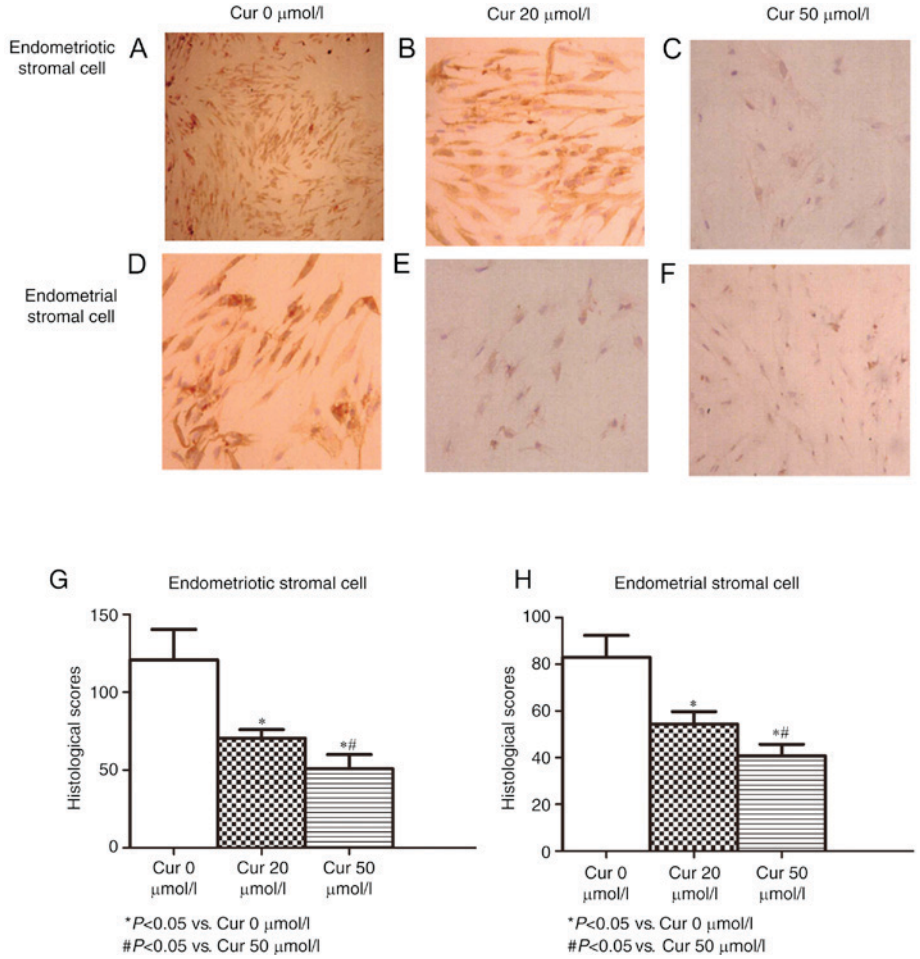

Figure 7. Expression of VEGF in human endometriotic stromal cells and human endometrial stromal cells assessed by immunohistochemistry staining. Endometriotic cells were treated with curcumin at (A) 0 μmol/l, (B) 20 μmol/l and (C) 50 μmol/l for 48 h prior to being fixed for immunostaining. Endometrial cells were treated with curcumin at (D) 0 μmol/l, (E) 20 μmol/l and (F) 50 μmol/l for 48 h. Treatment with 20 and 50 μmol/l curcumin decreased VEGF positive expression compared with the 0 μmol/l treatment and accordingly, the fluorescence intensities of VEGF staining in (G) endometriotic and (H) endometrial, were decreased. *P<0.05 vs. Cur 0 μmol/l and $^{\#}$P<0.05 vs. Cur 50 μmol/l. Magnification, x100. VEGF, vascular endothelial growth factor; Cur, curcumin.

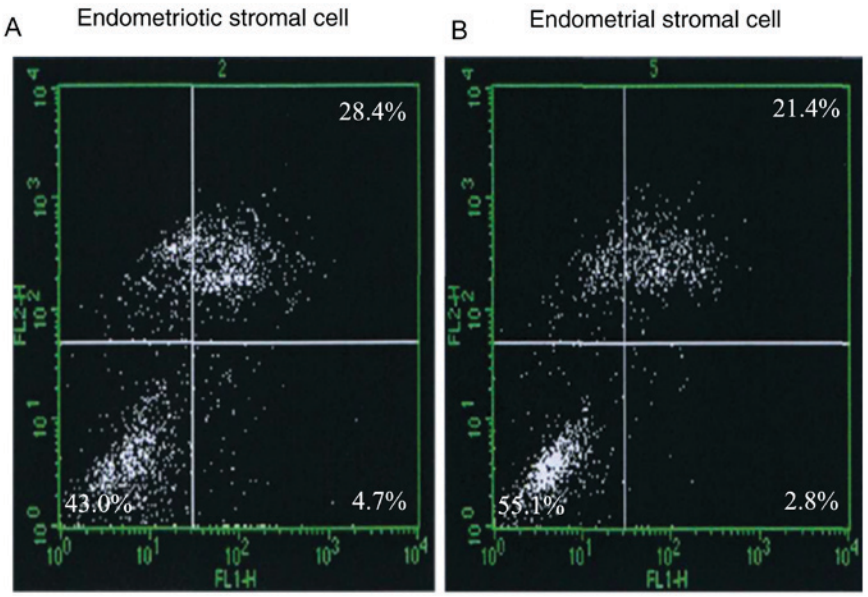

Figure 8. Annexin-V FITC/Propidium iodide dual staining was performed to study the distribution of early and late apoptotic cells following treatment of drugs. Following treatment with 50 μmol/l curcumin, (A) 4.7% early apoptosis (bottom right) and 28.4% late apoptosis (top right) was observed in endometriotic stromal cells and (B) 2.8% early apoptosis (bottom right) and 21.4% late apoptosis (top right) was revealed in endometrial stromal cells.

Acknowledgements

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