

Original Article

# Involvement of Bcl-2, Src, and ER $\alpha$ in gossypol-mediated growth inhibition and apoptosis in human uterine leiomyoma and myometrial cells

Yan ZHU<sup>1,\*</sup>, Shu-wu XIE<sup>1</sup>, Jian-feng ZHANG<sup>2</sup>, Ting-ting ZHANG<sup>3</sup>, Jie-yun ZHOU<sup>1</sup>, Yang CAO<sup>3</sup>, Lin CAO<sup>1</sup>

<sup>1</sup>Department of Reproductive Pharmacology, NPFPC Key Laboratory of Contraceptives and Devices, Shanghai Institute of Planned Parenthood Research, Shanghai 200032, China; <sup>2</sup>Shanghai Obstetrics and Gynecology Hospital, Fudan University, Shanghai 200011, China; <sup>3</sup>Department of Obstetrics and Gynecology, Shanghai Yueyang Hospital, Shanghai 200437, China

**Aim:** To investigate the effect of gossypol on the growth of cultured human uterine leiomyoma and myometrial cells, the level of Bcl-2 and the activity of Src and estrogen receptor (ER $\alpha$ ).

**Methods:** Human uterine leiomyoma and adjacent normal myometrial cells were cultured *in vitro*. Both cell types were treated with a graded concentration of gossypol. Cell viability was assayed using CCK-8. Morphological change was observed with optical and electronic microscopy. Apoptosis was evaluated using TUNEL assay. Levels of Bcl-2, ER $\alpha$  and Src were analyzed using Western blotting.

**Results:** Gossypol significantly inhibited growth and promoted apoptosis in cultured human uterine leiomyoma cells with the IC<sub>50</sub> value and its corresponding 95% confidence intervals (CI) of 6.5 (4.0–10.5), 9.0 (4.9–16.5), and 7.5 (4.0–14.1)  $\mu$ mol/L at 20, 40, and 60 h, respectively. Gossypol exerted inhibitory effects on the myometrial cells with the IC<sub>50</sub> value and its 95% CI of 49.1 (28.3–85.0), 14.5 (7.7–27.4), and 2.6 (1.2–5.6)  $\mu$ mol/L at 20, 40, and 60 h, respectively. Compared with control, gossypol 0.1–3.0  $\mu$ mol/L markedly decreased the protein expression of Bcl-2 ( $P < 0.05$ ) in both leiomyoma and myometrial cells in a concentration-dependent manner, and significantly suppressed the level of phospho-Tyr416Src ( $P < 0.05$ ) in both cell types at 3.0  $\mu$ mol/L without obvious alteration of c-Src and phospho-Tyr527Src levels ( $P > 0.05$ ). In addition, gossypol markedly reduced both the expression of ER $\alpha$  ( $P < 0.05$ ) at the low concentration of 0.1  $\mu$ mol/L in the myometrial cells and the level of phospho-ser167ER $\alpha$  ( $P < 0.05$ ) at the high concentration of 3.0  $\mu$ mol/L in the leiomyoma cells.

**Conclusion:** Gossypol inhibits proliferation and induces apoptosis in human uterine leiomyoma and myometrial cells. It is likely that the mechanisms of action involve reducing the protein level of Bcl-2 and the activity of Src and ER $\alpha$ .

**Keywords:** gossypol; uterine leiomyoma; myometrium; apoptosis; Bcl-2; c-Src; estrogen receptor  $\alpha$

Acta Pharmacologica Sinica (2010) 31: 1593–1603; doi: 10.1038/aps.2010.153; published online 22 Nov 2010

## Introduction

Uterine leiomyoma (fibroid or myoma) is a common benign tumor in premenopausal women. It occurs in more than 70% of American women of reproductive age<sup>[1]</sup>, and there is also a high occurrence in China. It is generally accepted that increased serum levels of estradiol (E2) and progesterone (P) and high expression of estrogen receptors (ERs) and progesterone receptors (PRs) act as predominant promoters in the development of the leiomyoma. A recent study shows that cytokines and growth factors are over-expressed in uterine leiomyoma compared with normal myometrium<sup>[2]</sup>. These

findings suggest that there are intricate pathways involved in the formation of the leiomyoma.

It is likely that an anti-apoptotic mechanism is involved in the development of uterine leiomyoma. Bcl-2 protein was reported to be over-expressed in leiomyomas when compared with homologous myometrium. Expression of Bcl-2 protein showed a strong correlation to ERs in leiomyoma<sup>[3,4]</sup>. Estrogen inhibits apoptosis in several cell types by inducing Bcl-2 expression<sup>[5,6]</sup>, and an anti-estrogen reagent induced apoptosis by down-regulating the expression of Bcl-2 protein<sup>[7]</sup>.

Moreover, c-Src, a member of the Src family of non-receptor protein tyrosine kinases, may also play a role in the formation of uterine leiomyoma. Src family kinases are involved in a number of signal transduction pathways<sup>[8]</sup>. Activation of Src family kinases protects cells from apoptosis<sup>[9]</sup> whereas

\* To whom correspondence should be addressed.

E-mail zhuyan@sippr.org or kmzhuyan@gmail.com

Received 2010-04-11 Accepted 2010-08-03

inhibition of Src tyrosine kinase reduces mitosis and induces apoptosis. Recent evidence indicates that there is an important feed-forward signaling loop involving E2, ER $\alpha$ , and Src<sup>[10]</sup>. Classical sex steroid receptors (ERs, PRs) can activate Src, and Src can drive expression of certain ER $\alpha$  target genes. Overexpression and aberrant activation of c-Src have been found in a variety of epithelial and non-epithelial cancers<sup>[11, 12]</sup>. We demonstrated that c-Src was also over-expressed in a guinea pig model of uterine leiomyoma induced by estradiol<sup>[13]</sup>. This evidence suggests that Src may be involved in the development of leiomyoma.

Gossypol, a product of the cotton plant, was initially investigated as an oral contraceptive for males<sup>[14, 15]</sup>. However, the anti-fertility research was abandoned because of unacceptably high rates of permanent infertility. There are, however, many studies on gossypol are still in progress. Gossypol has demonstrated potent anti-proliferative properties *in vitro* and *in vivo*<sup>[16–20]</sup>, and it is considered a promising anti-cancer candidate. In Chinese suburban hospitals, there has been interest in applying gossypol in the treatment of gynecological disease, such as endometriosis and uterine leiomyomas, because it is cheap and easily available. A preliminary clinical study showed that the volume of uterine leiomyoma was significantly reduced after patients were administered gossypol (10–20 mg/d) for 3 months<sup>[21, 22]</sup>. Unfortunately, there is a lack of sufficient experimental data to support this, and it is important to confirm gossypol's effectiveness in suppressing the growth of uterine leiomyoma before it is widely used. Therefore, in this study, we investigated the effect of gossypol on the growth of human uterine leiomyoma *in vitro* and the levels of Bcl-2, estrogen receptor alpha (ER $\alpha$ ), Src and its active form to explore the possible mechanism of action. In addition, we also investigated the effect of gossypol on the adjacent normal myometrial cells.

## Materials and methods

### Chemicals and reagents

Gossypol, C<sub>30</sub>H<sub>30</sub>O<sub>8</sub> (molecular weight 518.55, purity >98%) was a kindly gift of Prof ZHANG (Dongwu University, Jiangsu Province, China). The structure of gossypol is shown in Figure 1. PP2 (4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo-3,4-d] pyrimidine) was purchased from Calbiochem (EMD Biosciences, Inc, Merck KGaA, Darmstadt, Germany). Dextran-coated-charcoal treated fetal calf serum (D-FCS) was obtained from Biological Industries Ltd (Kibbutz Beit Haemek, Israel).

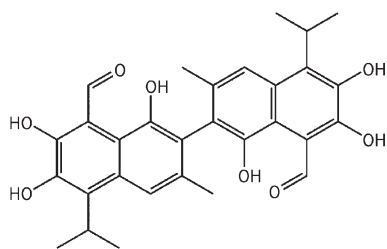


Figure 1. Structure of gossypol.

DMEM-F12 (1:1) and HEPES were purchased from Gibco, Invitrogen Co (Carlsbad, CA, USA). Dulbecco's modified Eagle's medium (DMEM): Nutrient Mixture F-12 (DMEM/F-12) Media and Hank's Balanced Salt Solution (HBSS), DMSO and type II collagenase were obtained from Sigma Chemical Co (St Louis, MO, USA). CCK-8 kit was obtained from Dojindo Molecular Technologies (Kumamoto, Japan). FragEL DNA fragmentation detection kit was obtained from Calbiochem (EMD Biosciences, Inc, Merck KGaA, Darmstadt, Germany). Mouse anti-ER $\alpha$  monoclonal antibody (65 kDa), c-Src rabbit polyclonal antibody (60 kDa), anti-phospho-Tyr416Src mouse monoclonal antibody (60 kDa), anti-phospho-Tyr527Src mouse monoclonal antibody (60 kDa) and Bcl-2 rabbit polyclonal antibody (26 kDa) were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-phospho-ER $\alpha$  (Ser167) antibody (66 kDa) was obtained from Upstate Cell Signaling Solution (Lake Placid, NY, USA). The enhanced chemiluminescence (ECL) detection kit and bicinchoninic acid (BCA) kit were purchased from Pierce (Rockford, IL, USA). Dharmacon siRNA transfection reagents, siCONTROL Non-Targeting siRNA Pools and siGENOME ON-Targeting SMART pool were purchased from Thermo Fisher Scientific Inc (MA, USA).

### Tissue collection

The leiomyoma and adjacent normal myometrial tissue samples were obtained from premenopausal women (38–45 years old) with regular menstrual cycles who were undergoing hysterectomies. None of the patients had received any hormonal therapy before surgery. Patients underwent surgery in 2008–2009 at Shanghai Gynecological and Obstetrics Hospital. Informed consent for the use of the tissue was obtained from each patient before surgery. The study was approved by the Ethical Committee for Clinical Research of the Hospital and Shanghai Institute of Planned Parenthood Research. The histological diagnosis of each uterine specimen was examined. Samples were collected from the scheduled surgeries at the patient's convenience during the proliferative phase of the menstrual cycle.

### Cell culture and treatment

The culture of leiomyoma and adjacent normal myometrial cells was performed as follows: each leiomyoma and myometrial tissue sample was rinsed in Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free HBSS, cut into small pieces, and then digested in pre-warmed HBSS containing 2.0% type II collagenase (*w/v*) and 25 mmol/L of HEPES at 37 °C for 4–5 h. The cells were collected by centrifugation at 460×g for 5 min and washed several times with HBSS. The isolated leiomyoma cells and myometrial cells were then transferred to a 75-cm<sup>2</sup> flask at a density of 1×10<sup>6</sup> cells/mL and cultured in phenol-red free DMEM-F12 (1:1) with 10% (*v/v*) D-FCS, 25 mmol/L HEPES (pH 7.4), 2 mmol/L glutamine, 1 mmol/mL sodium pyruvate, 10 mmol/mL non-essential amino acids and 1 mmol/mL insulin-transferrin-selenium-G supplement. The cells were cultured in a humidified atmosphere of 5% CO<sub>2</sub>/95% air. The culture medium was replaced every 3–4 d until the cells reached confluence (10–12

d). The cultured cells were then digested with 0.25% trypsin and subcultured under the same conditions as the primary cultures. The primary and subcultured smooth muscle cells were confirmed by immunohistochemistry staining of the muscle-specific protein desmin and  $\alpha$ -smooth muscle actin.

At approximately 70% confluence, the myometrial and leiomyoma cell cultures were treated with gossypol or DMSO alone as a control. Gossypol was dissolved in DMSO and diluted to a desired concentration. The final concentration of DMSO in culture media was 0.5% (*v/v*).

#### Cell viability assay

The viabilities of cultured leiomyoma and myometrial cells were determined by WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] assay using cell-counting kit-8 (CCK-8). The cells were cultured in 96-well plates and treated with DMSO or graded concentrations of gossypol (0.01, 0.05, 0.1, 0.5, 1.0, 5.0, 10, or 50  $\mu\text{mol/L}$ ) for 20, 40, and 60 h. Ten  $\mu\text{L}$  CCK-8 was added to each well, and cells were incubated at 37 °C for 2 h. Using a spectrophotometric plate reader (Bio-Tek ELX-800, USA), the absorbance (*OD*) at 450 nm was read to determine cell viability in each well. Results are presented as the percentage of cell growth inhibition rate (%) and the half maximal inhibitory concentration ( $\text{IC}_{50}$ ). Control cells were not treated with gossypol, and the cell growth inhibition rate (%) of cells treated with gossypol was calculated by the following equation: [(1-*OD* of gossypol-treated wells/*OD* of control wells)  $\times$  100%]. The  $\text{IC}_{50}$  was calculated from the dose response using the Bliss method. The experiments were performed in three different samples.

#### Transmission electron microscopy

Morphological changes in the leiomyoma and myometrial cells were observed by electron microscopy. Briefly, the cells treated with DMSO or graded concentrations (0.1, 1.0, and 3.0  $\mu\text{mol/L}$ ) of gossypol were collected by digesting with 0.25% trypsin and fixed in 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4) at 4 °C. The fixed cells were washed three times in Sabatini's solution (PBS with 6.8% sucrose) (pH 7.4). The pellets were cut into small (1 mm<sup>3</sup>) cubes, post-fixed with 1% osmium tetroxide for 1 h, washed three times in Sabatini's solution and dehydrated by passage through graded concentrations of ethylene alcohol. This process was followed by sequential treatments with propylene oxide, a 1:1 Epon-propylene oxide mix, and three washes in pure epon. Polymerization was performed at 60 °C overnight. Ultrathin sections were cut with a Leica Ultracut UCT Ultra Microtome with a Dupont diamond knife, stained with 1% uranyl acetate and lead citrate, and examined with a transmission electron microscope (JEM-1230, JEOL, Japan) at an accelerating voltage of 80 kV with a 40-mm objective aperture.

#### Terminal deoxynucleotidyl transferase-mediated digoxigenin-dUTP nick-end labeling assay (TUNEL)

Cells treated with DMSO or graded concentrations (0.1, 1.0,

and 3.0  $\mu\text{mol/L}$ ) of gossypol were collected and then stained by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) using the TdT-FragEL DNA fragmentation detection kit. Briefly, the adherent cells were cultured and fixed in a 96-well Nunc plate. Then, we performed the experiment according to the manufacturer's instructions. The cells were stained with DAB, and the numbers of stained positive cells were observed using a light microscope. A negative control was generated by substituting dH<sub>2</sub>O for the TdT in the reaction mixture during the labeling step. The present values were an average ratio of the number of positive cells against total cells in three different samples.

#### Western blotting

Western blotting was used to evaluate the levels of Bcl-2, ER $\alpha$  and Src and its activity. Cells treated with DMSO or graded concentrations (0.1, 1.0, and 3.0  $\mu\text{mol/L}$ ) of gossypol were collected. Western blotting was performed according to routine procedure. The protein concentration was determined using the bicinchoninic acid (BCA) method according to the manufacturer's instructions. Fifty- $\mu\text{g}$  samples were loaded onto 10% SDS-PAGE (30% acrylamide:bisacrylamide 29:1) gels for electrophoresis. The primary antibodies for Bcl-2, ER $\alpha$ , Src, phosphorylated-167ER, phosphorylated-416Src, and phosphorylated-527Src were diluted at 1:3000. The primary  $\beta$ -actin antibody was diluted at 1:10000. The relative levels of protein were semiquantitatively determined with Image-Pro Plus<sup>®</sup> Version 5.1.0 software (Media Cybernetics Inc). The relative levels of protein were obtained by taking the ratio of the band intensity of the target protein against that of  $\beta$ -actin. Statistical analysis was performed on the average protein level of three samples.

#### Src siRNA transfection

Specific siRNA oligonucleotides against Src and control scrambled siRNA oligonucleotides were transfected into the leiomyoma and myometrial cells cultured in media with 5% FBS using Dharmacon siRNA transfection reagents for 4 h. The cells were transferred to media containing D-FCS for 36 h and then collected for Western blot analysis to determine the levels of ER $\alpha$  and Src.

#### Statistical analysis

The data are presented as mean $\pm$ SD. The statistical significance of the inhibition rate at various times was determined by repeated measurement by ANOVA with SPSS 11.5 software (version 11.5 for Windows; SPSS, Chicago, Illinois, USA), and then a paired-sample *t* test was used between 2 groups. Other statistical significance among 3 or more groups was determined by one-way ANOVA. *P*<0.05 was considered statistically significant.

#### Results

##### Effects of gossypol on the growth of cultured uterine leiomyoma and myometrium cells

The effects of graded concentrations of gossypol on the num-

ber of viable cultured leiomyoma and myometrial cells were determined by CCK-8 assay and then presented as the percentage of viable cells inhibition rate and  $IC_{50}$ . In the myometrial cells, the  $IC_{50}$  of gossypol and its corresponding 95% confidence intervals was 6.5 (4.9–10.5), 9.0 (5.0–16.5), and 7.5 (4.0–14.1)  $\mu\text{mol/L}$ . There was no pronounced difference in the inhibition rates of viable cells observed among 20, 40, and 60 h ( $P>0.05$ ). In the leiomyoma cells, the  $IC_{50}$  of gossypol and its corresponding 95% confidence intervals was 49.1 (28.3–85.0), 14.5 (7.7–27.4), and 2.6 (1.2–5.6)  $\mu\text{mol/L}$  at 20, 40, and 60 h, respectively. There was a significant difference between the inhibition rates of viable cells detected between 20 and 60 h ( $P<0.05$ , Figure 2).

### Effects of gossypol on the morphological change of cultured uterine leiomyoma and myometrial cells

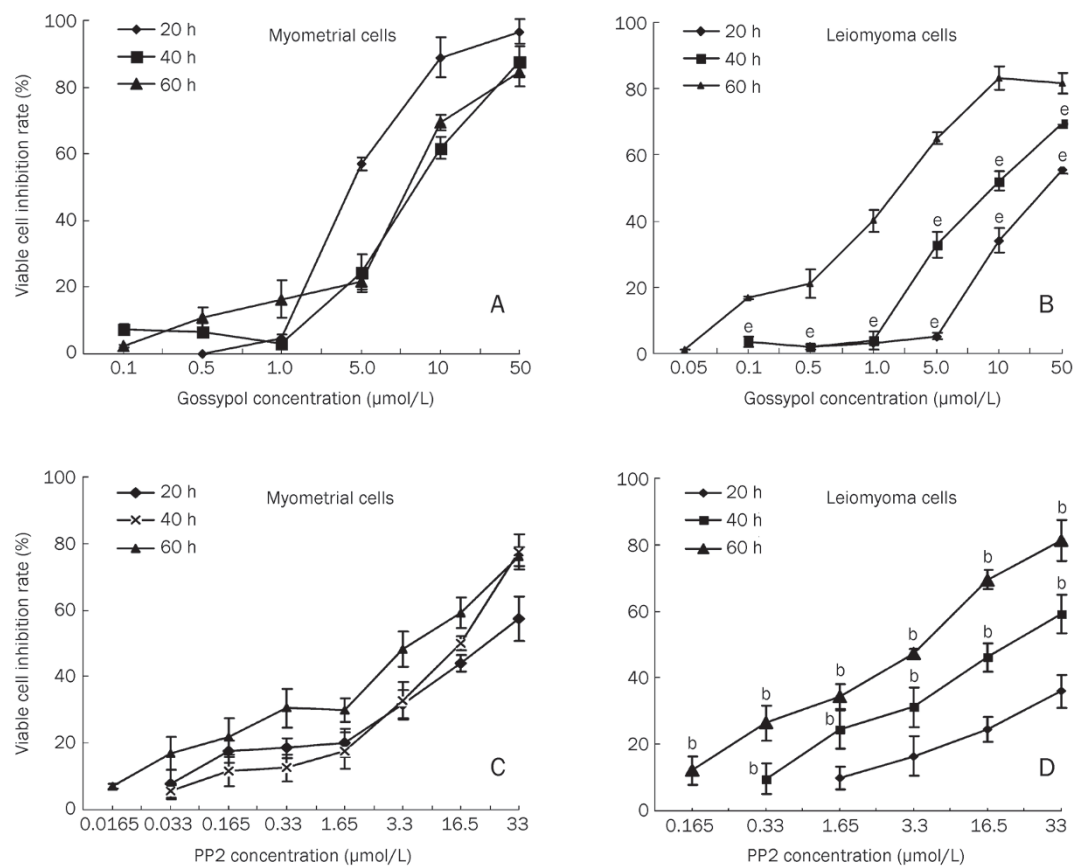
The morphological changes of both cell types were observed via optical and electronic microscope. Under the optical microscope, both cell types treated with DMSO appeared robust, with an elongated shape and abundant cytoplasm.

The leiomyoma cells were morphologically similar to the myometrial cells, but they lined up more densely and exhibited a whirlpool or fan-shape. In the presence of gossypol, both cell types appeared thin, and the cytoplasm atrophic and intercellular connection dwindled (Figure 3).

Under the electron microscope, a series of distinct morphological changes in both cell types was observed after treatment with a graded concentration of gossypol. Both cell types showed extensive cell death with features of apoptosis. The cells maintained membrane integrity but showed signs of cell shrinkage, nuclear deformation, increased cytoplasmic vacuolar degeneration, slight aggregation of chromatin, and damage of the cytoplasmic organelles (eg, mitochondrial swelling, the disappearance of mitochondrial cristae and enlargement of the endoplasmic reticulum) (Figure 4).

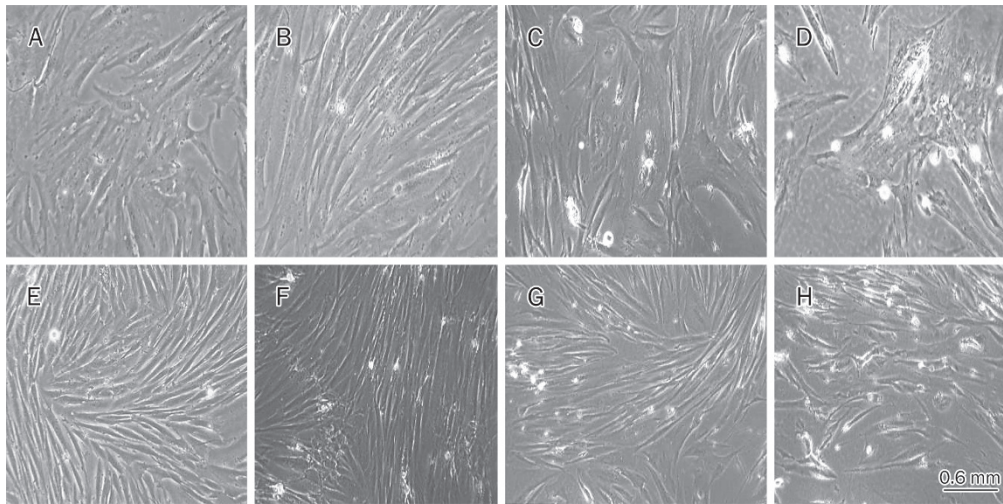
### Gossypol-induced apoptosis in the cultured uterine leiomyoma and myometrial cells

Apoptosis was assessed by TUNEL. Non-apoptotic cells were predominantly rounded and appeared counterstained, and

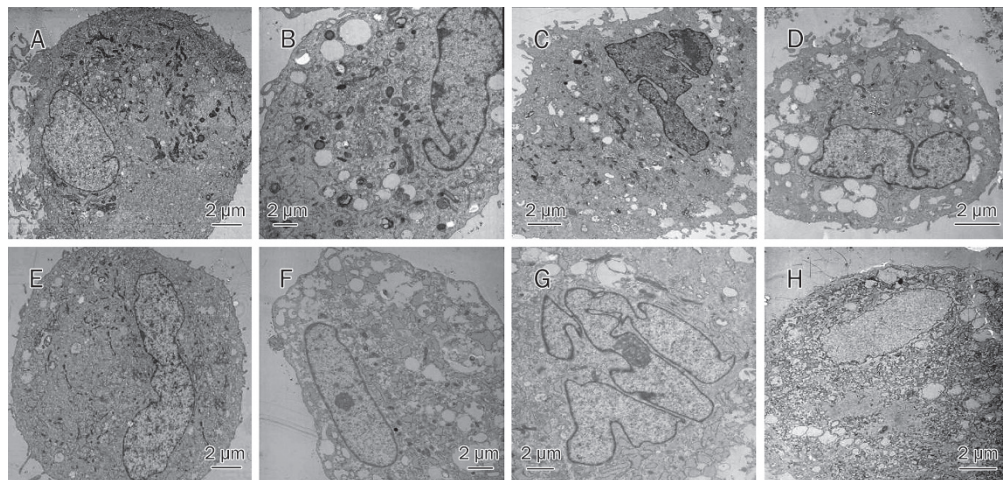


**Figure 2.** Inhibitory effect of graded concentrations of gossypol on the growth of cultured leiomyoma and myometrial cells at 20, 40, and 60 h, as assessed by CCK-8. PP2, a specific inhibitor of c-Src, was used as a positive control, suppressed the viability of both cells as well. The cells treated with DMSO as control, and the cell growth inhibition rate treated with gossypol was calculated by the following equation:  $[(1-OD \text{ of gossypol treated wells} / OD \text{ of control wells}) \times 100\%]$ . The experiments were performed in triplicate with three different cultured samples. (A) Myometrial cells treated with graded concentrations of gossypol (0.1 to 50  $\mu\text{mol/L}$ ); (B) Leiomyoma cells treated with graded concentration of gossypol (0.05 to 50  $\mu\text{mol/L}$ ); (C) Myometrial cells treated with graded concentration of PP2 (0.0165 to 33  $\mu\text{mol/L}$ ); (D) Leiomyoma cells treated with graded concentration of PP2 (0.165 to 33  $\mu\text{mol/L}$ ). Data are presented as means  $\pm$  SD of three independent samples. <sup>b</sup> $P<0.05$  vs treated for 20 h. <sup>e</sup> $P<0.05$  vs treated for 60 h.





**Figure 3.** Morphology of uterine leiomyoma and myometrial cells after treatment with graded concentrations of gossypol for 40 h, observed by optical microscopy. The myometrial cells were morphologically similar to that of the leiomyoma cells, except that the latter exhibited whirlpool or fan-shape and lined up more densely than the former. In the presence of gossypol, both of cells appeared thin, the cytoplasm atrophic and intercellular connection dwindled. (A) Myometrial cells treated with DMSO alone ( $\times 200$ ). (B) Myometrial cells treated with gossypol 0.1  $\mu\text{mol/L}$  ( $\times 200$ ). (C) Myometrial cells treated with gossypol 1.0  $\mu\text{mol/L}$  ( $\times 200$ ). (D) Myometrial cells treated with gossypol 3.0  $\mu\text{mol/L}$  ( $\times 200$ ). (E) Leiomyoma cells treated with DMSO alone ( $\times 200$ ). (F) Leiomyoma cells treated with gossypol 0.1  $\mu\text{mol/L}$  ( $\times 200$ ). (G) Leiomyoma cells treated with gossypol 1.0  $\mu\text{mol/L}$  ( $\times 200$ ). (H) Leiomyoma cells treated with gossypol 3.0  $\mu\text{mol/L}$  ( $\times 200$ ).



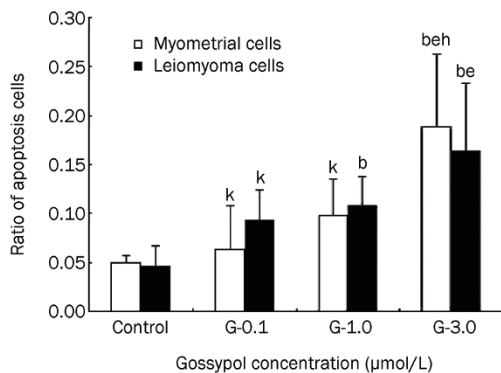
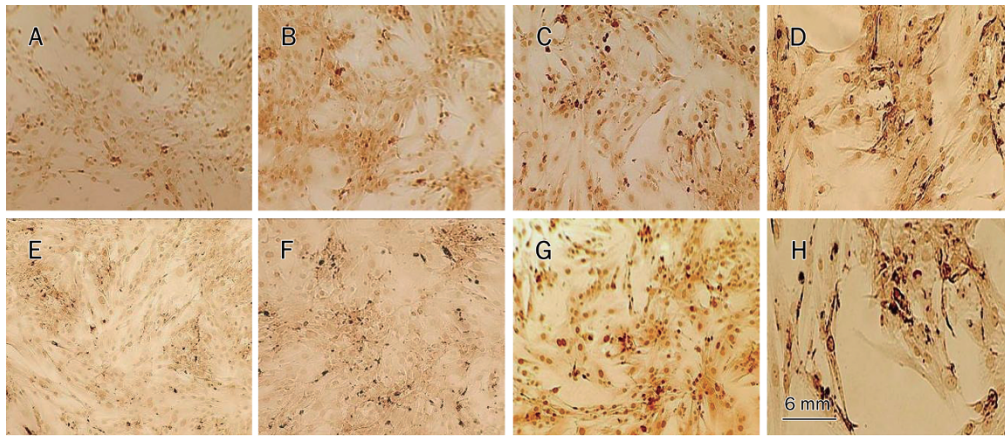
**Figure 4.** Effects of gossypol on the morphological changes of the leiomyoma and the myometrial cells, as assessed by electron transmission microscopy. There are varieties of distinct morphological changes in the cells after treatment with gossypol for 40 h. The changes of the cells include nuclear deformation, increasing of vacuolar degeneration in cytoplasm, chromatin margination and condensation, shrinkage of nuclear volume, membrane protuberance, and damage of the cytoplasmic organelles, eg. mitochondria swelling, mitochondria cristae disappearing and endoplasmic reticulum enlarging. (A) Myometrial cells treated with DMSO alone ( $\times 3000$ ). (B) Myometrial cells treated with gossypol 0.1  $\mu\text{mol/L}$  ( $\times 5000$ ). (C) Myometrial cells treated with gossypol 1.0  $\mu\text{mol/L}$  ( $\times 6000$ ). (D) Myometrial cells treated with gossypol at the concentration of 3.0  $\mu\text{mol/L}$  ( $\times 5000$ ). (E) Leiomyoma cells treated with DMSO alone ( $\times 2500$ ). (F) Leiomyoma cells treated with gossypol 0.1  $\mu\text{mol/L}$  ( $\times 5000$ ). (G) Leiomyoma cells treated with gossypol 1.0  $\mu\text{mol/L}$  ( $\times 3000$ ). (H) Leiomyoma cells treated with gossypol 3.0  $\mu\text{mol/L}$  ( $\times 4000$ ).

apoptotic cells appeared dark brown with a variety of distinctive morphological changes, including pyknosis, dark brown encircling, and dense-stained oval, crescent or ring-shaped nuclei or granules. In the presence of gossypol, there were more dark brown-stained cells observed in both leiomyoma and myometrial cells compared with the control cells. Compared with the control, there was a significant increase in the

ratio of apoptotic cells after treatment with 3.0  $\mu\text{mol/L}$  gossypol in both cell types ( $P < 0.05$ , Figure 5).

#### Effects of gossypol on the expression of Bcl-2 in uterine leiomyoma and myometrial cells

We further investigated the effect of gossypol on Bcl-2 expression to analyze the mechanism of apoptosis induction. The



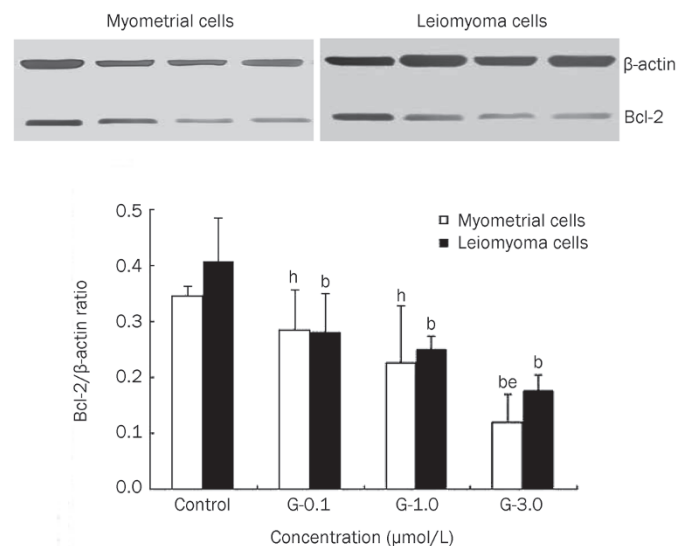
**Figure 5.** Effects of gossypol on apoptosis of the cultured uterine leiomyoma and myometrial cells. Both cells were cultured in the presence of DMSO or gossypol for 40 h. The apoptosis were detected using TUNEL method. Apoptotic cells with markedly circled, oval, crescent or ring-shape dense-stained nucleus or granule were easily distinguishable from non-apoptotic cells. (A) Myometrial cells treated with DMSO alone ( $\times 200$ ). (B) Myometrial cells treated with gossypol 0.1  $\mu\text{mol/L}$  ( $\times 200$ ). (C) Myometrial cells treated with gossypol 1.0  $\mu\text{mol/L}$  ( $\times 200$ ). (D) Myometrial cells treated with gossypol 3.0  $\mu\text{mol/L}$  ( $\times 200$ ). (E) Leiomyoma cells treated with DMSO alone ( $\times 200$ ). (F) Leiomyoma cells treated with gossypol 0.1  $\mu\text{mol/L}$  ( $\times 200$ ). (G) Leiomyoma cells treated with gossypol 1.0  $\mu\text{mol/L}$  ( $\times 200$ ). (H) Leiomyoma cells treated with gossypol 3.0  $\mu\text{mol/L}$  ( $\times 200$ ). Data are presented as means $\pm$ SD of three independent samples. <sup>b</sup> $P < 0.05$  vs control cells; <sup>e</sup> $P < 0.05$  vs gossypol 0.1  $\mu\text{mol/L}$  (G-0.1) treated cells; <sup>h</sup> $P < 0.05$  vs gossypol 1.0  $\mu\text{mol/L}$  (G-1.0) treated cells; <sup>h</sup> $P < 0.05$  vs gossypol 3.0  $\mu\text{mol/L}$  (G-3.0) treated cells.

results indicated a small increase in the expression of Bcl-2/ $\beta$ -actin in the leiomyoma in comparison to the myometrial cells, but no obvious difference was detected between the two types of cells ( $P > 0.05$ ). Gossypol inhibited the protein level of Bcl-2/ $\beta$ -actin in both cells in a concentration-dependent manner. Compared with the control, there was a pronounced decline in the expression of Bcl-2/ $\beta$ -actin in both cell types after treatment with 1.0 or 3.0  $\mu\text{mol/L}$  gossypol ( $P < 0.05$ , Figure 6).

#### Effects of gossypol on the level of ER $\alpha$ and phospho-ser167ER $\alpha$ in uterine leiomyoma and myometrial cells

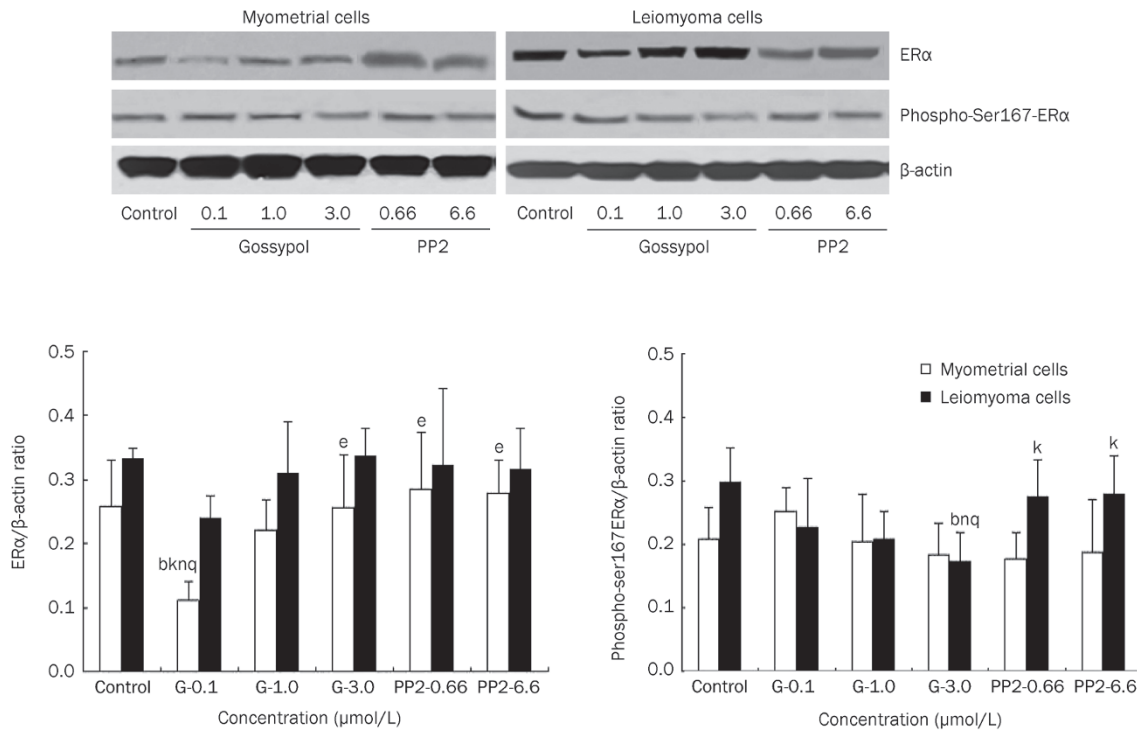
The effect of gossypol on the levels of ER $\alpha$  and phospho-ser167-ER $\alpha$  were analyzed by Western blotting. The results showed a small increase in the expression of ER $\alpha$ / $\beta$ -actin in the leiomyoma cells in comparison with the myometrial cells, but no pronounced difference was detected between the two cell types ( $P > 0.05$ ). Gossypol inhibited the expression of ER $\alpha$ / $\beta$ -actin in both leiomyoma and myometrial cells at of 0.1  $\mu\text{mol/L}$ , and there was a significant difference between the control and myometrial cells ( $P < 0.05$ , Figure 7).

One active form of ER $\alpha$  is phosphorylated at serine-167. The results indicated a small increase in the level of phospho-ser167ER $\alpha$ / $\beta$ -actin in the leiomyoma cells when compared with the myometrial cells, but no pronounced difference was



**Figure 6.** Effects of gossypol on the protein expression of Bcl-2 in the cultured leiomyoma and myometrial cells, as assessed by Western blotting. Both cells were cultured in DMSO (as control) or graded concentrations of gossypol (at 0.1, 1.0, and 3.0  $\mu\text{mol/L}$ ) for 40 h. Data was presented as means $\pm$ SD of three independent samples. <sup>b</sup> $P < 0.05$  vs control cells; <sup>e</sup> $P < 0.05$  vs gossypol 0.1  $\mu\text{mol/L}$  (G-0.1) treated cells; <sup>h</sup> $P < 0.05$  vs gossypol 3.0  $\mu\text{mol/L}$  (G-3.0) treated cells.





**Figure 7.** Effects of gossypol on the level of ER $\alpha$  and phospho-Ser167-ER $\alpha$  in cultured leiomyoma and myometrial cells, as assessed by Western blotting. Both cells were cultured in DMSO (as control) or graded concentrations of gossypol (at 0.1, 1.0, and 3.0  $\mu\text{mol/L}$ ) for 40 h. Data was presented as means $\pm$ SD of three independent samples. <sup>b</sup> $P < 0.05$  vs control cells; <sup>c</sup> $P < 0.05$  vs gossypol 0.1  $\mu\text{mol/L}$  (G-0.1) treated cells; <sup>d</sup> $P < 0.05$  vs gossypol 1.0  $\mu\text{mol/L}$  (G-1.0) treated cells; <sup>e</sup> $P < 0.05$  vs gossypol 3.0  $\mu\text{mol/L}$  (G-3.0) treated cells; <sup>f</sup> $P < 0.05$  vs PP2 0.66  $\mu\text{mol/L}$  (PP2-0.66) treated cells; <sup>g</sup> $P < 0.05$  vs PP2 6.6  $\mu\text{mol/L}$  (PP2-6.6) treated cells.

detected between the two cell types ( $P > 0.05$ ). In the presence of gossypol, there was a concentration-dependent decline in the level of phospho-ser167ER $\alpha$ / $\beta$ -actin in both cell types, and a pronounced difference was detected in the leiomyoma cells compared with the control cells ( $P < 0.05$ ) at a concentration of 3.0  $\mu\text{mol/L}$  (Figure 7).

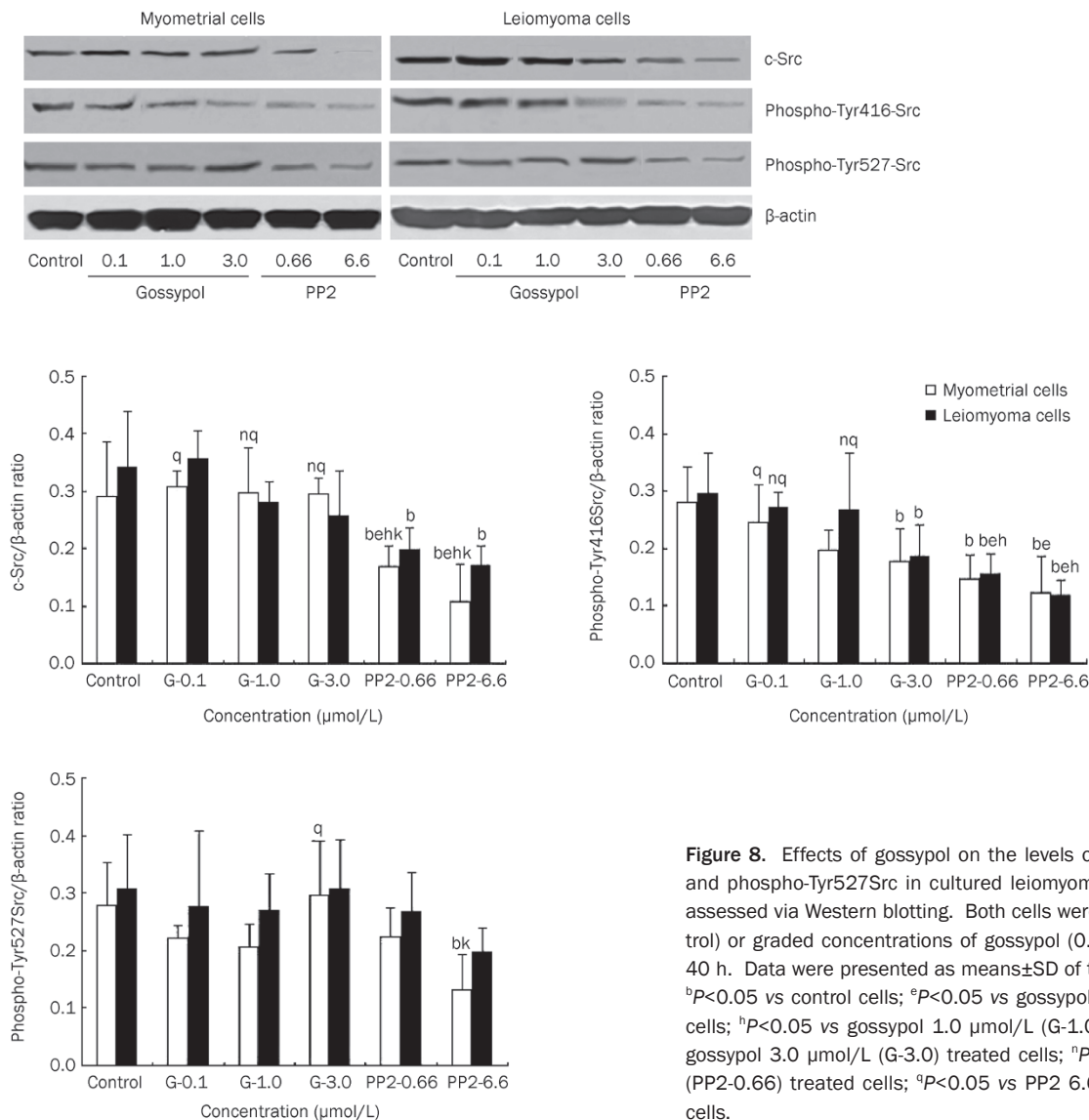
#### Effects of gossypol on the levels of c-Src, phospho-Tyr416Src, and phospho-Tyr527Src in leiomyoma and myometrial cells

The levels of Src, phospho-Tyr416Src and phospho-Tyr527Src in the leiomyoma and myometrial cells were analyzed by Western blotting. The results indicated a minor increase in the level of c-Src/ $\beta$ -actin, phospho-Tyr416Src, and phospho-Tyr527Src in the leiomyoma cells in comparison with the myometrial cells, but no significant difference was detected between the two cell types ( $P > 0.05$ ). Compared with the control, there was no significant change detected in the levels of c-Src/ $\beta$ -actin and phospho-Tyr527Src in both cell types after treatment with 0.1–3.0  $\mu\text{mol/L}$  gossypol ( $P > 0.05$ ). However, there was a concentration-dependent decline in the expression of phospho-Tyr416Src in both cell types, and there was a pronounced decrease observed in both cell types at 3.0  $\mu\text{mol/L}$  compared with control cells ( $P < 0.05$ , Figure 8).

#### The effect of Src inhibition on the growth of leiomyoma and myometrial cells

To determine whether Src kinase indeed plays a role in the growth of uterine leiomyoma and myometrial cells, we examined the effects of PP2, a specific Src inhibitor on both cell types. PP2 (0.66 and 6.6  $\mu\text{mol/L}$ ) inhibited the growth of leiomyoma and myometrial cells and decreased the levels of c-Src/ $\beta$ -actin, phospho-Tyr416Src, and phospho-Tyr527Src in both cell types in a concentration-dependent manner. There was a significant difference in the levels of c-Src/ $\beta$ -actin and phospho-Tyr416Src in the control in comparison with the PP2 treated cells ( $P < 0.05$ ). In addition, PP2 slightly increased the expression of ER $\alpha$ / $\beta$ -actin and phospho-ser167ER $\alpha$ / $\beta$ -actin in both cell types ( $P > 0.05$ ) compared with control (Figures 2, 7, and 8).

We further examined the specific role of Src in the leiomyoma and myometrial cells with Src siRNA. The growth of the leiomyoma and myometrial cells was impaired after treatment with Src siRNA (12.5, 25, and 50 nmol/L). In comparison with the control siRNA, reduced expression of Src and increased expression of ER $\alpha$  were observed in both Src siRNA-transfected cell types (Figure 9).



**Figure 8.** Effects of gossypol on the levels of c-Src, phospho-Tyr416Src, and phospho-Tyr527Src in cultured leiomyoma and myometrial cells, as assessed via Western blotting. Both cells were cultured in DMSO (as control) or graded concentrations of gossypol (0.1, 1.0, and 3.0  $\mu$ mol/L) for 40 h. Data were presented as means $\pm$ SD of three independent samples. <sup>b</sup> $P$ <0.05 vs control cells; <sup>q</sup> $P$ <0.05 vs gossypol 0.1  $\mu$ mol/L (G-0.1) treated cells; <sup>h</sup> $P$ <0.05 vs gossypol 1.0  $\mu$ mol/L (G-1.0) treated cells; <sup>k</sup> $P$ <0.05 vs gossypol 3.0  $\mu$ mol/L (G-3.0) treated cells; <sup>n</sup> $P$ <0.05 vs PP2 0.66  $\mu$ mol/L (PP2-0.66) treated cells; <sup>o</sup> $P$ <0.05 vs PP2 6.6  $\mu$ mol/L (PP2-6.6) treated cells.

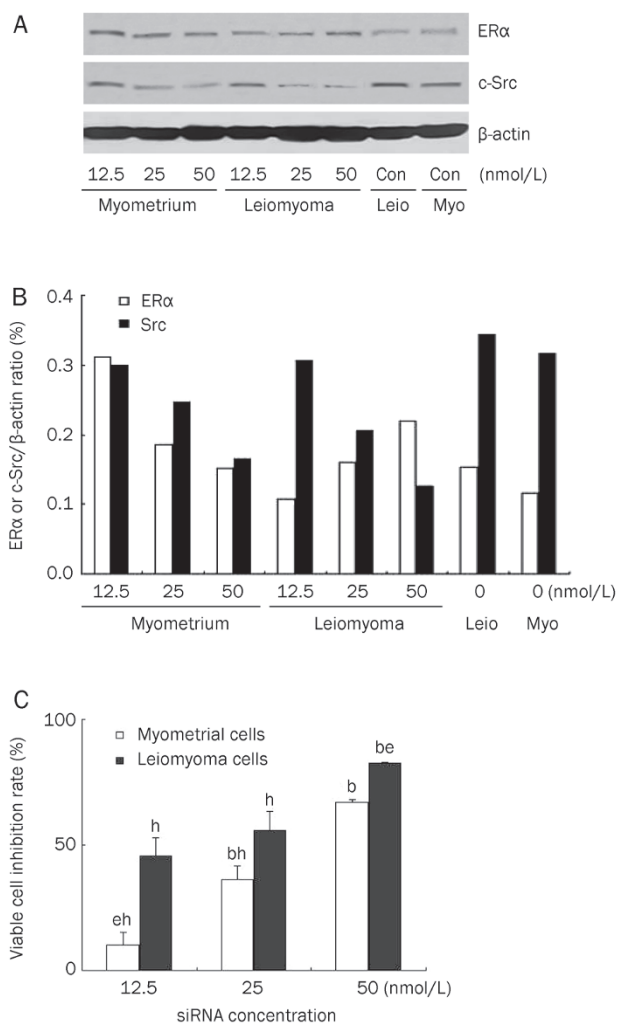
## Discussion

In the present study we demonstrated that gossypol inhibits growth and induces apoptosis in human uterine leiomyoma cells *in vitro* in a concentration- and time-dependent manner. The results support the therapeutic efficacy of gossypol in the treatment of uterine leiomyoma. Gossypol has inhibitory effects on myometrial cells as well. The mechanism of action involves the suppression of Bcl-2 expression and decreasing Src activity at a higher concentration. In addition, gossypol significantly reduces both the expression of ER $\alpha$  at a low concentration in the myometrial cells and the activity of ER $\alpha$  at a high concentration in the leiomyoma cells.

Most commonly, a leiomyoma is a partial lesion of the myometrium. Considering that gossypol may be administered to those who suffer from small leiomyomas and want to become pregnant, it is necessary to investigate the effects of gossypol on both leiomyoma and the adjacent normal myometrium. By comparing the IC<sub>50</sub> values and the percentage of inhibition

rate, we found that gossypol exerts different inhibitory effects on the leiomyoma and myometrial cells. In the myometrial cells, the inhibition rates at various times from 20 to 60 h were not noticeably different. However, gossypol caused a markedly stronger inhibition of the growth of leiomyoma cells at 60 h than at 20 and 40 h. At 20 h, a higher concentration of gossypol was required to inhibit the growth of leiomyoma cells than was required to inhibit the growth of myometrial cells. The results suggest that there was less response to gossypol in the leiomyoma cells than that in the myometrial cells before 40 h. It may imply that, clinically, more time is required for gossypol to effectively reduce the volume of the leiomyoma. In addition, the results suggest that gossypol suppresses not only the proliferation of leiomyoma cells but also the growth of adjacent myometrial cells, both of which might help to reduce the formation of leiomyoma. On the other hand, the findings also suggested that gossypol would lead to an adverse effect on the growth of normal myometrium.





**Figure 9.** (A&B) Effect of Src siRNA on the expression of ERα and Src in the leiomyoma and myometrial cells, as assessed by Western blotting. Both cells were cultured in control siRNA or graded concentrations of siRNA Src (12.5, 25, and 50 nmol/L) for 40 h. “Leio” stands for leiomyoma cells; “Myo” stands for myometrial cells; “Con” stands for control cells with non-target siRNA. (C) Inhibitory effect of Src siRNA on the growth of cultured leiomyoma and myometrial cells at 40 h, as assessed by CCK-8. The result was presented as percentage of the inhibition ratio, calculated as the following equation: [(1-OD of gossypol treated wells/OD of control wells) × 100%]. The experiments were performed in triplicate with three different cultured samples. Data were presented as means ± SD of three independent samples. <sup>b</sup>P < 0.05 vs siRNA 12.5 nmol/L treated cells; <sup>e</sup>P < 0.05 vs siRNA 25 nmol/L-treated cells; <sup>h</sup>P < 0.05 vs siRNA 50 nmol/L treated cells.

With electron microscopy and the TUNEL assay, we confirmed that gossypol induces apoptosis in a concentration-dependent manner in both cell types. The apoptotic characteristics induced by gossypol were similar to those previously reported in spermatocytes and cancer cells<sup>[23, 24]</sup>. To explore the mechanism of apoptosis induction by gossypol, we investigated the expression of Bcl-2 in both cell types. Bcl-2 has already been recognized as an inhibitor of the apoptotic pro-

cess. Related to sex steroid hormones, Bcl-2 protein expression in leiomyoma cells has been extensively reported<sup>[25]</sup>. In this study, we noticed that there was no significant difference in Bcl-2 expression in the leiomyoma and myometrial cells, and this is supported by data previously reported by Wu *et al*<sup>[3]</sup>. Gossypol induces apoptosis associated with decreased expression of Bcl-2. In fact, gossypol has also been identified as a potent Bcl-2/Bcl-X<sub>L</sub> small molecule inhibitor in other tumors and spermatocytic cells<sup>[24, 26]</sup>. Our findings are similar to those in malignant tumor cells.

Recent evidence shows there is a correlation between the activity of members of the Bcl-2 family and c-Src. c-Src tyrosine kinase plays an important role in the regulation of the cell cycle and cell proliferation<sup>[8]</sup>. Src acts not only as a potent mitogenic signaling element but also as an antiapoptotic signaling protein<sup>[27, 28]</sup>. Activation of Src family kinases plays a significant role in enhanced Bcl-2 expression<sup>[29]</sup>, and the inhibition of Src reduces the expression of Bcl-2 and Bcl-XL and induces apoptosis<sup>[30-32]</sup>. Since gossypol induces apoptosis as an inhibitor of Bcl-2, we investigated the impact of gossypol on the expression of Src. In a preliminary immunohistochemical experiment, we found that c-Src was expressed in the cultured human leiomyoma cells (data not shown). Here, we demonstrated that gossypol has no obvious impact on the expression of c-Src in both cell types. We speculate that apoptosis induced by gossypol is not directly correlated with the protein expression of Src.

To verify that the inhibition of Src kinase indeed plays a role in the gossypol-mediated cell death, we also examined the effects of Src siRNA and PP2, a specific Src inhibitor, on both cell types. In another study, PP2 was shown to induce apoptosis in both cell types (data not shown). Here, we demonstrated that the growth of both cell types was suppressed by either PP2 or Src siRNA in a concentration-dependent manner. This indicates that c-Src plays a vital role in the growth of leiomyoma cells, and the inhibition of c-Src may be effective in uterine leiomyoma therapy.

Because c-Src is a tyrosine kinase, the regulation of its activity is very important. Tyrosine phosphorylation regulates activity or inactivity at different sites. Phosphorylation of a conserved tyrosine in the activation loop (Tyr-416) up-regulates Src kinase activity<sup>[33]</sup>, and phosphorylation of a conserved tyrosine (Tyr527) in the carboxyl-terminal tail by Csk inactivates such activity<sup>[34]</sup>. To better understand the role of c-Src in the growth of leiomyoma cells, we further assayed the activity of c-Src in both cell types by detecting the level of phospho-Tyr416Src and phospho-Tyr527Src. We found that gossypol decreases the level of phospho-Tyr416Src in both cell types, especially at the high concentration of 3.0 μmol/L. However, there was no significant alteration of the level of phospho-Tyr527Src after treatment with gossypol. The results indicate that gossypol reduces the activity of c-Src and has no impact on the inactivity of Src. Accordingly, we presumed that gossypol might be an active regulator of Src. Although gossypol was unable to directly reduce the expression of Src, decreasing the activity of Src may suppress the growth of uterine lei-

myoma and induce apoptosis.

Moreover, because reducing the expression of ER $\alpha$  is a vital mechanism of action in the treatment of uterine leiomyoma, it is necessary to investigate the effect of gossypol on the expression of ER $\alpha$ . In this study, we showed that there is no pronounced difference in the expression of ER $\alpha$  in the two types of cells. These results agree with those previously reported by Jakimiuk *et al* and Strissel *et al*<sup>[35,36]</sup>. We found that gossypol decreases the expression of ER $\alpha$  at the low concentration of 0.1  $\mu\text{mol/L}$  but not at other concentrations; in addition, gossypol decreases the level of phospho-ser167ER $\alpha$  in a concentration-dependent manner. These observations suggest that gossypol down-regulates the activity of ER $\alpha$ . We hypothesize that this down-regulation may be correlated with the decreased Src activity. More studies need to be done.

Although gossypol has been extensively studied as an anti-proliferative agent in breast and prostate cancer, little is known about its relationship to the expression of ER $\alpha$ . This may be related to the data presented by Gilbert *et al* in 1995<sup>[37]</sup>. They demonstrated that co-incubation of MCF-7 cells with gossypol (5  $\mu\text{mol/L}$ ) and estradiol (10 nmol/L) did not alter the effects of gossypol, and they therefore presumed that the anti-proliferative effects of gossypol were not influenced by the ER mechanism. However, Huang *et al*<sup>[38]</sup> demonstrated that gossypol reduces the binding capacity to ER in normal endometrial cells at the concentration of 1  $\mu\text{mol/L}$ . Wang *et al*<sup>[39]</sup> presumed that gossypol exhibits a non-specific weak binding capacity to ER as a result of reducing protein synthesis. Accordingly, we speculated that, due to its weak binding capacity to ER, gossypol suppresses the expression of ER $\alpha$  only at a low concentration of 0.1  $\mu\text{mol/L}$  and not at higher concentrations of 1.0 and 3.0  $\mu\text{mol/L}$ .

We also noticed that pretreatment with PP2, the potent inhibitor of Src, elevated the level of ER $\alpha$ . There is an important feed-forward signaling loop involving estrogen, the ER $\alpha$  and Src. On the one hand, crosstalk between ER $\alpha$  and c-Src improve ER $\alpha$  transcriptional activity; on the other hand, the crosstalk also activates ER $\alpha$  proteolysis. Increased Src activity correlates with a shortened ER $\alpha$   $t_{1/2}$ . Inhibition of cellular Src impairs ER $\alpha$  ubiquitylation and ER $\alpha$  loss, resulting in the accumulation of ER $\alpha$ <sup>[10]</sup>. As a result, pretreatment with either PP2 or Src siRNA elevates the level of ER $\alpha$ . These results agree with those previously reported by Chu *et al* in breast cancer cells<sup>[10]</sup>. Thus, we presume that another reason why pretreatment with gossypol could not down-regulate the level of ER $\alpha$  may be that high concentrations of gossypol reduce Src activity.

In summary, our findings provide evidence that gossypol inhibits the growth of leiomyoma cells and induces apoptosis. Unlike other hormone-based medicine used in clinical practice, gossypol reduces the level of ER $\alpha$  only at a low concentration. Besides the effect on Bcl-2, one of gossypol's mechanisms of action may involve decreasing the activity of Src and ER $\alpha$ . These results may open new avenues for the therapy of uterine leiomyoma. The precise mechanism of action of gossypol remains to be elucidated, and more investigation is needed.

## Acknowledgements

This work was supported by a grant from the Shanghai Leading Academic Discipline Project (grant No S30303).

## Author contribution

Yan ZHU and Lin CAO designed the research; Shu-wu XIE, Jian-feng ZHANG, Ting-ting ZHANG, and Jie-yun ZHOU performed the research; Yang CAO analyzed the data; and Yan ZHU wrote the paper.

## References

- Walker CL. Role of hormonal and reproductive factors in the etiology and treatment of uterine leiomyoma. *Recent Prog Horm Res* 2002; 57: 277–94.
- Walker CL, Stewart EA. Uterine fibroids: the elephant in the room. *Science* 2005; 308: 1589–92.
- Wu X, Blanck A, Olovsson M, Henriksen R, Lindblom B. Expression of Bcl-2, Bcl-x, Mcl-1, Bax, and Bak in human uterine leiomyomas and myometrium during the menstrual cycle and after menopause. *J Steroid Biochem Mol Biol* 2002; 80: 77–83.
- Kovács KA, Lengyel F, Környei JL, Vértés Z, Szabó I, Sümegi B, *et al*. Differential expression of Akt/protein kinase B, Bcl-2 and Bax proteins in human leiomyoma and myometrium. *J Steroid Biochem Mol Biol* 2003; 87: 233–40.
- Perillo B, Sasso A, Abbondanza C, Palumbo G. 17Beta-estradiol inhibits apoptosis in MCF-7 cells, inducing bcl-2 expression via two estrogen-responsive elements present in the coding sequence. *Mol Cell Biol* 2000; 20: 2890–901.
- Gohel A, McCarthy MB, Gronowicz G. Estrogen prevents glucocorticoid-induced apoptosis in osteoblasts *in vivo* and *in vitro*. *Endocrinology* 1999; 140: 5339–47.
- Lim KB, Ng CY, Ong CK, Ong CS, Tran E, Nguyen TT, *et al*. Induction of apoptosis in mammary gland by a pure anti-estrogen ICI 182780. *Breast Cancer Res Treat* 2001; 68: 127–38.
- Schlessinger J. New roles for Src kinases in control of cell survival and angiogenesis. *Cell* 2000; 100: 293–6.
- Johnson D, Agochiya M, Samejima K, Earnshaw W, Frame M, Wyke J. Regulation of both apoptosis and cell survival by the v-Src oncoprotein. *Cell Death Differ* 2000; 7: 685–96.
- Chu I, Arnaout A, Loiseau S, Sun J, Seth A, McMahon C, *et al*. Src promotes estrogen-dependent estrogen receptor alpha proteolysis in human breast cancer. *J Clin Invest* 2007; 117: 2205–15.
- Irby RB, Yeatman TJ. Role of Src expression and activation in human cancer. *Oncogene* 2000; 19: 5636–42.
- Wiener JR, Windham TC, Estrella VC, Parikh NU, Thall PF, Deavers MT, *et al*. Activated SRC protein tyrosine kinase is overexpressed in late-stage human ovarian cancers. *Gynecol Oncol* 2003; 88: 73–9.
- Zhu Y, Qiu XY, Wang L, Wu JH, Liu GM, He GL, *et al*. Effects of gestrinone on uterine leiomyoma and expression of c-Src in a guinea pig model. *Acta Pharmacol Sin* 2007; 28: 685–94.
- Liu GZ. Clinical study of gossypol as a male contraceptive. *Reproduction* 1981; 5: 189–93.
- Wu D. An overview of the clinical pharmacology and therapeutic potential of gossypol as a male contraceptive agent and in gynaecological disease. *Drugs* 1989; 38: 333–41.
- Moon DO, Kim MO, Lee JD, Kim GY. Gossypol suppresses NF- $\kappa$ B activity and NF- $\kappa$ B-related gene expression in human leukemia U937 cells. *Cancer Lett* 2008; 264: 192–200.
- Zhang M, Liu H, Tian Z, Griffith BN, Ji M, Li QQ. Gossypol induces apoptosis in human PC-3 prostate cancer cells by modulating

- caspase-dependent and caspase-independent cell death pathways. *Life Sci* 2007; 80: 767–74.
- 18 Macoska JA, Adsule S, Tantivejkul K, Wang S, Pienta KJ, Lee CT. (–)Gossypol promotes the apoptosis of bladder cancer cells *in vitro*. *Pharmacol Res* 2008; 58: 323–31.
- 19 Xu, L, Yang D, Wang S, Tang W, Liu M, Davis M, et al. (–)Gossypol enhances response to radiation therapy and results in tumor regression of human prostate cancer. *Mol Cancer Ther* 2005; 4: 197–205.
- 20 Jiang J, Kulp SK, Sugimoto Y, Liu S, Lin YC. The effects of gossypol on the invasiveness of MAT-LyLu cells and MAT-LyLu cells from the metastasized lungs of MAT-LyLu-bearing Copenhagen rats. *Anticancer Res* 2000; 20: 4591–7.
- 21 Zhang Y, Wang Y, Li C, Zhou J, Zhang D, Han M. Estrogen and progesterone cytosol receptor concentrations in patients with endometriosis and their changes after gossypol therapy. *Chin Med J* 1996; 109: 814–6.
- 22 Han ML, Wang YF, Tang MY, Ge QS, Zhou LF, Zhu PD, et al. Gossypol in the treatment of endometriosis and uterine myoma. *Contrib Gynecol Obstet* 1987; 16: 268–70.
- 23 Zhang M, Liu H, Guo R, Ling Y, Wu X, Li B, et al. Molecular mechanism of gossypol-induced cell growth inhibition and cell death of HT-29 human colon carcinoma cells. *Biochem Pharmacol* 2003; 66: 93–103.
- 24 Teng CS. c-fos protein expression in apoptotic rat spermatocytes induced by gossypol. *Contraception* 1998; 57: 281–6.
- 25 Maruo T, Ohara N, Wang J, Matsuo H. Sex steroidal regulation of uterine leiomyoma growth and apoptosis. *Hum Reprod Update* 2004; 10: 207–20.
- 26 Oliver CL, Miranda MB, Shangary S, Land S, Wang S, Johnson DE. (–)Gossypol acts directly on the mitochondria to overcome Bcl-2- and Bcl-X<sub>L</sub>-mediated apoptosis resistance. *Mol Cancer Ther* 2005; 4: 23–31.
- 27 Iravani S, Zhang HQ, Yuan ZQ, Cheng JQ, Karl RC, Jove R, Coppola D. Modification of insulin-like growth factor 1 receptor, c-Src, and Bcl-X<sub>L</sub> protein expression during the progression of Barrett's neoplasia. *Hum Pathol* 2003; 34: 975–82.
- 28 Karni R, Jove R, Levitzki A. Inhibition of pp60c-Src reduces Bcl-X<sub>L</sub> expression and reverses the transformed phenotype of cells overexpressing EGF and HER-2 receptors. *Oncogene* 1999; 18: 4654–62.
- 29 Dai Y, Rahmani M, Corey SJ, Dent P, Grant S. A Bcr/Abl-independent, Lyn-dependent form of imatinib mesylate (STI-571) resistance is associated with altered expression of Bcl-2. *J Biol Chem* 2004; 279: 34227–39.
- 30 Jia HY, Wu JX, Zhu XF, Chen JM, Yang SP, Yan HJ, et al. ZD6474 inhibits Src kinase leading to apoptosis of imatinib-resistant K562 cells. *Leuk Res* 2009; 33: 1512–9.
- 31 Ohigashi T, Ueno M, Nonaka S, Nakanoma T, Furukawa Y, Deguchi N, et al. Tyrosine kinase inhibitors reduce bcl-2 expression and induce apoptosis in androgen-dependent cells. *Am J Physiol Cell Physiol* 2000; 278: C66–72.
- 32 Lee M, Kim JY, Koh WS. Apoptotic effect of PP2 a Src tyrosine kinase inhibitor, in murine B cell leukemia. *J Cell Biochem* 2004; 93: 629–38.
- 33 Boulet I, Fagard R, Fischer S. Correlation between phosphorylation and kinase activity of a tyrosine protein kinase: p56lck. *Biochem Biophys Res Commun* 1987; 149: 56–64.
- 34 Thomas JE, Soriano P, Brugge JS. Phosphorylation of c-Src on tyrosine 527 by another protein tyrosine kinase. *Science* 1991; 254: 568–71.
- 35 Jakimiuk AJ, Bogusiewicz M, Tarkowski R, Dziduch P, Adamiak A, Wróbel A, et al. Estrogen receptor  $\alpha$  and  $\beta$  expression in uterine leiomyomas from premenopausal women. *Fertil Steril* 2004; 82: 1244–9.
- 36 Strissel PL, Swiatek J, Oppelt P, Renner SP, Beckmann MW, Strick R. Transcriptional analysis of steroid hormone receptors in smooth muscle uterine leiomyoma tumors of postmenopausal patients. *J Steroid Biochem Mol Biol* 2007; 107: 42–7.
- 37 Gilbert NE, O'Reilly JE, Chang CJ, Lin YC, Brueggemeier RW. Antiproliferative activity of gossypol and gossypolone on human breast cancer cells. *Life Sci* 1995; 57: 61–7.
- 38 Huang HF, Wang M. Effects of gossypol acetate, danazol, progesterone and GnRH-A on estrogen and progesterone receptors of human endometrial cells. *Zhongguo Zhong Xi Yi Jie He Za Zhi* 1994; 14: 352–3, 325.
- 39 Wang NG, Guan MZ, Li HP, Lei HP. Effect of gossypol acetic acid (GAA) and 15-methyl PGF<sub>2</sub> alpha-methyl ester (PG05) on estrogen receptors of rat uteri. *Yao Xue Xue Bao* 1987; 22: 103–6.