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Original Research Article

Forsythia suspensa extract has inhibitory effect on proliferation and apoptosis of A549 lung cancer cells

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Abstract

Purpose: *To investigate the effect of Forsythia suspensa extract (FSE) on apoptosis and proliferation in A549 human lung cancer cells.*
Methods: *Inverted microscope was employed to observe morphological changes in A549 cells after exposure to FSE. Trypan blue staining of living cells was used to construct the cell growth curve after treatment with varying concentrations of FSE. The influence of FSE on cell proliferation, apoptosis and cell cycle was determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay, while protein expressions of key apoptosis-related enzymes were evaluated by immuno-cytochemical method.*
Results: *FSE inhibited the growth of A549 lung cancer cells at a concentration range of 10 - 150 μg/mL. Flow cytometry results showed that FSE induced apoptosis in A549 cells. The proportion of cells in G0/G1-phase increased significantly ($p < 0.01$), while the proportion of cells in S- and G2/M-phase decreased correspondingly, indicating that the cells were in G0/G1-phase arrest. Cell cycle arrest and apoptosis-inducing effect gradually rose with increase in FSE concentration. With increasing concentrations of FSE, there was also significant increase in the expressions of caspase-3 ($p < 0.05$), caspase-8 ($p < 0.01$) and caspase-9 ($p < 0.05$), but significant decrease in Ki-67 ($p < 0.01$) and p21 ras protein ($p < 0.01$).*
Conclusion: *FSE exerts significant inhibitory effect on the proliferation of A549 lung cancer cells. Therefore, the plant can potentially be developed for the treatment of lung cancer.*

Keywords: *Forsythia suspensa, Antitumor activity, Lung cancer A549 cells, Apoptosis, Cell cycle arrest, Caspase*

INTRODUCTION

Morbidity and mortality from lung cancer account for 13 and 19.4 %, respectively, worldwide [1]. Lung cancer is classified into small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC). The latter (NSCLC) accounts for most (about 80 %) lung tumors. At present, early-stage NSCLC is treated by surgical intervention, while mid-late NSCLC is treated with radiotherapy and chemotherapy. Although effective for some patients in the short run, radiotherapy and chemotherapy are associated with adverse effects, toxicity, high recurrence of tumor and poor prognosis [2]. Therefore, it is necessary to reduce the side effects of chemotherapy drugs by searching for more effective anticancer drugs with lower toxicity. This has been a major challenge to medical science.

Forsythia suspensa is a very valuable plant in Wuzhou of China. Studies have shown that *Forsythia suspensa* has anti-aging, blood pressure-lowering, hypoglycemic and immunity-enhancing properties [3]. It also has antioxidant activity [4], and exerts cytotoxic effects on various malignant tumors [5,6]. This study was carried out to investigate the effect of *Forsythia suspensa* extract on proliferation and apoptosis in A549 human lung cancer cells.

EXPERIMENTAL

Materials

Human lung cancer cell line (A549) was purchased from the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences. *Forsythia suspensa* was got from Wuzhou, China. RPMI1640 culture medium was product of GIBCO Company, USA). Calf serum and phosphate buffered saline (PBS, American hyclone); dimethylthiozol-2-yl-2,5-diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), propidium iodide (PI) and trypsin were obtained from *Sigma-Aldrich* (America). Caspase-3, caspase-8, caspase-9, Ki-67, bcl-2 p21 ras monoclonal antibodies, and SP generic kits were purchased from American NeoMarkers (San Francisco, USA).

Samples of *Forsythia suspensa* were collected from Wuzhou City, Guangxi Province, China in September 2017. Taxonomic identification of the plant was done by Professor Xia Cheng of Henan University of Traditional Chinese Medicine in China. A voucher specimen (no. FSE 20170323) was deposited in the herbarium of College of Pharmacy, Henan University of Traditional Chinese Medicine, China for future reference.

Instrumentation

The instruments used were 96-well plates (Corning Company, Wuhan, China); automatic CO_2 incubator (DuPont Company of America Napoc6100); 450 enzyme standard instrument (American BioRad); inverted microscope (Olympus CX41-32C02, Japan); 4 % hope blue mother liquor (Japanese BigWood Industrial Co. Ltd) and LKB- ultrathin sections machine (Sweden). Others were YZ-1450 laminar flow ultranet work station (Suzhou Purification Equipment Company, Suzhou, China); elite flow cytometer (Coulter, USA); blood cell counting plate; vacuum drying oven (DZF-6021 type, Shanghai Fine Macro Experiment Equipment Limited), and ultrasonic cleaner CQ25-12 type (Shanghai Scientz Biotechnology Research Institute).

Preparation of FSE

Dry leaves of *Forsythia suspensa* (80 g) were ground and extracted three times by refluxing with 90 % ethanol, each time for 2 h. The extracts were pooled and concentrated under reduced pressure to obtain FSE.

Cell culture

The A549 cells were cultured in RPMI-1640 medium (Gibco, Rockville, MD) containing 10 %(v/v) fetal bovine serum (FBS; Hyclone, Logan, UT) and 1 % Penicillin Streptomycin (100 U/mL and 100 µg/mL, respectively) at 37 °C in a humidified atmosphere containing 5 % CO_2, with 0.25 % trypsin digestion batches. The logarithmic phase of the cells were used for the study.

Drug sensitivity test

Cells at the logarithmic growth phase (in RPMI1640 medium) at a concentration of 1.0×10^5 cells/mL of cell suspension, were seeded in 48-well plates and cultured at 37 ^{o}C for 24 h, until the cells became adherent. FSE at different final concentrations (30, 60, 120, 240 µg/mL) were added to different wells, and the wells were incubated for different periods (24, 48 and 72 h) at 37 ^{o}C. A well containing 20 µg/mL cisplatin served as positive control, while another well containing saline in place of FSE served as negative control.

Cell morphology assay

A suspension of A549 cells at the logarithmic growth phase in RPMI 164 medium was adjusted to a concentration of 1.0×10^5 cells/mL in a 48-well culture plate, and cultured for 24 h at 37 ℃ to achieve adherence. The wells were grouped into two. One group (experimental group) received different concentrations of FSE (62.5, 125 or 250 ug/mL) in the culture medium, while the other group (control group) received 0.04 % DMSO in place of FSE. The cells were observed under inverted microscope after 24, 48 and 72 h for changes in cell size, cell membrane and nucleus.

Growth curve and doubling time (TD) of A549 lung cancer cells

The A549 cells at logarithmic growth phase at a concentration of 1 × 10^4 cells/mL were seeded in 24-well plates. One milliliter was taken from each of three wells at the same time point daily, and digested. The number of cells per well was counted using a hemocytometer, and the mean number of cells was determined. Counting was done for 8 days. A growth curve was plotted with mean cell count as vertical axis, and time as horizontal axis. Doubling time (TD) for the human lung cancer A549 cells was calculated using Eq 1.

$TD = t\{log2/(logN_t - logN_o)\}$ ………………. (1)

where *t* is time interval, while N_o, and N_t are the initial mean count and mean count after time *t*, respectively.

MTT assay

A suspension of the A549 cells (0.2 mL) at logarithmic phase was seeded in a 96-well plate with each well containing 5 × 10^3 cells, and cultured overnight for cell adhesion. In the experimental group, the original culture medium was drained, and replaced with different concentrations of freshly-prepared FSE (one concentration in 24 wells, and 6 wells per group). The wells of the control group medium contained 0.04 % DMSO, while wells without cells served as blank control. All wells were incubated for 24, 48, 72 and 96 h at 37 ^oC in a humid atmosphere containing 5 % CO_2. The supernatant was discarded, and 20 µL of MTT solution (5 g/L) was added to each well and incubated at 37 ^oC for 4 h. Then, the supernatant was carefully removed by suction, and 150 µL DMSO was added to each well to dissolve the crystals. The absorbance of each solution was read at 490 nm against blank control, and the results were used for calculating the growth inhibition rate.

FCM assay

A suspension of A549 human lung cancer cells (5.0 × 10^4 cells/mL) at logarithmic growth phase was seeded in 24-well plates (100 µL in each well), and cultured in RPMI 1640 medium for 24 h for adherence. Thereafter, FSE was added to a final concentration of 40 µg/mL. Wells in the control group contained an equivalent volume of normal saline. The plates were cultured at 37 ^oC in an atmosphere of 5 % CO_2. At 24 and 48 h, the cells in each group were collected (cell number > 1 × 10^6), washed twice in cold PBS (pH 7. 2 ~ 7. 4), and centrifuged at 1000 rpm for 5 min. Then, 1 mL of cold precipitation PBS (pH 7.2 ~ 7.4) was added to the cell suspension, followed by 3 mL of pre-cooled 70 % ethanol.

The cell suspension was kept overnight at 4 ^oC, and then centrifugal at 1000 rpm for 5 min. The supernatant was discarded; and the cells were washed twice in 1 mL of cold PBS (pH 7.2 ~ 7.4), and centrifuged at 1000 rpm for 5 min. The cell count was adjusted to 1 × 10^6 / mL, and 200 uL RNase was added, followed by incubation in a 37 ^oC water bath for 30 min. Iodide C ingot (PI) staining fluid 800 µL was added, mixed and kept at 4 ^oC in the dark for 30 min. DNA single parameter analysis was carried out to determine cell distribution and the proportion of cells in G0/G1, S, and G2/M phases, as a function of time. The results were analyzed using Modifit LT software.

Immunocytochemistry staining

The A549 cells at logarithmic phase were digested with 0.25 % trypsin, and 2 × 10^5 cells were seeded into a 6-well plate and cultured overnight for cell adhesion. The culture medium was removed, and 3 mL of FSE was added to different cells to final concentrations of 25, 50, 100 and 200 µg/mL. Each FSE concentration was replicated in six parallel wells. In the control wells, FSE was replaced with an equivalent volume of 0.04 % DMSO. The plates were incubated for 24 and 48 h; the glass slides were taken out, dried and fixed with 4 % paraformaldehyde for 10 min at 4 ^oC according to instructions contained in the operation manual of S-P kit. The plates were observed for appearance of clear brown granules which is indicative of positive cells.

The presence of Bcl-2 and p21 ras in cytoplasm is indicative of positive expression; Ki67 antigen is indicative of positive expression in the nucleus, caspase-9 is an index of positive expression in the cytoplasm and the nucleus; while caspase-3 and caspase-8 indicate positive expression in the cytoplasm or cytoplasm and nuclei. They are expressed less in cells without changes in color, or lightly-colored cells. The cells in the FSE plates and control plates were observed at high magnification (× 400) in 5 fields, and positive cells were counted and expressed as percentage of the total number of cells.

Statistical analysis

All data are expressed as mean ± standard error of mean (SEM), and processed using SPSS 16.0 (SPSS Inc., Illinois, Chicago, USA). They were analyzed by one-way analysis of variance

(ANOVA) followed by Dunnett's t-test. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of FSE on proliferation of A549 cells

FSE at different concentrations significantly inhibited the proliferation of A549 cells after 48 h when compared with control group ($p < 0.05$). The inhibitory effect was concentration-dependent. These results are shown in Table 1.

Effect of FSE on cell morphology

Under the inverted microscope, the control group of lung cancer A549 cells grew densely, with overlapping clusters, as a long fusiform or polygon, without direction and with clear cellular edges. On the other hand, A549 cells treated with FSE at concentrations of 10 and 20 µg/mL showed reduction in number of cells, and no obvious morphological changes. When the concentration of FSE reached 40 µg/mL, the cell volume shrank, becoming smaller; and the cells were fewer and separated, with many floating particles in the culture medium. At concentrations of 160 and 320 µg/mL of FSE, there were very clear and obvious changes in cell morphology, and evidence of cell disruption and higher cell death.

Inhibitory activity of FSE

Results from MTT assay showed that FSE exerted a concentration and time-dependent inhibitory effects on the growth of human lung cancer A549 cells (Table 2). The inhibitory effect was highest at a concentration of 320 µg/mL. In addition, for each FSE concentration, the inhibitory effect increased with time and became maximum at 96 h.

Effect of FSE on A549 cell apoptosis and cell cycle

FSE at a concentration of 40 µg/mL brought about arrest of cell cycle at G0 / G1 phase in 24 and 48 h, and blocked the transition from G1 phase to S phase (Table 3). With increase in the G0 - G1 phase of the cell cycle, there was reduction in the number of cells in the S phase. The increases in percentage of cells in the G0/G1 phase, and decrease in cells in S phase, as concentration-dependent and time-dependent ($p < 0.01$).

Table 1: Inhibitory effect of FSE on the proliferation of A549 cells

Group	Concentration (µg/mL)	Absorbance (250 nm)	Inhibition (%)
Negative control	—	0. 326±0. 002	—
Positive control	25	0. 048±0. 004^{2}	92. 17±0. 14
FSE	50	0. 188±0. 002^{1}	47. 08±0. 22
	100	0. 179±0. 004^{1}	52. 14±0. 25
	150	0. 156±0. 003^{2}	49. 19±0. 26
	200	0. 128±0. 004^{2}	62. 27±0. 23

Values are mean ± SEM (n = 4); $^{1}p < 0.05$, $^{2}p < 0.01$ relative to control

Table 2: Inhibitory effects of FSE on A549 lung cancer cell growth

Group	Concentration (µg/mL)	24h	48h	72h	96h
Control	—	—	—	—	—
FSE	8	1.25	2.18	2.12	2.18
FSE	16	5.39	13.56	20.54	23.29
FSE	32	20.32	25.24	36.34	43.28
FSE	64	29.72	35.16	44.28	52.15
FSE	128	37.43	56.29	61.28	70.83
FSE	256	40.16	54.28	72.46	75.17

Table 3: Effect of FSE on cell cycle distribution of A549 cells at 24 and 48 h

Group	G0/G1 (%)	S (%)	G2/M (%)
Control	49.26±3.14	44.25±3.02	9.14±1.05
FSE 24 h	55.76±3.08*	32.49±4.07*	4.56±1.27*
FSE 48 h	60.76±4.07*	19.56±5.05*	3.26±1.01*

Values are mean ± SEM (n = 8); $^{*}p < 0.01$ compared to control

DISCUSSION

The results obtained in this study show that FSE has significant time- and concentration-dependent inhibitory effects on the proliferation of A549 cells. The extract also exerted strong apoptotic effects on the A549 cells, as seen from the morphological changes in the cells. The dynamics of tumor cell proliferation are due to loss of control of cell cycle, resulting in uncontrolled G1 / S and G2 / M transformations. The cells were mainly in the active DNA synthesis S phase, which led to abnormal cell proliferation [5].

Thus, blockage of the G1/S phase and G2/M phase can effectively control the tumor cell cycle and inhibit tumor proliferation. In this study, results from cell cycle analysis showed that FSE reduced the number of A549 human lung cancer cells in G1, S and G2/M phases, leading to stagnation of the cell cycle and restraining of cell proliferation. Increased concentrations of aqueous fractions of FSE brought about enhanced effects on cell cycle arrest. This indicates that FSE causes lung cancer cell cycle arrest by regulation and inhibition of tumor cell mitosis.

Ki-67 antigen is a more positive marker of nuclear proliferation, and Ki-67 may be used as a biomarker for determining high risk individuals in pre-cancer population [7-9]. Ki-67 antigen assay results showed that FSE significantly decreased the expression of Ki-67 in A549 cells. This implies that a large number of the A549 cancer cells were in the stationary phase of cell cycle. In effect, FSE may contain active principles that regulate the gene which inhibits cancer cell mitosis, thereby inhibiting the growth of tumor cells. P21 ras cancer gene is involved in transmission of intracellular information which regulates the cell cycle, and it is the "initiation factor" in the occurrence of tumors [10-13]. The expression of p21ras antigen was also significantly decreased in the FSE-treated A549 cells, in a time- and concentration-dependent manner, when compared with untreated controls. Again, this is an indication that most of the cancer cells were in the S phase of the cell proliferation cycle. This supports the presence of anti-proliferative and mitosis-inhibiting agents in FSE.

Caspases are proteolytic enzymes which constitute key components of the apoptosis system. They are a family of cysteine proteases involved in regulation of apoptosis, which is considered to be key to cancer cell death [14–17]. In this study, FSE treatment led to significantly lowered expression of Bcl-2, and significant increases in the expression of caspase-9. These results demonstrate that the anti-proliferative effects FSE also involve the mitochondrial apoptotic pathway. Caspase-3 is a pro-apoptosis caspase. Its expression in the FSE-treated A549 human lung cancer cells was significantly higher than in the untreated control cells, indicating that FSE promotes apoptosis in these cells. Thus, FSE induced apoptosis by inhibiting the signal transduction pathway enzymes to achieve elevated expressions of the pro-apoptosis caspases 3, 8 and 9; as well as the apoptosis-related gene Bcl-2.

CONCLUSION

The findings of this study reveal that FSE has significant effect on the inhibition of proliferation and induction of apoptosis in human lung cancer A549 cells. These findings suggest that FSE might be a potent source of drugs for the treatment of lung cancer patients.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Wei-guo Zhang designed all the experiment and revised the paper. Qin Liu and Cai-peng Lei performed the experiment and wrote the manuscript.

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