Herbal formula Renshenwuweizi decoction induces p53-mediated cell cycle arrest and apoptosis in A549 cells

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Abstract

OBJECTIVE: To investigate the effect of Renshenwuweizi decoction (RSWWZ decoction) on the growth of non-small cell lung cancer cells in vitro.

METHODS: A549 non-small cell lung cancer cells were divided into two groups: control and RSWWZ decoction treatment groups. Cell Counting Kit-8 was used to measure the inhibitory effect of RSWWZ decoction on the growth of A549 cells. 4', 6-diamidino-2-phenylindole staining and Annexin V-fluorescein isothiocyanate/propidium iodide double staining were used to investigate apoptosis in A549 cells following RSWWZ decoction treatment, and the mitochondrial membrane potential of treated cells was detected with Rhodamine 123. Cell cycle progression was analyzed by flow cytometry. The mRNA levels of p53, Bax, B-cell lymphoma-2 (Bcl-2) and p21 were measured by quantitative real-time reverse transcription polymerase chain reaction. The protein expressions of p53, Bax, Bcl-2, p21, cyclin-dependent kinases 2 (CDK2), and cyclin A were detected by Western blot.

RESULTS: RSWWZ decoction reduced the viability of A549 cells in a dose-dependent manner by inducing apoptosis and decreased mitochondrial membrane potential. RSWWZ decoction increased p53 and Bax expression and decreased Bcl-2 expression in a dose-dependent manner. RSWWZ decoction also induced an S-phase cell cycle arrest by increasing p21 and decreasing cyclin A and CDK2 expression.

CONCLUSION: In vitro experiments revealed that the Renshenwuweizi decoction-induced decrease in A549 cell proliferation was achieved by inducing apoptosis and S-phase cell cycle arrest via the p53 pathway. These findings provide the experimental basis for Renshenwuweizi decoction treatment of lung cancer.

INTRODUCTION

Lung cancer is a malignant tumor that originates from bronchial lung cells. Based on histology, lung cancers are generally divided into two categories, non-small cell lung cancer (NSCLC) and small cell lung cancer, the respective ratio of which is approximately 4:1. Over the past decades, lung cancer has become one of...
the world’s leading causes of cancer-related death. In 2012, more than 1.6 million people died of lung cancer, and the number of global lung cancer deaths is expected to reach 3 million by 2035. With continuous improvements in medical standards, the mortality rate of lung cancer has decreased; however, lung cancer still has one of the highest mortality rates among all cancers.

The usual treatments for lung cancer include surgery, chemotherapy, radiotherapy, and immunotherapy. However, most patients have adverse reactions to treatments, which are also very expensive and place a heavy economic burden on the family and generally lead to poor outcomes.

The multiple clinical applications and pharmacological research indicate that Traditional Chinese Medicine (TCM) has been used for treating lung cancer for a long time. TCM has the advantages of wide sources, low cost, and few side effects, which underscore why TCM has become a new form of cancer treatment. According to TCM, lung cancer is a systemic disease associated with the general state of the body. The concepts and strategies of TCM treatment emphasize systemic adjustments that improve the whole body and produce therapeutic effects through multiple targets instead of removing tumors or killing cancer cells. Therefore, in TCM decoctions, a herbal formula is generally used to treat the disease rather than a single herb.

One particular herbal formula, the Renshenwuweizi (RSWWZ) decoction, has been used to treat cancer for many years. RSWWZ consists of seven Chinese herbal medicines, including Renshen (Radix Ginseng), Wuweizi (Fructus Schisandrae Chinensis), Sangbaipi (Cortex Morus Albae Radici), Digupi (Cortex Lycii Radicis), Zhimu (Rhizoma Anemarrhena), Jiegeng (Radix Platycodi), and Gancaco (Radix Glycyrrhizae). Each herb contains many chemical constituents with anticancer effects. Ginsenoside inhibits lung cancer invasion and reverses tumor cisplatin resistance. Schisandin B induces apoptosis in A549 cells and decreases lung cancer invasion. Platycodin D, an active ingredient in P. grandiflorum, induces apoptosis in NSCLC cells and blocks cell cycle progression. Both licorice chalcone and glycyrrhetic acid in licorice can induce endoplasmic reticulum stress and induce apoptosis in NSCLC cells. However, the efficacy of RSWWZ decoction for treatment of lung cancer are still lacking.

In this study, we investigated the effects and potential mechanism of RSWWZ decoction in A549 NSCLC cell line. Our findings demonstrated that RSWWZ decoction may inhibit the growth of A549 cells by inducing apoptosis and cell cycle arrest via the p53 pathway.

**MATERIALS AND METHODS**

**Preparation of RSWWZ decoction**

RSWWZ decoction consists of seven TCMs: Renshen (Radix Ginseng), Wuweizi (Fructus Schisandrae Chinensis), Sangbaipi (Cortex Morus Albae Radici), Digupi (Cortex Lycii Radicis), Zhimu (Rhizoma Anemarrhena), Jiegeng (Radix Platycodi), and Gancaco (Radix Glycyrrhizae) at a 1:1:1:1:1:1:1 ratio. All components were purchased from Hebei Kaida Pharmaceutical Co., Ltd. (Jiaozuo, China). The decoction was extracted twice with distilled water for 2 h and then the supernatant extract was combined, filtered, and lyophilized into a powder using a freeze dryer. The powder was dissolved in DMEM (HyClone, Logan, UT, USA) before the experiments to obtain a 5 mg/mL solution, which was diluted before use.

**Cell culture conditions**

A549 cells were purchased from the Shanghai Institute of Life Science, Chinese Academy of Sciences (Shanghai, China). Cells were grown in DMEM (HyClone) containing 10% fetal bovine serum (Cellmax, Sunnyvale, CA, USA), 100 U/mL penicillin, and 100 mg/mL streptomycin (HyClone) in a humidified atmosphere with 5% CO₂ at 37 °C.

**Reagents, kits and antibodies**

Cell Counting Kit-8 (CCK-8) was purchased from Boster Biological Technology Co., Ltd. (Wuhan, China). The Cell Cycle and Apoptosis Analysis Kit, 4,6-diamidino-2-phenylindole (DAPI), 2-(6-amino-3-imino-3H-xanthen-9-yl)-benzoic acid methyl ester (Rhadmine 123), BCA Protein Assay Kit, and enhanced chemiluminescence (ECL) kit were purchased from Beyotime (Nanjing, China). The Annexin V-FITC Apoptosis Detection Kit I was purchased from Becton Biosciences (San Diego, CA, USA). Antibodies against p53 (#2524T), Bax (#5023T), B-cell lymphoma-2 (Bcl-2) (#15071), p21 (#2947T), cyclin A (#4656T), cyclin-dependent kinases 2 (CDK2) (#2546T), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (#2118) were purchased from Cell Signaling Technology (Danvers, MA, USA).

**Cell viability assay**

The effects of RSWWZ decoction on the proliferation and viability of A549 cells were assessed using the CCK-8 assay. A549 cells were seeded in 96-well plates at a density of 2 x 10^4 cells per well for 24 h and then treated with various concentrations of RSWWZ decoction for 24, 48, or 72 h. CCK-8 was then added to the cells and cells were incubated for 1 h at 37 °C. The numbers of viable cells were calculated by detecting the optical density (OD) at 450 nm using an enzyme-labeled instrument (Tecan, Switzerland). At least three independent experiments were performed. Cisplatin (DDP; Shuyaanye, Shanghái, China) was used as a positive control, and the experimental concentration (2.9 µg/mL) was calculated from preliminary testing.

**DAPI staining**

The effect of RSWWZ decoction on the nuclear morphology of A549 cells was detected by DAPI staining.
A549 cells were seeded in 6-well plates at a density of $1 \times 10^4$ cells per well for 24 h. Cells were treated with various concentrations of RSWWZ decoction for 48 h, washed once with cold PBS, and then fixed with 4% paraformaldehyde for 15 min at room temperature. The cells were then washed once with cold PBS and stained with DAPI solution (1 μg/mL) for 5 min at room temperature in the dark. Nuclear morphology was analyzed on an Olympus IX73 fluorescence microscope (Olympus, Tokyo, Japan).

**Annexin V-FITC/propidium iodide (PI) double staining**
Apopotosis rates were measured by flow cytometry following Annexin V-FITC/PI double staining. A549 cells were seeded in 6-well plates at a density of $1 \times 10^5$ cells per well for 24 h. Cells were then treated with various concentrations of RSWWZ decoction for 24 h. Cells were harvested and washed twice with cold PBS. The cells were resuspended in 195 μL of binding buffer, to which 5 μL of Annexin V-FITC and 5 μL of PI were added. The mixture was incubated for 15 min at room temperature in the dark. Apoptotic cells were analyzed by flow cytometry (Amnis, MilliporeSigma, Burlington, MA, USA).

**Mitochondrial Membrane Potential (MMP) assay**
MMP was measured by flow cytometry following Rhodamine 123 staining. A549 cells were seeded in 6-well plates at a density of $1 \times 10^5$ cells per well for 24 h and then treated with various concentrations of RSWWZ decoction for 8 h. As a positive control, 10 μM CCCP was added to the medium of the positive control well, and cells were incubated for 20 min at 37 °C. Cells were then harvested and washed once with cold PBS. Cells were then resuspended in 500 μL 2 μM Rhodamine 123 for 30 min at 37 °C in the dark. The stained cells were washed twice with PBS, resuspended in 300 μL PBS, and then measured by flow cytometry within 24 h.

**Cell cycle analysis by flow cytometry**
PI was used as a fluorescent dye for flow cytometry-based determination of cell cycle distribution. A549 cells were seeded in 6-well plates at a density of $1 \times 10^5$ cells per well for 24 h. Cells were treated with various concentrations of RSWWZ decoction for 48 h and fixed with 70% ice-cold ethanol at 4 °C overnight. The cells were then washed once with cold PBS and resuspended in 500 μL of PI solution for 30 min at 37 °C in the dark. Cell cycle progression was measured by flow cytometry within 24 h and analyzed with FlowJo v7.6 software (FlowJo LLC, Ashland, OR, USA).

**Quantitative real-time (qRT)-PCR**
A549 cells were seeded in 6-well plates at a density of $1 \times 10^5$ cells per well for 24 h and then treated with various concentrations of RSWWZ decoction for 48 h. Total RNA was extracted using TRIsol (Sigma-Aldrich, St. Louis, MO, USA). cDNA was synthesized from total RNA using the PrimeScript™ RT Kit (DRR047A, Takara, Shiga, Japan). The primers were used: GAPDH forward 5′-GGTGAAGTGGAGTCAACGG-GA-3′ and reverse 5′-GGTGGGATGCTGCTTCTG-GAAAG-3′; p53 forward 5′-GCCATCTCAAGCA-TGCACAGCAT-3′ and reverse 5′-GGACAAA-CAGGCACCTCAAAGC-3′; Bax forward 5′-GGAT-GATGTTGCGCCGGT-3′ and reverse 5′-CCCAGTT-GAAGGTGCCTG-3′; Bel-2 forward 5′-TTTGTGA- GTTCGTTGGGG-3′ and reverse 5′-CCCTAG-GAGGGACCCAGG-3′; and p21 forward 5′-TGG-GGATGTCCGTCAAGAA-3′ and reverse 5′-CCCTGG-GGGAAAGGTAGACGA-3′. PCR was performed as follows: 30 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) was performed using iQ™ SYBR Green Supermix (Takara Biotechnology, Osaka, Japan) and an iCycler-iQ real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA).

**Western blotting**
A549 cells were seeded in 6-well plates at a density of $1 \times 10^5$ cells per well for 24 h and then treated with various concentrations of RSWWZ decoction for 48 h. Cells were harvested and lysed in RIPA buffer (Bio-world, China) supplemented with 1% PMSF (Beyotime, Haimen, China) for 30 min on ice and then centrifuged at 10000 × g for 15 min at 4 °C. Protein concentration of the collected supernatant was measured by the BCA Assay (Beyotime). Equal amounts of protein were subjected to SDS-PAGE and then transferred to nitrocellulose membranes. The membranes were blocked with 3% skim milk in PBS for 1 h and incubated with primary antibodies overnight at 4 °C. The membranes were then washed twice with PBST and incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Immunoreactive bands were detected using an ECL system (Takara, Shiga, Japan) and an iCycler-iQ real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA).

**Statistical analysis**
All assays were performed in triplicate, and results are expressed as mean ± standard deviation. Statistical significance was analyzed by one-way analysis of variance using GraphPad Prism software v6.0 (GraphPad Software, San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

**RESULTS**

**RSWWZ decoction inhibited the cell viability of A549 cells**
The cell viability of A549 cells treated with RSWWZ decoction was tested with CCK-8 assay. As shown in
Figure 1A, RSWWZ decoction inhibited the viability of A549 cells in both time- and dose-dependent manners. The inhibition of A549 cell proliferation was greater after 48 h compared with after 24 h, and cell viability was further significantly decreased at 72 h ($P < 0.001$). Furthermore, more cell death was observed under the microscope at 72 h. Therefore, RSWWZ decoction treatment for 48 h was selected for subsequent experiments.

The inhibition of cell growth by 50% (IC50) of A549 cells was 1.2 mg/mL after treatment with RSWWZ decoction at 48 h, as shown in Figure 1B. Compared with DDP, RSWWZ decoction at 1.2 mg/mL showed a similar inhibition of A549 cell viability while RSWWZ decoction at 1.6 mg/mL showed a higher inhibition.

**RSWWZ decoction induced apoptosis in A549 cells**

The effect of RSWWZ decoction on apoptosis of A549 cells was evaluated by DAPI staining and Annexin V-FITC/PI double staining, A549 cells were treated with RSWWZ decoction for 48 h at different concentrations and then stained with DAPI staining (Figure 2A-D). Nuclear chromatin was condensed, blue fluorescence was enhanced, and some nuclei had ruptured, forming fragments (nuclear disintegration) after treatment. To confirm that RSWWZ decoction induced apoptosis, we performed Annexin V-FITC/PI double staining and flow cytometry (Figure 2E-H). As shown in Figure 2I, the percentage of apoptotic cells in three groups of RSWWZ decoction was significantly higher than that of in the control group ($P < 0.001$) and increased in a dose-dependent manner.

**RSWWZ decoction induced loss of MMP in A549 cells**

Apoptosis can be caused by mitochondrial dysfunction, therefore, we next examined MMP using Rhodamine 123 and flow cytometry. As shown in Figure 3A-F, MMP was significantly decreased in a dose-dependent manner after 8 h of RSWWZ decoction treatment compared with controls ($P < 0.01$). This result indicated that RSWWZ treatment-induced apoptosis of A549 cells may involve the mitochondrial pathway.

**RSWWZ decoction induced the p53 pathway in A549 cells**

To further confirm that RSWWZ decoction-induced apoptosis in A549 cells was associated with the mitochondrial pathway, we next examined levels of apoptosis-associated proteins and mRNA by western blot and qRT-PCR, respectively. As shown in Figure 4, p53 and Bax were increased at both mRNA and protein levels after 48 h of RSWWZ decoction treatment, while Bcl-2 was decreased. These results suggested that RSWWZ decoction-induced apoptosis in A549 cells may be mediated by the p53 pathway.

**RSWWZ decoction induced an S-phase cell cycle arrest**

To further investigate the mechanism of cell growth inhibition by RSWWZ decoction, we used flow cytometry to detect changes in cell cycle progression of A549 cells after 48 h treatment with RSWWZ decoction. As shown in Figure 5A-E, the number of S-phase A549 cells was significantly increased in a dose-dependent manner upon 48 h RSWWZ decoction treatment compared with controls ($P < 0.001$), while the proportion of A549 cells in G0 decreased. We then examined levels of proteins that affect S-phase progression. As shown in Figure 5F-I, p21 levels were increased after 48 h treatment with RSWWZ decoction, while cyclin A and CDK2 levels were decreased. These results indicated that the inhibition of cell proliferation by RSWWZ decoction was partly associated with the induction of S-phase arrest in A549 cells.

**DISCUSSION**

Lung cancer is one of the most malignant tumors with the fastest growth in morbidity and mortality rates, making it one of the greatest threats to human health. Recently, TCM has been increasingly used for lung cancer treatment. RSWWZ decoction is regarded as has...
search into its mechanism of action in lung cancer has potential therapeutics for treating lung cancer, but research into its mechanism of action in lung cancer has been lacking. In our examinations, the effect of RSWWZ decoction on A549 cells were showed immensely inhibited on cell viability and in a dose- and time-dependent manner compared with control groups. More-
Apoptosis is the predominant form of programmed cell death. Apoptosis is regulated by intracellular and extracellular signals and is characterized by morphological changes, including nuclear fragmentation and condensation, mitochondrial outer membrane permeabilization, membrane blebbing, and cell contraction. The extrinsic death receptor pathway and the intrinsic mitochondria pathway are the two core apoptotic pathways. The p53 tumor suppressor plays a major role in apoptosis. p53 promotes the expression of a variety of pro-apoptotic proteins, mediates death receptors, and regulates mitochondrial pathways, usually in the cytoplasm. p53 translocates to the mitochondrial surface and directly binds Bcl-2 family proteins, leading to apoptosis and decreased MMP. RSWWZ decoction induced apoptosis in A549 cells. These data indicated that the p53-mediated mitochondrial apoptosis pathway might be involved in the apoptotic mechanism induced by RSWWZ decoction in A549 cells. We found that treating A549 cells with RSWWZ decoction for 48 h significantly increased the protein and mRNA levels of p53 and Bax and decreased those of Bcl-2. Moreover, exposure to RSWWZ decoction resulted in decreased MMP in A549 cells. These data indicated that the p53-mediated mitochondrial apoptosis pathway might be involved in the apoptotic mechanism induced by RSWWZ decoction in A549 cells. Cell viability and cell cycle progression are inseparable. Cell cycle analysis of A549 cells revealed that the percentage of S-phase cells increased with increasing dose of RSWWZ decoction, along with a decrease in the G1 population. p53 plays an important regulatory role in both cell cycle progression and apoptosis. Both p53 and its downstream target p21 (a cell cycle-dependent kinase inhibitor) inhibit the cell cycle-dependent

Figure 4 RSWWZ decoction activated the p53 pathway in A549 cells
A: p53, Bcl-2, and Bax levels were determined by Western blotting after 48 h treatment with various concentrations of RSWWZ decoction (0.4, 0.8, and 1.2 mg/mL). B-D: the quantification of p53, Bcl-2, and Bax protein levels were performed by ImagePro program after 48 h treatment with various concentrations of RSWWZ decoction (0.4, 0.8, and 1.2 mg/mL). B: relative density of p53; C: relative density of Bcl-2; D: relative density of Bax. E-G: p53, Bcl-2, and Bax mRNA levels were analyzed by qRT-PCR after 48 h treatment with various concentrations of RSWWZ decoction (0.4, 0.8, and 1.2 mg/mL). E: relative p53 mRNA level; F: relative Bcl-2 mRNA level; G: relative Bax mRNA level. All experiments were repeated three times. RSWWZ decoction: Renshenwuweizi decoction; Bcl-2: B-cell lymphoma-2; qRT-PCR: quantitative real-time reverse transcription polymerase chain reaction. *P < 0.05, **P < 0.01 and ***P < 0.001 vs Control group.
kinase CDK2. Cyclin A is expressed in S-phase and binds CDK2, thereby promoting DNA replication and S-phase transition. This is consistent with the results of this study, which found that p21 mRNA expression was increased in RSWWZ decoction-treated cells compared with controls. Furthermore, cyclin A and CDK2 protein and mRNA expression were decreased in cells treated with RSWWZ decoction. Therefore, RSWWZ decoction may cause an S-phase cell cycle arrest in A549 cells.

In conclusion, RSWWZ decoction inhibited the proliferation of A549 cells, and its mechanism may be through inducing apoptosis and cell cycle arrest through the p53 pathway. Our findings provide a hard evidence on RSWWZ decoction for treating lung cancer in vitro and establish a foundation for exploring the precise in vivo effectiveness in the following research.

REFERENCES