



ORIGINAL RESEARCH ARTICLE

Anti-tumor Effect of Shikonin on S180 tumor - Bearing Mice

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ABSTRACT

Objective: To investigate the changes of cell cycle and the content of P53 protein in S180 cells by using the specific fluorescent probe to identify the biological target to be measured according to the change of the fluorescence signal. The results showed that Shikonin had good anti-tumor effect. **Methods:** Cell cycle changes and P53 protein content in S180 cells were detected by flow cytometry. **Results:** With the increase of Shikonin dose, S180 cells G1 and S phase content gradually increased, it can be inferred Shikonin action in cells, and the cells can be blocked in G1 and S phase. With the increase of the dose of Shikonin, the expression of P53 protein increased. This suggests that Shikonin may up-regulate P53 activity and induce apoptosis. **Conclusion:** From the experimental results, we can see that Shikonin has a good antitumor effect in vivo.

KEYWORDS: Shikonin; apoptosis; Caspase-3,9

1. Preface

Tumor is a serious harm to human life and health of the disease, its harmful second only to cardiovascular disease, is the primary enemy of mankind. With the changes in human living environment and living habits, in the adverse external environment and some unfavorable factors, the incidence of cancer is gradually increasing. In the face of this serious social problem, the main goal of cancer control in the world today is to reduce cancer mortality and improve survival. But so far all the anti-tumor chemotherapy drugs almost no beyond the scope of cytotoxicity, toxic side effects greatly limits its wide range of applications and efficacy play, therefore, looking for low toxicity and effective natural anti-tumor drugs has been a hot topic in recent years [1].

Most of the drugs are through the induction of tumor cell apoptosis and then to achieve the purpose of tumor control. Mitochondria play an important role in this process of apoptosis. In the experiments of various stimuli induced apoptosis, it was found that the permeability of the mitochondrial membrane was increased and the various proteins in the mitochondria were released. Through a series of reactions ultimately induce cell apoptosis.

It is found from the literature that the current research on the yield of Shikonin at the level of cell in vitro has studied the antitumor activity of Shikonin on different tumor cells and its mechanism. At the animal level, only the crude product of *Lithospermum erythrorhizon* (S180 mice), the antitumor activity of Shikonin and its in vivo prophylaxis mechanism were studied systematically. The mechanism of apoptosis of tumor cells was studied mainly from the mitochondrial pathway.

2. Literature review

In recent years, the field of tumor research has made great progress, especially the development of modern molecular biology technology, so that people understand the nature of the tumor has a new breakthrough, the completion of the human genome project has greatly promoted the new tumor treatment methods and the development of some new antitumor drugs. At present, anti-tumor drug research is from the traditional cytotoxic drugs to gene therapy products and cancer-specific genes for the development of specific antagonists. It is expected that some new drugs such as antiangiogenic drugs, antisense drugs, protein kinase C inhibitors, oncogene inhibitors, apoptotic agonists and telomerase inhibitors, anticancer vaccines, gene drugs, new monoclonal antibodies products and updates on existing products, will become the mainstream of anti-cancer drug market [2].

Mitochondria play an important role in the process of apoptosis. In the experiments of various stimuli induced apoptosis, it was found that the permeability of the mitochondrial membrane was increased and the various proteins in the mitochondria were released. These proteins include cytochrome c and apoptosis factor AIF (apoptosis inducing factor) and so on. When the cells are involved in the apoptotic signal, the mitochondria release cytochrome C into the cytoplasm and form the apoptosome (apoptosome) with the apolipoprotein-1 and caspase-9 precursors, and the dATP

present in the cytoplasm, Activated caspase-9 precursor, start caspase cascade reaction, and ultimately induce cell apoptosis.

P53 also plays an important role in the regulation of apoptosis. Its main function is to monitor the G1 and G2 / M correction points. P53 downstream gene P21 protein is a cyclin-dependent kinase inhibitor, which can inhibit the activity of corresponding protein kinase by binding to cyclin-cdk, a cyclin-dependent kinase (CDK) and cyclin complex. Block P53 down-regulated the expression of cyclin B 1, cells cannot enter the M phase.

Caspase family plays an important role in the regulation of apoptosis. Caspase is mainly regulated by its activating factor and inhibitory factor. An apoptotic signal can often lead to the regulation of cofactor, caspase initiation factor and inhibitory factor. In the process of cofactor activation, Bax promotes the release of cyto c, while Bcl -2 is to prevent the release of cyto c [3]. Bcl-2 interacts with Apaf-1 to prevent cyto c activation of caspase and delay cell apoptosis. But when Bcl-2 and Bax form heterodimer, then start the death cycle [4]; regulation of caspase initiation factor and cofactor interaction can also achieve the regulation of apoptosis; apoptosis inhibitor (Inhibitors of apoptosis, IAPs) family is to inhibit the activity of caspase-induced molecules to prevent apoptosis [5].

The anti-tumor effect of Shikonin and its derivatives has been paid more and more attention. Singh [6] and other evidence that Shikonin can significantly inhibit the growth of human epidermal cancer cells (A431), inhibition of intensity and drug treatment time and drug concentration was dependent, Wu Zhen [7] also proved that Shikonin (A375-S2), human cervical cancer cells (hela), human breast cancer cells (MCF-7) and mouse fibrosarcoma cells (L929) were significantly inhibited in a dose-and time-dependent manner, and IC50 values of four kinds of cells were less than 90uM, indicating that Shikonin on a variety of tumors have a strong cytotoxic activity.

The chemical structure is naphthoquinone, English name: Shikonin, molecular weight: 288.2994, molecular formula: C₁₆H₁₆O₅, chemical name: (+) - 5,8-dihydroxy-2- (1-hydroxy-4 -methyl-3-pentenyl) -1,4-naphthoquinone. (Structure as shown in Figure 1-1) Shikonin is purple with metallic luster of the needle crystal, the melting point of 147 to 149°C, slightly soluble in water, soluble in benzene, ethanol, ether and other organic solvents, due to the ability to absorb 500 -560nm wavelength of light and red, and the absorption spectrum will migrate with the pH. It has acid-base stability, and the solution color changes with pH, acidic conditions under the red, neutral purple, alkaline blue.

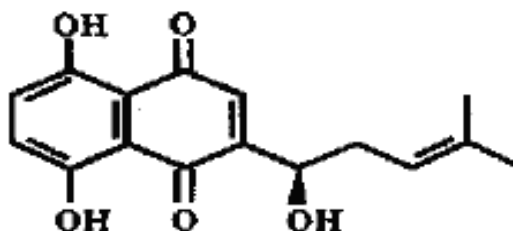


Figure 1. structure of Shikonin

At present, there are many studies on the anti-cancer mechanism of Shikonin. The possible mechanism is to selectively inhibit DNA topoisomerase I. Topoisomerase plays an important role in cell division and proliferation. Topoisomerase I reduces the DNA of the negative supercoil while the topoisomerase II is introduced into the DNA of the supercoil. Their synergies control the topological structure of DNA. Reproduction requires a higher level of negative supercoiling, which facilitates DNA unwinding. At the end of replication, it is necessary to reduce the negative supercoil level of DNA in order to carry out transcription at the active chromatin (often chromatin) site [8]. Shikonin has a strong inhibitory effect on topoisomerase I. It and alkannin is a potential inhibitor of topoisomerase I, which has a strong inhibitory effect on topoisomerase I. Mitochondrial Pathways: Lithospermum-induced apoptosis of A375-S2 cells is mediated by mitochondrial pathways. Mitochondria play an important role in the process of apoptosis. In the experiments of various stimuli induced apoptosis, it was found that the permeability of the mitochondrial membrane was increased and the various proteins in the mitochondria were released. These proteins include cytochrome c and AIF (apoptosis inducing factor) and so on. When the cells are involved in the apoptotic signal, the mitochondria release cytochrome c into the cytoplasm and form apoptosome with Apaf-1 and caspase-9 precursors. Under the combined action of dATP in the cytoplasm, caspase-9 precursor, start caspase cascade reaction, and ultimately induce cell apoptosis.

Cancer is an important disease that threatens human health and life safety. The treatment of its use of surgical therapy [9], radiation therapy [10], chemical therapy [11], interventional therapy and traditional Chinese medicine oral, rescue and other methods [12]. In recent years, the field of tumor research has made great progress, especially the development of modern molecular biology technology, so that people understand the nature of the tumor has a new breakthrough, and promote the new method of tumor therapy and new anti-tumor drugs development. But so far almost

all anti-tumor chemotherapy drugs exist cytotoxicity, so looking for low toxicity and efficient natural anti-tumor drugs become a hot research.

Shikonin exists in the roots of Lithospermaceae Lithospermum roots, isolated from the roots of Lithospermum to get the bioactive ingredients, with antibacterial, anti-inflammatory, anti-tumor [13] and other biological activity, there are many reports of anti-cancer medicinal research.

Modern scientific research confirmed that the anti-cancer effect of Shikonin exact, significant, but the current experiment using in vitro experiments to carry out anti-tumor effect research, in-vivo anti-cancer mechanism has not been reported. In this study, the anti-tumor effect of Shikonin in vivo and anti-tumor effect was studied, which provided effective pharmacological and theoretical basis for human cancer, which was beneficial to further drug research and clinical application.

3. Materials and Methods

3.1. Experimental materials

Experimental animal and animal tumor strains

Kunming mice, 18-22g, male and female, provided by the Harbin Cancer Hospital Animal Breeding Center. Animal transplanted tumor mice S180, provided by the Affiliated Tumor Hospital of Harbin Medical University.

Experimental apparatus

Optical Microscope (Leica) Sartorius BS110S One-tenth Electronic Balance (Germany BSS)

LD4-2A desktop low speed centrifuge (Beijing Medical centrifuge plant) micro pipette (Gilson SAS) vortex oscillator (Jiangsu Haimen Kirin Medical Instrument Factory) HH-S constant temperature water bath (Tianjin Tai Site Instrument Co., Ltd.) CLOUTER EPICS-XL Flow Cytometry (Beckman-Coulter, USA)

Experimental drugs and reagents

Shikonin Xuzhou Hongkang Technology Co., Ltd., content (30%)

Anhydrous ethanol Tianjin chemical reagents plant six plants

5 - fluorouracil Zhejiang Haizheng Pharmaceutical Co., Ltd

Healthy salt water Harbin Pharmaceuticals six plants hematoxylin Wuhan Dr. Biological Engineering Co., Ltd

Calf serum albumin (BSA) Sigma company paraformaldehyde Wuhan Boster Biological Engineering Co., Ltd.

95% ethanol Tianjin Tianxin Fine Chemical Development Center Formaldehyde Tianjin Tianxin Fine Chemical Development Center 30% Hydrogen Peroxide Shenyang Xinxin Reagent Factory Citrate Buffer Wuhan Boster Biological Engineering Co., Ltd. TritonX-100 Shanghai Huashun Biological Engineering Co., Ltd. Rabbit anti-P53 Wuhan Boster Biological Engineering Co., Ltd. FITC mark goat anti-rabbit IgG Wuhan Boster Biological Engineering Co., Ltd.

3.2. Experimental methods

Cell cycle and apoptosis were measured by flow cytometry [14, 15]

Mouse grouping and administration:

Grouped: Mice were randomly divided into 5 groups, 10 in each group. The rats in the experimental group were: high dose group, middle dose group, and low dose group, positive control group: 5-Fu; negative control group: 10% ethanol.

Administration: mice were inoculated with the corresponding tumor cells 24 hours after the experimental group was given intraperitoneal administration of shikonin 10 mg · kg⁻¹ · d, 5 mg · kg⁻¹ · d, 2.5 mg · kg⁻¹ · d, positive control group 5-Fu 20 mg · kg⁻¹ · d, negative control group with the same volume of 10% ethanol. Sterile continuous administration for 10 days.

Preparation of tumor cells: S180 mice were sacrificed under aseptic conditions. The tumor was then removed and placed on a 300 mesh cell sieve. The cells were washed with PBS solution, 1000 r · min⁻¹, centrifuged for 10 min, repeated twice, and finally add PBS into the concentration of 1 × 10⁶ L⁻¹, mix to make cell suspension.

Preparation of cell smears [16,17]: Take appropriate centrifugal sediment, place the appropriate position on the slide, and then use the slide (the slide must be a very smooth edge of the gap), contact the precipitate on the slide, where the precipitate will immediately penetrate the edge of the slide and the slide, depending on the degree of dilution of the sediment, select the appropriate angle (referring to the slide and slide between the angle) and the speed forward until the sediment is pushed up. In the process of pushing the film, cannot change the angle and speed, but cannot stop halfway, must be at one go, otherwise the introduction of the smear thickness uneven. When the sediment is thick, it is appropriate to choose the angle is small, slow to push the film, the precipitate is more dilute when it is.

Fixed: The volume fraction of 4% paraformaldehyde at room temperature for 5-10 minutes.

Dye: Hematoxylin staining 5min, tap water for 1min, hydrochloric acid ethanol differentiation 30s, and tap water soak 15min, with eosin complex 2min.

Conventional dehydration, transparent, sealed: washed with 95% ethanol twice, each time 1min. Then, washed with ethanol twice, each time 1min. And transparent with xylene 1min, and then repeat the transparent once. Finally, with a neutral resin seal, observed under a microscope.

Flow cytometry detection of P53 protein [18]

Mice were grouped and administered: same as (5.2.1).

Preparation of tumor cells: same as (5.2.1).

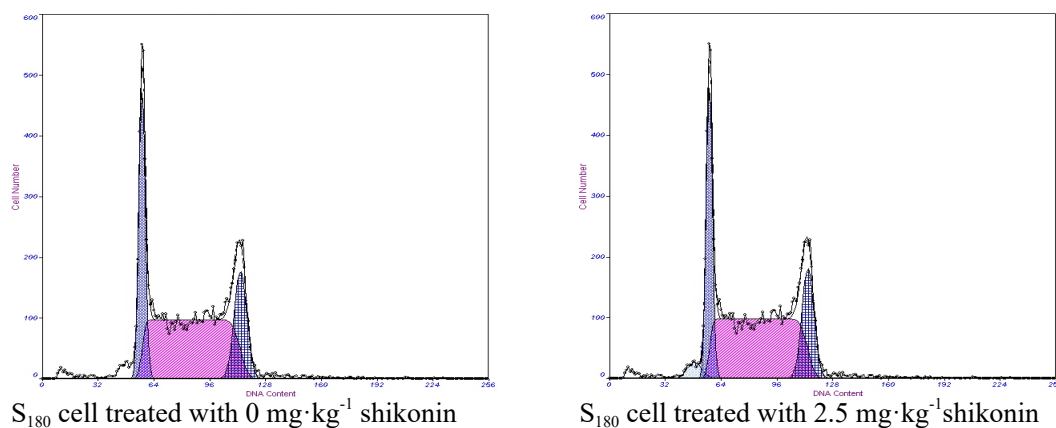
Immunofluorescence labeling of cells: PBS supplemented with 4% paraformaldehyde for 5 min. After centrifugation, rinse with 0.1% Triton X-100 in PBS and wash twice with PBS containing 5% BSA. After discarding the supernatant, rabbits were incubated with anti-P53 (1:50) and incubated on ice for 30 min. After washing with PBS, FITC labeled goat anti - rabbit antibody (1: 200) was incubated for 30 min. After washing with PBS, the shock was suspended in 500 μ L PBS and the 300 mesh sieve was filtered.

Flow cytometry parameters: 488nm excitation light, observed at 515nm fluorescence.

4. Results

4.1. Cell cycle and apoptosis were detected by flow cytometry

Flow cytometry (FCM) is a new technique for the quantitative analysis of cells or cell pellets and the classification of cells by integrating fluid injection technology, laser optics, electronic technology and computer technology. The principle is to use specific fluorescent probe marker to be measured biological target, according to the fluorescence signal strength reflects the detection target changes. Cell apoptosis, DNA degradation and DNA degradation, DNA-binding fluorescent probe can be directly on the cell DNA staining FCM analysis, detection of cell cycle changes; cell each mutation can lead to cell Of the apoptosis, which can also detect the cell apoptosis rate.



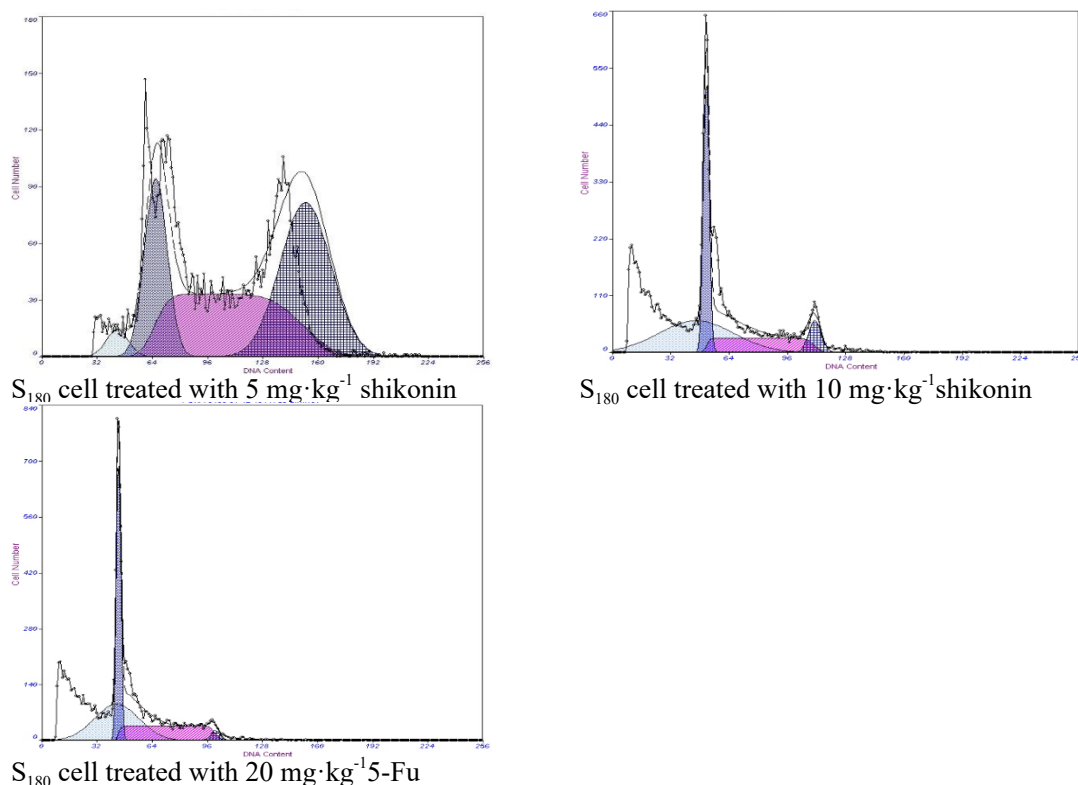


Figure 2. Effect of shikonin on cell cycle of S180 mice

Table 1. Effects of shikonin on cell cycle and apoptosis in S180 mice (n = 10, x ± S)

Group	G ₀ /G ₁ (%)	S(%)	G ₂ /M(%)	Apoptosis(%)
Control	10.0925±0.93224	19.6363±0.80479	70.2713±0.33396	—
Shikonin-Low	18.2500±0.77332**	29.7700±0.84099**	51.9800±0.74529**	9.723±0.27912
Shikonin-Mid	39.2425±0.85433**	37.3463±0.71276**	23.4113±0.66919**	16.210±0.36376
Shikonin-High	47.3513±0.83498**	40.3463±0.66322**	12.3025±1.22080**	28.813±0.15279
5-Fu	51.0350±0.74606**	42.4638±0.55923**	6.5013±0.58060**	33.175±0.67328

* Compared with the control group P <0.01

It can be seen from the experimental results in Table 6-1 that Shikonin dose group and 5-fluorouracil group can induce tumor cell apoptosis (P <0.01) compared with the negative control group, and can affect the cycle of tumor cells. In the G₀ / G₁ and S phases, the percentage of tumor cells was significantly higher in each dose group and 5-fluorouracil group compared with the negative control group (P <0.01), and with the increase of the dose, the tumor cells (P <0.01), and the proportion of tumor cells was significantly decreased with the increase of dose. In the G₂ / M phase, the proportion of tumor cells was significantly decreased (P <0.01).

4.2. Detection of P53 protein by flow cytometry

The expression of P53 protein in tumor cells was detected by immune-compromised tumor cells and the positive rate of tumor cells was detected by flow cytometry.

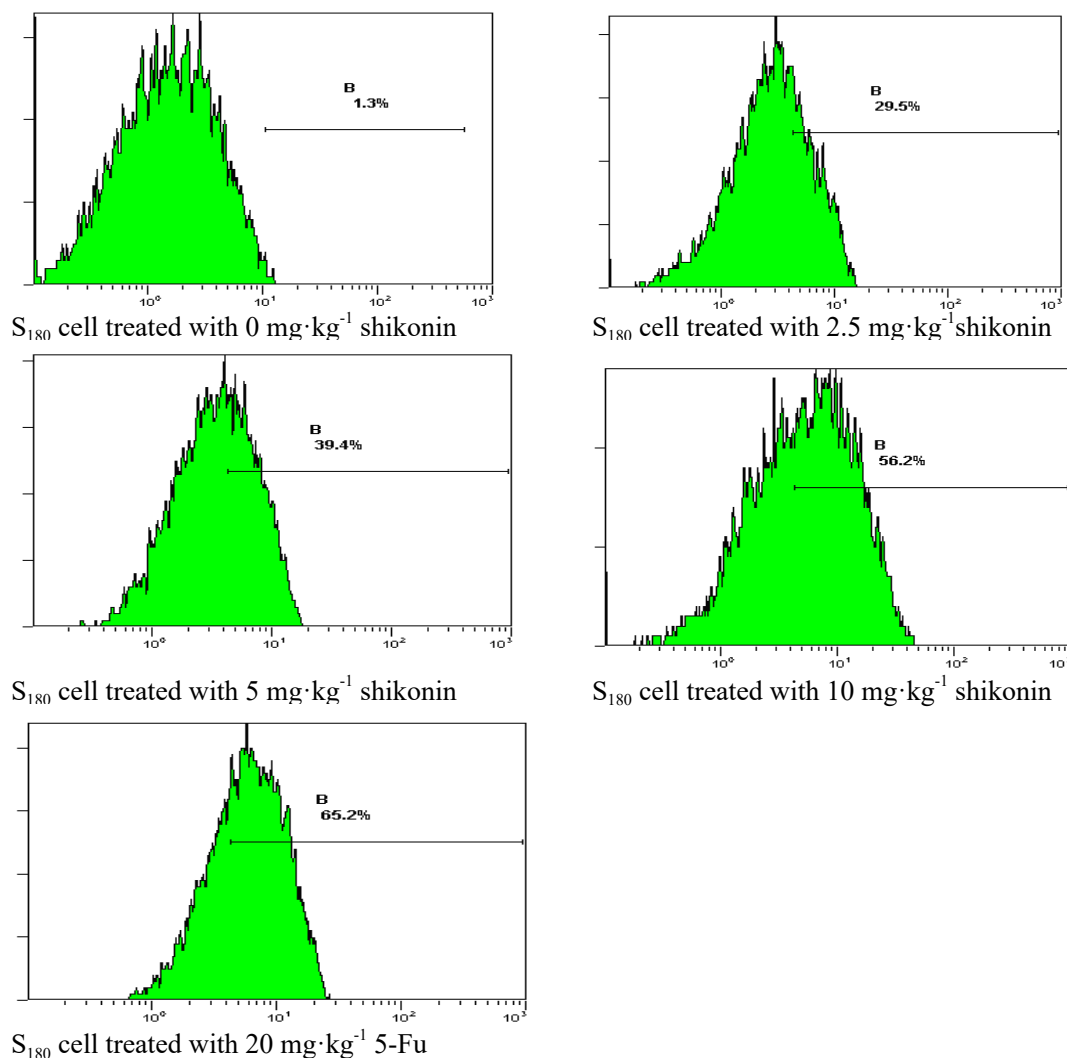


Figure 3. Effect of shikonin on the expression of P53 protein in S180 tumor cells

It can be seen from Figure 6-2 that the low, medium and high dose groups of shikonin have different levels of P53 protein expression in S180 cells compared with the negative control group. The expression rate of P53 in high dose group up to 56.2%. The expression of P53 was not higher in the dose group than that in the shampoo group, but the expression rate of P53 increased with the increase of the dose. Therefore, it is speculated that shikonin may induce apoptosis of S180 cells by up-regulating the expression of P53 protein.

5. Discussion

5.1. Effects of Shikonin on tumor inhibition rate in S180 tumor - bearing mice

The results showed that Shikonin had different antitumor effects on S180 mice ($P < 0.01$). There was a significant difference between the high dose group and the control group ($P < 0.05$). Compared with the positive control group, the tumor inhibition rate of the high dose group was slightly lower.

It can be seen from the experiment, Shikonin in mice in vivo S180 have a certain inhibitory effect, in a certain dose range, with the dose of Shikonin increased, the more obvious anti-tumor effect, and a certain dose-dependent relationship.

5.2. Cell cycle was measured by flow cytometry

From the experimental results, it can be seen that Shikonin has an effect on the changes of cells, and the proportion of G1 phase cells increased, the proportion of S phase cells increased, the proportion of G2 cells decreased, and the dose-dependent, indicating that Shikonin on G1 phase and S phase cells to G2 phase shift has a certain blocking effect.

Therefore, it can be inferred that Shikonin cells block cells in G1 phase and S phase, making it impossible to repair apoptosis.

5.3. On the expression of P53 protein

The results showed that the expression of P53 protein in S180 cells was higher than that in the negative control group, and the expression rate of P53 in the high dose group was 56.2%. The expression of P53 was not higher in the dose group than that in the shampoo group, but the expression rate of P53 increased with the increase of the dose. Therefore, it is speculated that Shikonin may induce apoptosis of S180 cells by up-regulating the expression of P53 protein. In addition to promoting apoptosis, the main function of P53 is also reflected in the block cell cycle and maintain genomic stability. In the experiment, we also investigated the effect of Shikonin on cell cycle. Shikonin can significantly block the cell cycle from G1 to S phase of the transition, which may make Shikonin inhibit the main reason for cell growth.

6. Conclusions

In vivo experiments showed that Shikonin significantly inhibited the survival of tumor cells in S180 mice. The mechanism of antitumor effect of Shikonin may be related to the following aspects. Induced apoptosis of tumor cells; the cells were blocked in the G1 and S phases with longer duration of action; it could induce the apoptosis of S180 cells by up-regulating the expression of P53 protein, which could play an anti-tumor effect.

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