


# Fuzheng Qingjie Granules Inhibit Growth of Hepatoma Cells via Inducing Mitochondria-Mediated Apoptosis and Enhancing Immune Function

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## Abstract

Fuzheng Qingjie (FZQJ) granules, a compound Chinese medicine, have been used as an adjuvant therapy for alimentary tract cancers. However, the underlying anticancer mechanisms are still not well understood. In the present study, HepG2 cells were treated with FZQJ-containing serum. Cell proliferation was evaluated using MTT assay. Apoptosis was analyzed using a flow cytometer. Cell ultrastructure was observed under a transmission electron microscope. The mitochondrial membrane potential ( $\Delta\psi$ ) was examined with JC-1 dye. In H22 tumor-bearing mice, CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, CD3<sup>+</sup> T cells, and natural killer (NK) cells in peripheral blood were evaluated cytometrically. Interleukin (IL)-2 and tumor necrosis factor (TNF)- $\alpha$  levels were measured using radioimmunoassay. The mRNA levels of Bax and Bcl-2 were examined by reverse transcription-polymerase chain reaction. The protein levels of Bax, Bcl-2, cytochrome C, caspase 3 and 9, PARP, and CD69 were examined by Western blotting. The apoptotic cells in tissues were observed using TUNEL method. Alanine transaminase (ALT), aspartate transaminase (AST), blood urea nitrogen (BUN), and creatinine (CRE) were detected by an automatic biochemical analyzer. The results showed that FZQJ-containing serum remarkably inhibited proliferation of HepG2 cells in dose- and time-dependent manners, induced HepG2 cell apoptosis and caused a decrease of  $\Delta\psi$ . Analysis of tumor tissue showed that FZQJ-induced apoptosis was accompanied by downregulation of Bcl-2 and upregulation of Bax, release of cytochrome c, activation of caspase 3 and 9, and cleavage of PARP. In addition, FZQJ increased the percentages of CD4<sup>+</sup> T and NK cells, the ratio of CD4<sup>+</sup>/CD8<sup>+</sup> T cells as well as the levels of serum TNF- $\alpha$ . FZQJ also increased CD69 expression in tumor tissue. No hepatorenal toxicity was observed in H22 tumor-bearing mice. These results indicated that FZQJ could inhibit the growth of hepatoma cells via regulating immune function and inducing mitochondria mediated apoptosis.

## Keywords

Fuzheng Qingjie granules, hepatoma, apoptosis, mitochondrial membrane potential, immune function

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## Introduction

According to the concept of tumor immunoediting, tumor immunoediting includes 3 phases, that is, elimination, equilibrium, and escape.<sup>1</sup> In the elimination phase, the immune system detects and eliminates tumor cells. Tumor cells may be completely cleared in the elimination phase. If only a part of tumor is eliminated, a temporary state of equilibrium can then develop between the immune system and tumor. If the immune response still fails to completely eliminate the tumor, some tumor cells that are able to resist, avoid, or suppress the

antitumor immune response may appear, leading to escape phase.<sup>2</sup> In addition, tumor cells may cause local or systemic

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immune deficiency.<sup>3</sup> Immune deficiency in cancer patients is well documented, as indicated by a lower percentage of CD4<sup>+</sup> T cells, dysfunction of dendritic cells, and higher levels of immunosuppressive factors (such as transforming growth factor [TGF]- $\beta$ ).<sup>4</sup> Therefore, immunotherapy has been advocated as a promising cancer treatment. A variety of immunotherapy agents have been developed, including tumor cell vaccine, antigen vaccine, monoclonal antibodies (mAb), and cellular immunotherapy.<sup>5-8</sup>

Traditional Chinese medicine (TCM) emphasizes the importance of boosting immune function in cancer patients. Accordingly Fuzheng Guben herbs and Qingre Jiedu herbs are widely used as adjuvant treatments for cancers. In TCM theory, Fuzheng Guben means to supplement vital energy, nourish yin and tonify kidney. Herbs for Fuzheng Guben, such as *Ganoderma lucidum*, *Astragalus membranaceus* and *Ginseng*, are found to be capable of strengthening the body's immune response by activating T lymphocytes, B lymphocytes, macrophages, natural killer (NK) cells, and dendritic cells, and promoting the productions of cytokines (such as interleukins [IL], tumor necrosis factors [TNF], interferon) and alexins.<sup>9,10</sup> For example, *Ginseng* is found to have anticancer activity, which may be attributed to modulation of macrophages, NK cells, dendritic T cells and B cells.<sup>11</sup> Another kind of herbal therapy is Qingre Jiedu herbs (such as *Hedyotis diffusa* Willd, *Prunella vulgaris*, *Lobelia chinensis*-Lour, and *Sophora flavescens*). Qingre Jiedu means clearing heat and detoxifying the body. In TCM, heat and toxin are considered to be an important causative factor of malignant cancers. Pharmacological studies demonstrate that Qingre Jiedu herbs contain ingredients that can inhibit tumor proliferation, induce cell apoptosis, and suppress angiogenesis, such as anthraquinones, polysaccharides, flavonoids, and triterpenoids.<sup>12-14</sup> For instance, matrine in *Sophora flavescens* can induce apoptosis of HepG2 cells via upregulation of p53, Bax, and Fas and downregulation of Bcl-2 and c-myc.<sup>15</sup>

Fuzheng Qingjie (FZQJ) granules are composed of both Fuzheng Guben and Qingre Jiedu herbs. It contains four Fuzheng Guben herbs (*Astragalus membranaceus*, *Ligustrum lucidum*, *Ganoderma lucidum* and *Rhizoma dioscorea*) and two Qingre Jiedu herbs (*Hedyotis diffusa* Willd and *Prunella vulgaris*). It has been used to treat cancer patients with Yin and Qi insufficiency for more than a decade. However, its anticancer mechanism is still not well understood. In the present study we showed that FZQJ granules could suppress the growth of hepatoma cells at least via enhancing immune function and inducing mitochondria-mediated apoptosis.

## Materials and Methods

### Preparation of FZQJ-Containing Sera

FZQJ granules were provided by the Department of Pharmacy, the Second Affiliated Hospital of Fujian University

of Traditional Chinese Medicine (Fuzhou, Fujian, China). Forty SPF (specific pathogen free) male Sprague-Dawley rats were randomly divided into 4 groups (n = 10 in each), that is, FZQJ high-dose group, FZQJ intermediate-dose group, FZQJ low-dose group, and control group, which received 5.4 g/kg/d, 2.7 g/kg/d, 1.35 g/kg/d FZQJ solution, and saline, respectively, by gavage twice each day for 7 days. On day 8, 2 hours after the last administration, rats were sacrificed and peripheral blood was collected and centrifuged at 2000  $\times$  g for 15 minutes. For each group, the sera were pooled, filtered through a 0.22- $\mu$ m cellulose membrane filter, heat inactivated at 56°C for 30 minutes, and stored at -80°C until use. Animal experiment protocol was approved by the Ethics Committee of Fujian University of Traditional Chinese Medicine.

### Cell Culture

Human HepG2 hepatoma cell line was obtained from the Shanghai Institute of Life Science, Chinese Academy of Sciences (Shanghai, China). Cells were grown in PRMI-1640 culture medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA) in 5% CO<sub>2</sub> atmosphere at 37°C.

To evaluate the effects of sera from the different groups, cells were seeded at a density of 2.5  $\times$  10<sup>5</sup> cells per well in 2 mL of RPMI medium in 6-well plates. Afterward, 0.5 mL of serum from the different groups was added. After 24 or 48 hours of incubation, cells were subjected to the following assays.

### Cell Viability Assay

For viability assay, 20  $\mu$ L of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Invitrogen-Life Technologies, Carlsbad, CA, USA) was added and incubated for 4 hours. Then 100  $\mu$ L dimethyl sulfoxide (DMSO) was added to dissolve the purple-blue formazan precipitate. The optical densities (OD) were measured at a wavelength of 490 nm with a microplate reader (Biotek Instruments Inc, Winooski, VT, USA). The percentage of cell viability was calculated according to the following formula: cell viability (%) = average OD<sub>sample</sub> / average OD<sub>control</sub>  $\times$  100%. At least 8 replicates of each test were performed in triplicate.

### Cell Apoptosis Assay

Cells were digested and resuspended with cold phosphate buffered saline (PBS). The cell apoptotic rate was determined by a flow cytometry (BD Biosciences, FranklinLakes, NJ, USA) after cells were stained with Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI). The percentage of apoptosis was calculated with CellQuest software.

### **Mitochondrial Membrane Potential Assay**

Mitochondrial membrane potential ( $\Delta\psi$ ) was measured by the flow cytometer with 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl benzimidazol carbocyanine iodide (JC-1) staining according to the instructions of the JC-1 Mitochondrial Membrane Potential Assay Kit (BD Biosciences).

### **Transmission Electron Microscope Examination**

Cells were collected after they were cultured in the presence of different sera for 48 hours. The cells were fixed in 2.5% glutaraldehyde for 2 hours and washed triple with PBS, then fixed in 1% osmium tetroxide for 2 hours, dehydrated in graded alcohols, embedded in epon 618, sectioned with an ultramicrotome (Leica Microsystems, Wetzlar, Germany), and stained with uranyl acetate and lead citrate. Sections were examined with a transmission electron microscope (TEM, Hitachi, Tokyo, Japan).

### **Mouse Xenograft Experiments**

Male SPF imprinting control region (ICR) mice (22-25 g) were purchased from the Guangdong Animal Center (Guangzhou, Guangdong, China). Mice were inoculated subcutaneously with  $5 \times 10^6$  H22 cell suspension in Matrigel/medium (BD Biosciences) on the back. The mice were randomly divided into 2 groups ( $n = 10$  for each group), that is, vehicle group and FZQJ group. Mice in the vehicle group were given saline, and those in FZQJ group FZQJ granules 5.4 g/kg/d for 7 days. Another 10 mice without H22 cells served as control. At day 7, all mice were sacrificed and peripheral blood was harvested. Tumors were dissected and weighed.

### **Evaluation of T and NK Cells of Peripheral Blood in H22-Bearing Mice**

Anticoagulated whole blood of mice was stained with FITC-conjugated CD3 mAb in combination with PE-conjugated CD8a mAb, or in combination with PE-conjugated CD4 mAb, or in combination with PE-conjugated CD49b mAb (all from Biolegend, San Diego, CA, USA), incubated for 15 minutes in the dark and then lysed with lysing solution. Flow cytometry was conducted to analyze CD3<sup>+</sup> T cells, CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, and NK cells. All experiments were performed at least 3 times.

### **Evaluation of Hepatorenal Function in H22-Bearing Mice**

In order to evaluate the safety of FZQJ, the levels of alanine transaminase (ALT), aspartate transaminase (AST), urea nitrogen (BUN), and creatinine (CRE) in the sera were

evaluated by an automatic biochemical analyzer (Toshiba, Kawasaki, Japan).

### **Cytokine Assays**

The levels of IL-2 and TNF- $\alpha$  in sera of H22-bearing mice were determined by  $\gamma$ -radioimmunoassay according to the manufacturer's instructions (Dongya Co, Beijing, China).

### **Hematoxylin and Eosin Staining and TUNEL Assay**

Briefly, tissues were fixed in 4% paraformaldehyde. After being embedded and dewaxed, tissues were characterized by hematoxylin and eosin (HE) staining. The apoptotic cells of tumors were detected with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay according to the manufacturer's instructions (Maxim, Fuzhou, Fujian, China). TUNEL-positive cells had dark brown staining. They were counted in ten random fields at 200 $\times$  magnification. Apoptotic index was reported as number of TUNEL-positive cells / number of total cells.

### **Reverse Transcription–Polymerase Chain Reaction Assay**

Total RNA was extracted using Trizol (Invitrogen-Life Technologies). Single-strand cDNA was synthesized with reverse transcriptase (Promega, Madison, WI, USA). The primers of Bax, Bcl-2, and  $\beta$ -actin are as follow: Bcl-2, forward 5'-TACCGTCGTGACTTCGCAGAGAT-3' and reverse 5'-AGGAGAAATCAAACAGAGGTCGC-3'. Bax, forward 5'-TTTGCTACAGGGTTTCATCCAGG-3' and reverse 5'-CAAAGTAGAAGAGGGCAACCACG-3'.  $\beta$ -actin, forward 5'-GTCCCTCACCCCTCCAAAAG-3' and reverse 5'-GCTGCCTCAACACCTCAACCC-3'. Polymerase chain reaction (PCR) amplification was performed as follow: 94 $^{\circ}$ C for 5 minutes followed by 40 cycles of 94 $^{\circ}$ C for 30 seconds, 60 $^{\circ}$ C for 30 seconds, 72 $^{\circ}$ C for 30 seconds, and an extension for 10 minutes at 72 $^{\circ}$ C. The amplification products were separated on 1.5% agarose gel electrophoresis containing ethidium bromide, and examined using a Gel Doc 2000 Imaging System (Bio-Rad, Hercules, CA, USA).

### **Western Blotting Analysis**

Tissues were lysed with lysis buffer (Beyotime Inc, Shanghai, China) followed by collection of cell protein. Protein concentration was determined using a BCA (bicinchoninic acid) protein kit (Invitrogen-Life Technologies). Equal amounts of denatured protein were separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and transferred into nitrocellulose membranes. These membranes were blocked in Tris-buffered saline containing

0.1 % Tween 20 for 1 hour. Then the membranes were incubated with primary antibodies against Bax, Bcl-2 (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA), cytochrome *c*, cleaved caspase 3 and 9, cleaved PARP, or CD69 (Cell Signaling Technology, Inc, Danvers, MA, USA) overnight at 4°C with shaking. After the membranes were washed 3 times, horseradish peroxidase (HRP)-conjugated secondary antibodies (Beyotime Inc) were added for 1 hour. After ECL chemiluminescence staining, bands were scanned and quantified using a chemiluminescence imaging system (Bio-Rad).

### Statistical Analysis

Statistical analysis was performed using SPSS version 18.0 software. Data were represented as mean  $\pm$  SD. For multiple comparisons, data were analyzed using a 1-way analysis of variance. For 2 comparisons, data were analyzed using independent-sample *t* test.

## Results

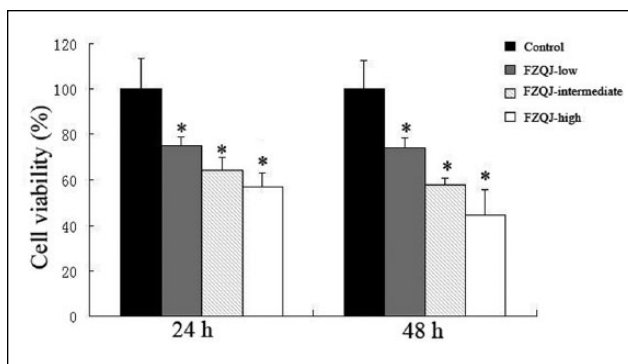
### FZQJ-Containing Serum Inhibits the Viability of HepG2 Cells In Vitro

Cell viability was assessed by MTT. As shown in Figure 1, compared with the control group, cell viabilities were inhibited by FZQJ-containing serum in dose- and time-dependent manners. The results indicated that serum contained ingredients and/or metabolites of FZQJ that could effectively inhibit the proliferation of HepG2 cells.

### FZQJ-Containing Serum Induces Apoptosis of HepG2 Cells In Vitro

Apoptotic cells were evaluated by Annexin V-FITC/PI double staining and TEM. Annexin-V is a cellular protein used to detect apoptotic cells, and PI dye is an intercalating agent commonly used for identifying dead cells. Annexin V-FITC<sup>-</sup>/PI<sup>-</sup> cells are considered to be intact cells, Annexin V-FITC<sup>+</sup>/PI<sup>-</sup> represents early apoptotic cells, and Annexin V-FITC<sup>+</sup>/PI<sup>+</sup> means late apoptotic cells.<sup>16</sup> Total apoptosis ratio (%) = early apoptosis ratio (%) + late apoptosis ratio (%). As shown in Figure 2A, the early apoptosis ratio and the late apoptosis ratio were both increased in the presence of FZQJ-containing serum in a dose-dependent manner. And the percentages of total apoptotic cells in the FZQJ-low group, FZQJ-intermediate group and FZQJ-high group were all notably increased compared with the control group 10.01%  $\pm$  1.72%, 12.28%  $\pm$  2.08%, and 16.79%  $\pm$  1.81% versus 7.89%  $\pm$  1.49% (respectively, *P* < .05 for all).

To further confirm that FZQJ-containing serum can induce cell apoptosis, the ultrastructure of cells was observed under a TEM (Figure 2B). The cells treated with



**Figure 1.** The effect of Fuzheng Qingjie (FZQJ)-containing serum on HepG2 cell viability. HepG2 cells were treated with FZQJ-containing serum or control serum for 24 or 48 hours. The cell viability was determined by MTT assay. Percentage cell survival in the control group was assumed 100. \**P* < .05, compared with the control.

the control serum were ellipse-shaped with abundant microvilli around the surface. However, the cells treated with the intermediate dose of FZQJ serum exhibited the characteristic ultrastructure of apoptosis: microvilli rarefaction, swelling of endoplasmic reticulum, increased number of lipid droplets, mitochondria degeneration, nucleus atypia, and chromatin condensation. Thus, FZQJ-containing serum could induce apoptosis of HepG2 cells.

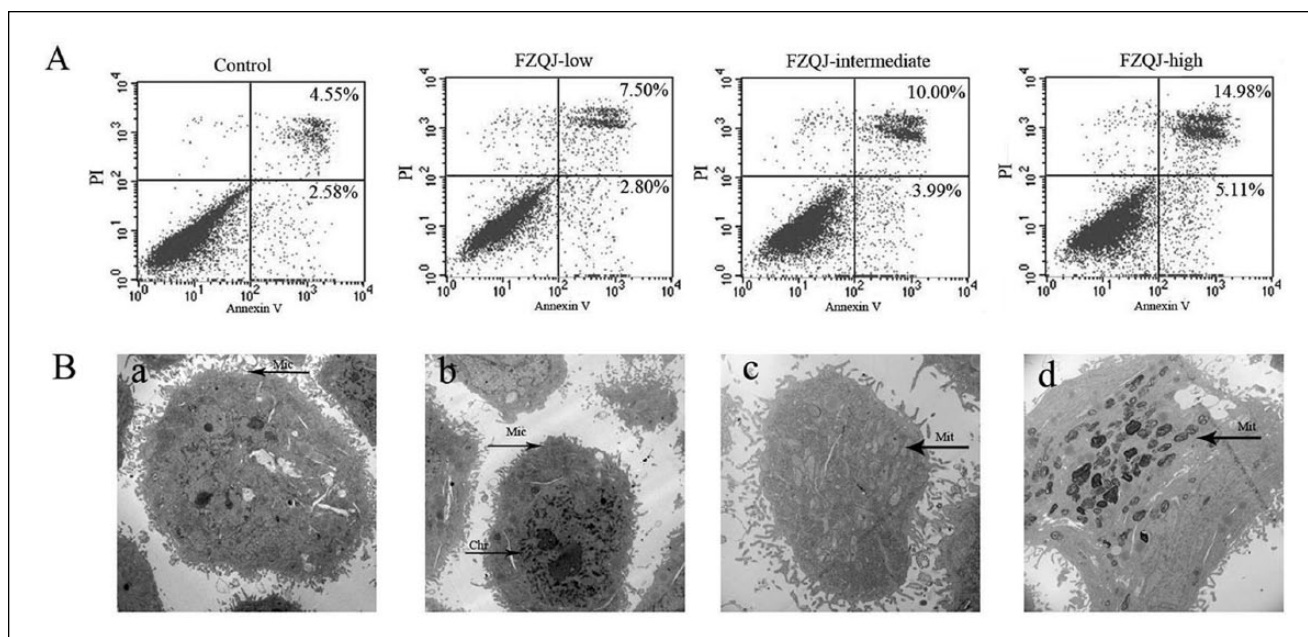
### FZQJ-Containing Serum Induces the Loss of $\Delta\psi$ In Vitro

Loss of  $\Delta\psi$  is one of the major consequences of apoptosis. In order to determine whether FZQJ-containing serum-induced apoptosis was mediated by the mitochondrial-dependent pathway,  $\Delta\psi$  was investigated using JC-1 staining. JC-1 is a membrane permeable dye that can selectively enter mitochondria. Depolarized  $\Delta\psi$  was indicated by decreased red fluorescence. As shown in Figure 3, the loss of  $\Delta\psi$  (R3 region) was observed when HepG2 cells were treated with FZQJ-containing serum. The loss rate of  $\Delta\psi$  was 5.38% in the control group and 14.90% in the FZQJ-high group.

### FZQJ Granule Inhibits the Growth of H22 Tumor In Vivo

To evaluate the effects of FZQJ granules on the growth of tumor, we examined tumor xenograft weight at sacrifice. As shown in Figure 4A, compared with the vehicle group, FZQJ granules significantly inhibited tumor growth (0.53  $\pm$  0.12 g in the FZQJ group vs 1.18  $\pm$  0.48 g in the vehicle group), with an inhibitory rate of 55.08%.

HE staining is shown in Figure 4B. Polygon- or irregular-shaped H22 tumor cells with large nuclei had invaded the entire muscle in the vehicle group. In the FZQJ-treated



**Figure 2.** The effect of Fuzheng Qingjie (FZQJ)-containing serum on apoptosis of HepG2 cells. The cell apoptotic rate was determined by flow a cytometer using Annexin V–fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining and the ultrastructure of cells was observed under a transmission electron microscope (TEM). (A) Results of Annexin V/PI staining after FZQJ-treated HepG2 cells. The early apoptotic portion (right lower quadrant) and the late apoptotic portion (right upper quadrant) were both increased in a dose-dependent manner. (B) The ultrastructure of HepG2 cells in the control group (a and c) and the FZQJ-intermediate group (b and d) were observed under a TEM. a and b magnifications, 8000 $\times$ ; c and d magnifications, 15000 $\times$ . The changes of microvilli, chromatin and mitochondria were marked by arrows. Mic, microvilli; Chr, chromatin; Mit, mitochondria.

group, many vacuoles appeared in the swollen cytoplasm of neoplastic cells. Remarkable karyopyknosis was seen in the nuclei.

In addition, TUNEL assay was performed to assess apoptotic cells in neoplastic tissue. TUNEL-positive cells displayed dark brown staining (Figure 4C). Apoptosis index in the FZQJ-treated group was significantly higher (29.33%  $\pm$  4.04%) than that of the vehicle group (5.67%  $\pm$  2.15%, Figure 4D).

Taken together, these data showed that FZQJ granules could inhibit the growth and induce apoptosis of subcutaneous tumor in H22-bearing mice.

### FZQJ Induces Mitochondria-Mediated Apoptosis of Subcutaneous Tumor

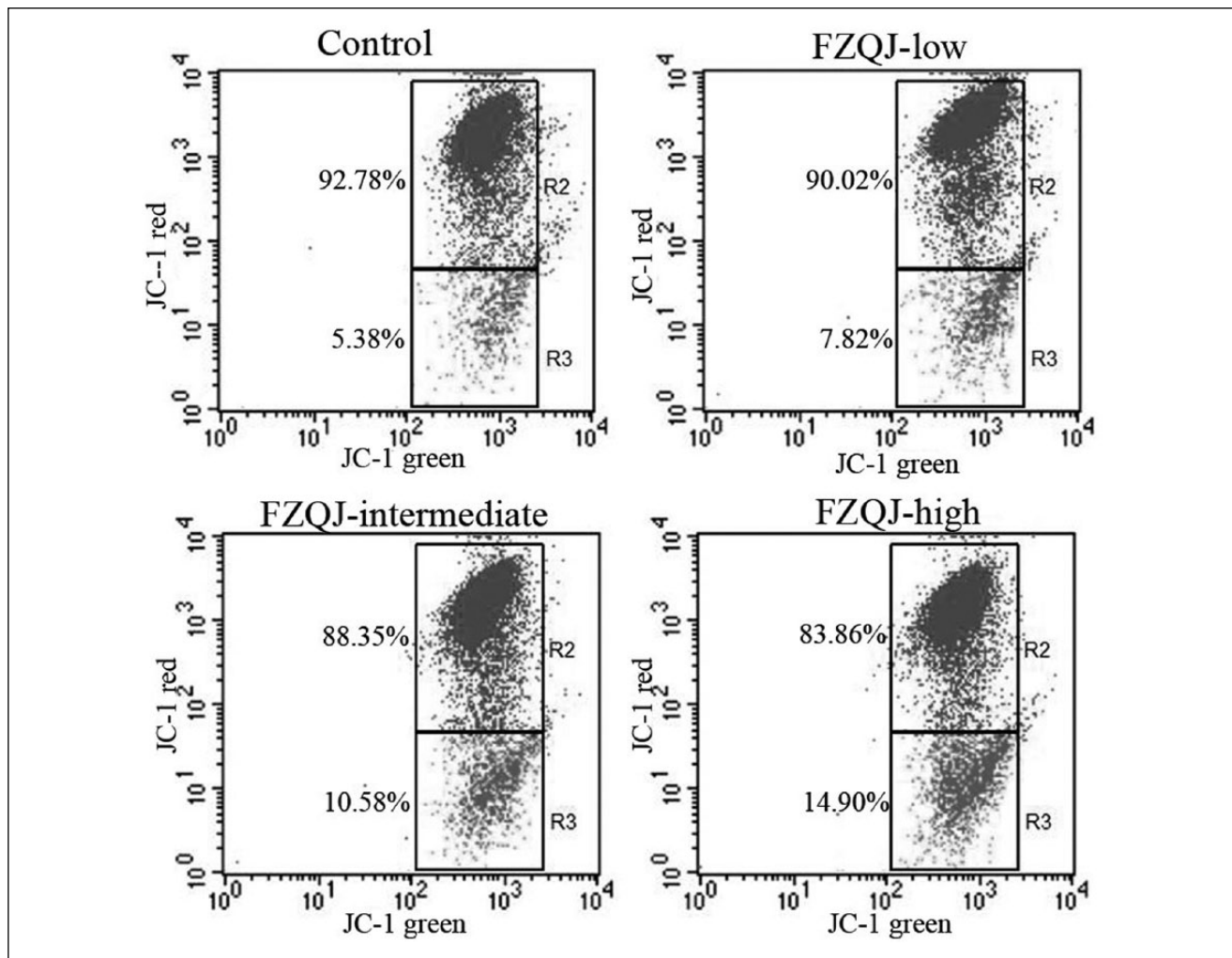
As shown above, FZQJ treatment could induce loss of  $\Delta\psi$  in vitro. Here we further investigated the expressions of cytoplasm cytochrome *c*, active caspase 9 and 3 as well as cleaved PARP, which are key molecules responsible for mitochondria mediated apoptosis by Western blot in tumor tissues. As shown in Figure 5A and Table 2, FZQJ remarkably increased the levels of cytoplasm cytochrome *c*, activated caspase 9 and 3, and increased the cleavage of PARP. In addition, we also investigated the expressions of anti-apoptotic Bcl-2 and

pro-apoptotic Bax, both of which are critical in mitochondrion-dependent apoptosis. The expressions of Bcl-2 and Bax were evaluated by RT-PCR and Western blot, respectively. As shown in Figure 5B, compared with the vehicle group, FZQJ remarkably decreased Bcl-2 mRNA and increased Bax mRNA expression. Similar results were seen in Western blot analysis (Figure 5C). These results showed that FZQJ indeed could induce mitochondria mediated apoptosis.

### FZQJ Regulated the Cellular Immunity of H22-Bearing Mice

To evaluate the effect of FZQJ on the cellular immunity of H22-bearing mice, the subpopulations of lymphocyte cells, thymus index, serum IL-2 and TNF- $\alpha$  levels and expression of CD69 in tumor tissue were examined.

As expected, the percentage of CD4<sup>+</sup> T cells and ratio of CD4<sup>+</sup>/CD8<sup>+</sup> T cells in the vehicle group were notably lower than that of the normal group, which could be reversed by FZQJ. In addition, the percentage of NK cells was also increased in the FZQJ-treated mice (Table 1). The thymus index of the mice also increased when treated with FZQJ, consistent with the change tendency of lymphocyte cells (Figure 6A).



**Figure 3.** The effect of Fuzheng Qingjie (FZQJ)-containing serum on the loss of  $\Delta\psi$  in vitro. HepG2 cells were treated with FZQJ-containing serum or control serum. The loss of  $\Delta\psi$  was measured by a flow cytometer with JC-1 staining. R2 region represents intact cells and R3 region indicates a depolarized  $\Delta\psi$ .

Next CD69 expression in tumor tissue was investigated as a marker of the early activation of T lymphocytes. As shown in Figure 5A and Table 2, CD69 expression in the tumor tissue in the FZQJ group was significantly increased compared with the vehicle control group, indicating that T lymphocytes were activated by FZQJ. We further evaluated serum levels of IL-2 and TNF- $\alpha$ , which are involved in the regulation of apoptosis and immunity.<sup>17,18</sup> No significant difference was observed in the levels of IL-2 among the 3 groups. But the level of TNF- $\alpha$  was highest in the FZQJ group (Figure 6B).

The above results showed that FZQJ could improve the cellular immune function of the mice via stimulating T lymphocytes activation.

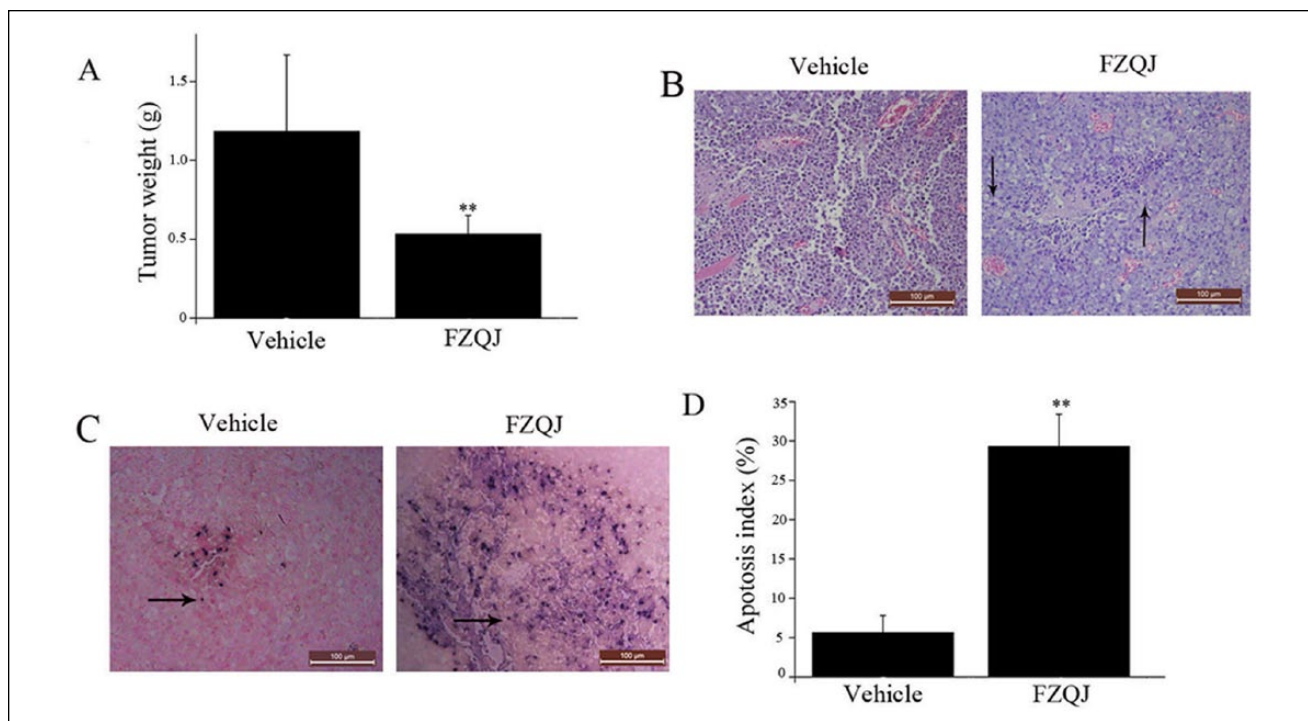
#### *FZQJ Has No Overt Toxicity in Kidney and Liver*

As shown in Table 3, no significant difference was observed among the 3 groups in terms of hepatic and renal function.

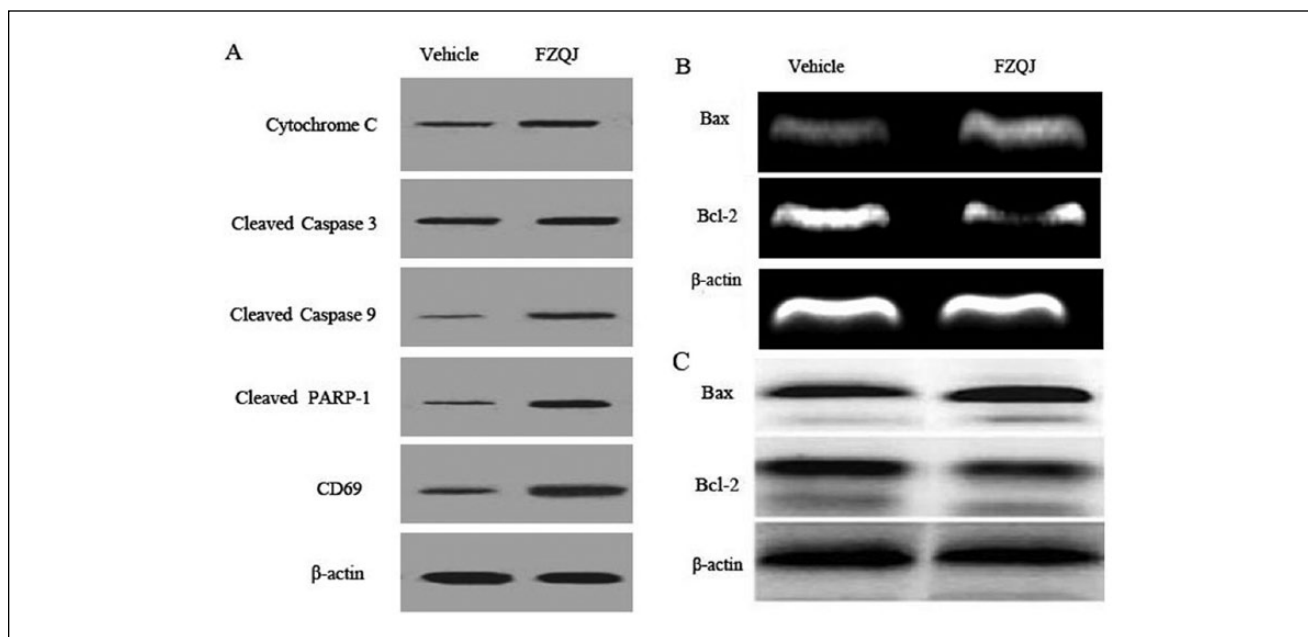
## Discussion

FZQJ granules have been used as an adjuvant therapy for alimentary tract cancers for a decade. The present study showed that FZQJ-containing serum remarkably inhibited proliferation of HepG2 cells in dose- and time-dependent manners and induced HepG2 cells apoptosis. In addition, FZQJ increased the percentages of CD4<sup>+</sup> T and NK cells, the ratio of CD4<sup>+</sup>/CD8<sup>+</sup> T cells as well as the level of TNF- $\alpha$  in the serum of H22 tumor-bearing mice. Thus FZQJ granules can boost the host's immune function, which is described as Fuzheng in TCM.

According to the practice of TCM, *Astragalus membranaceus* and *Ligustrum lucidum* are capable of tonifying Qi and nourishing Yin. *Ganoderma lucidum* and *Rhizoma dioscorea* can supplement vital energy and promote circulation of Qi, which will further strengthen the therapeutic efficacy of *Astragalus membranaceus* and *Ligustrum lucidum*.



**Figure 4.** Fuzheng Qingjie (FZQJ) granules inhibit the growth and induce the apoptosis of subcutaneous H22 tumor. Tumor xenograft weight at sacrifice was examined to evaluate the effects of FZQJ granules on the growth of tumor. Hematoxylin and eosin (HE) staining and TUNEL assay were performed to assess the apoptotic cells in neoplastic tissue. (A) Comparison of tumor weight in the vehicle and the FZQJ group. (B) HE staining of the xenograft (magnification ×200). The H22 cell which appeared karyopyknosis and vacuoles was marked by arrows. (C) TUNEL assay (magnification ×200). The positive cells were marked by arrows. (D) Apoptosis index was calculated by dividing the number of TUNEL-positive cells by the total number of cells in the field. \*\* $P < .01$ , compared with the vehicle.



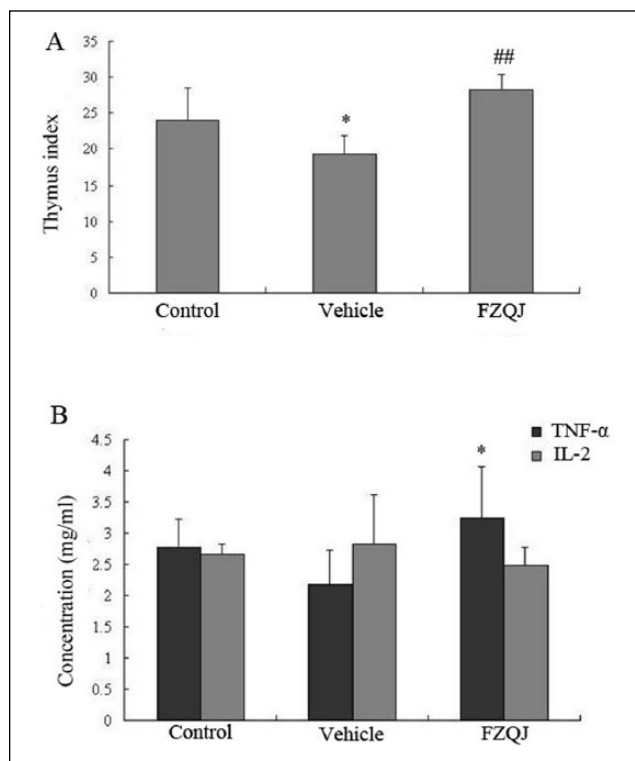
**Figure 5.** Fuzheng Qingjie (FZQJ)-induced mitochondria mediated apoptosis of subcutaneous H22 tumor is regulated by Bcl-2 family. The levels of cytochrome c, cleaved caspase 9 and 3, CD69 and cleaved PARP were analyzed by Western blot (A). The levels of Bax and Bcl-2 were analyzed by (B) reverse transcription-polymerase chain reaction (RT-PCR) and (C) Western blot.

**Table 1.** Percentage of Lymphocyte Cells in the Peripheral Blood of H22 Tumor-Bearing Mice.

Group	Percentages of Lymphocyte Cells (%)				
	CD3 <sup>+</sup>	CD4 <sup>+</sup>	CD8 <sup>+</sup>	NK	CD4 <sup>+</sup> /CD8 <sup>+</sup>
Normal	55.48 ± 5.41	42.88 ± 3.24	12.59 ± 3.02	8.96 ± 2.20	3.53 ± 0.64
Vehicle	51.95 ± 8.59	34.33 ± 6.92 <sup>a</sup>	13.69 ± 2.48	7.77 ± 2.73	2.58 ± 0.73 <sup>b</sup>
Fuzheng Qingjie (FZQJ)	52.30 ± 4.21	39.79 ± 2.29 <sup>b,c</sup>	11.91 ± 2.92	10.05 ± 0.85 <sup>c</sup>	3.51 ± 0.83 <sup>c</sup>

<sup>a</sup>P < .01 versus the normal group.<sup>b</sup>P < .05 versus the normal group.<sup>c</sup>P < .05 versus the vehicle group.**Table 2.** The Related Protein Expression of Cytochrome c, Cleaved Caspase 9 and 3, CD69, Cleaved PARP, Bax, and Bcl-2 Compared With β-Actin.

Group	Intensity of Protein Expression/β-Actin						
	Cytochrome c	Cleaved Caspase 9	Cleaved Caspase 3	Cleaved PARP	CD69	Bax	Bcl-2
Vehicle	0.28 ± 0.01	0.22 ± 0.02	0.41 ± 0.01	0.48 ± 0.05	0.30 ± 0.01	0.59 ± 0.03	0.77 ± 0.03
Fuzheng Qingjie (FZQJ)	0.42 ± 0.03 <sup>a</sup>	0.40 ± 0.04 <sup>a</sup>	0.49 ± 0.01 <sup>a</sup>	0.24 ± 0.03 <sup>a</sup>	0.55 ± 0.06 <sup>a</sup>	0.92 ± 0.01 <sup>a</sup>	0.45 ± 0.03 <sup>a</sup>

<sup>a</sup>P < .01 versus the vehicle group.**Figure 6.** Fuzheng Qingjie (FZQJ) granules regulate the immunity of H22-bearing mice. (A) Thymus index. Thymus index was calculated according to the following formula: thymus index = thymus weight (mg)/body weight (g) × 10. \*P < .05, compared with the control group and ##P < .01, compared with the vehicle group. (B) Levels of interleukin (IL)-2 and tumor necrosis factor (TNF)-α in sera by radioimmunoassay. \*P < .05, compared with the vehicle group.

*Hedyotis diffusa* Willd and *Prunella vulgaris* have abilities of clearing heat and toxin, eliminating cancer pain and inhibiting tumor growth.<sup>19,20</sup> Therefore, the combination of the 6 herbs can not only inhibit cancer growth more efficiently but also alleviate the adverse effects of chemoradiotherapy.<sup>21</sup>

Pharmacological studies have demonstrated that *Astragalus membranaceus*, *Ligustrum lucidum*, *Ganoderma lucidum*, and *Rhizoma dioscorea* contain potent immune stimulants which stimulate the production of many cytokines in vivo, such as IL-2, IL-12 and TNF-α.<sup>22-25</sup> *Hedyotis Diffusa* Willd and *Prunella vulgaris* have also been found to be effective in inducing cancer cell apoptosis and metastasis, and suppressing angiogenesis.<sup>12-14</sup>

Major histocompatibility complex (MHC) class II molecules present epitopes to T cell receptors of CD4<sup>+</sup> T cells. CD4<sup>+</sup> T helper cells secrete cytokines (such as IL-2, IL-4, and TNF-α) that activate T and B cells, and help other cells recognize tumor antigens.<sup>26,27</sup> MHC class I molecules present epitopes to T cell receptors of CD8<sup>+</sup> T cells, causing activation of CD8<sup>+</sup> T cells which express both TNF-related apoptosis-inducing ligand (TRAIL) and granzyme B to induce apoptosis of cancer cells.<sup>28,29</sup> Normally, the ratio of CD4<sup>+</sup>/CD8<sup>+</sup> keeps a dynamic balance. Low CD4<sup>+</sup>/CD8<sup>+</sup> ratio is associated with immune suppression.<sup>30</sup> NK cells are capable of killing tumor cells via antibody-dependent cellular cytotoxicity (ADCC).<sup>31</sup> However, immune deficiency is well documented in patients with malignant tumors, with decreased numbers of CD4<sup>+</sup> T cells and NK cells, decreased serum level of TNF-α and reversal of CD4<sup>+</sup>/CD8<sup>+</sup> ratio. Pharmacological studies have demonstrated that many herbs can increase the numbers of these cells. For example,



**Table 3.** Hepatorenal Toxicity of Normal, Vehicle, and FZQJ.

Group	ALT (IU/L)	AST (IU/L)	BUN (mmol/L)	CRE ( $\mu$ mol/L)
Normal	45.25 $\pm$ 9.36	223.75 $\pm$ 23.14	7.73 $\pm$ 1.52	18.53 $\pm$ 2.10
Vehicle	51.50 $\pm$ 6.76	211.00 $\pm$ 24.26	7.50 $\pm$ 1.78	20.00 $\pm$ 3.17
FZQJ	50.75 $\pm$ 10.14	220.00 $\pm$ 25.97	6.55 $\pm$ 1.18	16.85 $\pm$ 2.09

Abbreviations: FZQJ, Fuzheng Qingjie; ALT, alanine transaminase; AST, aspartate transaminase; BUN, blood urea nitrogen; CRE, creatinine.

in a randomized controlled trial, *Ganoderma lucidum* was shown to increase the percentages of CD3<sup>+</sup>, CD4<sup>+</sup>, NK-cell activity, as well as CD4<sup>+</sup>/CD8<sup>+</sup> ratio in cancer patients.<sup>32</sup>

Our data showed that FZQJ granules could raise the levels of CD4<sup>+</sup> T helper cells and NK cells, indicating that FZQJ might stimulate T lymphocytes activation to fight cancers via increasing CD4<sup>+</sup> T helper cells. In addition, FZQJ also could improve NK cells level to directly kill tumor cells. In order to further verify if T lymphocytes were activated by FZQJ, CD69 was detected using Western blot. CD69 expression will increase after T lymphocyte activation. As expected, we found FZQJ could significantly increase CD69 expression, indicating that T lymphocytes were activated by FZQJ.

Mitochondria-mediated apoptosis begins with the permeabilization of the mitochondrial outer membrane. On apoptotic stimuli, cytochrome *c* and other pro-apoptotic factors are released from intermembrane space into the cytosol, which promote proteolytic cleavage of procaspase 9 and 3. Subsequently, endogenous caspase 3 substrate PARP is cleaved. These events finally lead to inevitable apoptotic cell death.<sup>33</sup> The process is governed by pro- and anti-apoptotic members of Bcl-2 family.<sup>34</sup> In the present study, we found that FZQJ granule could induce loss of  $\Delta\psi$ , promote release of cytochrome C, activate procaspase 9 and 3, promote PARP cleavage, increase the expression of Bax, and decrease that of Bcl-2, indicating that FZQJ induces the apoptosis of hepatoma cells at least via mitochondrial mediated apoptotic pathway. Most important, we investigated the effect of FZQJ granule on apoptosis of the HepG2 cells in vitro by the serum pharmacology method. As we know, the compositions of herbs are quite complex, especially Chinese compound decoctions. Biotransformation in the gastrointestinal tract is needed for some herbs to exert their therapeutic action.<sup>35</sup> Therefore it is believed that the application of serum from herb(s)-treated animals, so-called “serum pharmacology,” can more truly simulate the in vivo therapeutic effects.<sup>36</sup> The in vitro results showed that FZQJ could induce the apoptosis of HepG2 cells via mitochondria-dependent apoptotic pathway, which was verified in animal experiments.

In conclusion, FZQJ granules can not only enhance host immunity but also induce mitochondria-mediated apoptosis of hepatoma. FZQJ granules might be a promising adjuvant treatment against hepatoma.

### Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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