

## **Effects of Kanglaite on Apoptosis and Apoptotic Protein Procaspase-3 and Capase-9 Expression in Hepatic Carcinoma BEL-7404 Cells**

Currently, surgery is a major approach for curing PHC. However, only 25% of the cases can be treated by surgical resection. The 5-year survival rate for patients after being resected with focus is only around 40%. Research shows that the main reasons influencing the curative effect are transfer and relapse. Thus, traditional Chinese medicine, as an auxiliary approach for comprehensively treating hepatic carcinoma has increasingly been valued and acknowledged. KLT boasts wide biological effect and apparent anti-tumor effect. Procaspase-3 and capase-9 are key regulatory proteins in apoptosis. The research aims at examining the effects of KLT on inhibiting the proliferation and inducing apoptosis of human liver cell strain BEL-7404, and discussing the action mechanism of inducing apoptosis through the influence on procaspase-3 and capase-9 expression.

### **1. Materials and Method**

#### **1.1 Reagent**

Hepatic carcinoma BEL-7404 cell line is presented by the Medical Research Center of the First Affiliated Hospital of Xinjiang Medical University. KLT injection is produced by Zhejiang Kanglaite Pharmaceutical Co., Ltd. DMEM culture solution is manufactured by American Gbco Corporation. FCS is produced by Hangzhou Sijiqing Biological Engineering Materials Co., Ltd.. MTT is supplied by American Amresco Corporation. Hoechst 33258 stain is provided by American Sigma Corporation. Rabbit anti human procaspase-3 Pab, rabbit anti human casepase-9 Pab, HRP mark sheep anti rabbit antibody and DAB developing kit are all produced by Beijing Zhongshan Goldenbridge Biotechnology Co., Ltd.. Annxien-V and PI double staining kit is produced by American BM Corporation. CO<sub>2</sub> incubator is the product of German FORMA Corporation. The invert fluorescence microscope is provided by Japanese Olympus Corporation. Leica DC200 image acquisition system is produced by Leica Corporation. Half dry transfer printing instrument and ELIASA detector are produced by Biorad Corporation.

#### **1.2. Method**

##### **1.2.1 Cell culture**

BEL-7404 cells are cultured in DMEM culture solution including 10% FCS and 0.1% NaHCO<sub>3</sub>, 100mg/mL Gln and penicillin and SM of 1,000,000u/L respectively. Place it in the incubator with CO<sub>2</sub> volume fraction of 5% at 37°C for culture. Take cells at exponential phase for experiment. During that time, add KLT injection into fresh culture solution and adjust the concentration of medicine volume for later experiment.

##### **1.2.2 Detect the inhibitory rate of cell proliferation with MTT**

Digest BEL-7404 cells at exponential phase with pancreatin, and prepare into monoplast suspension. The cell density is adjusted into  $5 \times 10^4$ /mL. Inoculate at 96-pore culture plate, with each pore of 200 $\mu$ L. After the cells enter exponential phase, add KLT injection of different volume concentrations to make the KLT injection achieve volume concentration of 10, 20, 40, 80 and 160 $\mu$ L/ mL. Each group is set with 4 multiple pores. Repeat the experiment for 3 times for each; Set DDP as positive control group (mass concentration: 10 $\mu$ L/ mL). Solvent control group

and blank control group are negative control. After being cultured for 24, 48, 72 and 96 h, add 5mg/mL MTT solution of 20 $\mu$ L into each pore, and continue to culture for 4 h. Absorb all the culture solution in the plates. Add DMSO solution of 150 $\mu$ L into all pores, and oscillate for 10 min. Determine the absorbance ( $D$ ) value of each pore at ELIASA wavelength of 570nm. Calculate pursuant to formula, the inhibitory rate of cell growth (%)=  $(1 - D_{\text{experimental group Value}} / D_{\text{control group Value}}) \times 100\%$ .

### 1.2.3 Observe apoptosis with Hoechst33258 fluorescent staining

Put the cover glass (1 cm<sup>2</sup>) in the 6-pore culture plate. Take BEL-7404 cells at exponential phase. The cell density is adjusted into 5 $\times 10^4$ / mL. Inoculate at 6-pore culture plate, with each hole of 2mL. Set blank control group, positive control group DDP 10 $\mu$ g/ mL group and KLT injection 80 $\mu$ L/ mL. After function for 48 h, fix the cells for 10 min with 2% glutaraldehyde solution, and rinse the cells with PBS buffer solution for 3 times. Finally, drop Hoechst33258 for staining for 5 min, and place under fluorescence microscope at wavelength of 450nm for observation and recording.

### 1.2.4 Detect apoptosis with FCM

Take BEL-7404 cells at exponential phase, and prepare into monoplast suspension. The cell density is adjusted into 5 $\times 10^4$ / mL. Inoculate at culture flask of 25 mL, with each flask of 5 mL. After the cells enter exponential phase, set blank control group, positive control group DDP 10 $\mu$ g/ mL group and KLT injection 80 $\mu$ L/ mL. Repeat 3 flasks for each group. After culturing for 48 h, regularly collect cells; rinse the cells with PBS buffer solution for 3 times. The cell density is adjusted into 1 $\times 10^6$ / mL. Add Annexin-V of 5 $\mu$ L and PI of 10 $\mu$ L in each flask respectively. Keep in dark place at 4 $^{\circ}$ C for 5 and 10 min respectively, and immediately detect with up-flow coulter.

### 1.2.5 Detect expression of procaspase-3 andcaspase-9 with Western blot analysis

Digest BEL-7404 cells at exponential phase with pancreatin. The cell suspension density is adjusted into 5 $\times 10^4$ / mL. Inoculate cells of 2 mL for each pore. Set blank control group, positive control group DDP 10 $\mu$ g/ mL group and KLT injection 80 $\mu$ L/ mL. After treating for 48 h, collect the cells. Extract protein after treating cells with cell lysis solution. Add equal volume of loading buffer, and mix well. Boil for 10 min, and immediately separate protein with 12% SDS-PAGE. Transfer the separated protein to nitrocellulose membrane with half dry transfer membrane instrument. Transfer with 5% skim milk powder in an enclosed way. Add rabbit anti human procaspase-3 and caspase-9 Pab respectively (dilute as per 1:250). Place on table react over night at 4 $^{\circ}$ C. Wash film and add HRP (dilute as per 1:2500). React at room temperature for 1.5 h. Then develop with DAB, and analyze after gray scan. Products relative intensity expression= Total light density value of the product/ Corresponding  $\beta$ -tubulin total light density value.

### 1.2.6. Statistical method

SPSS13.0 statistics software is introduced to make statistical analysis. The metering data is expressed with  $\bar{x} \pm s$ . Analyze through One-way ANOVA. If the variances are not even, look for similar  $F$  value by means of Welch method. If the variances are even, detect by  $LSD$  method. It is statistically significant if the difference  $P < 0.05$ .

## 2. Result

### 2.1 KLT injection has inhibitory effect on BEL-7404 cells

After KLT injection of different volume concentrations (10, 20, 40, 80 and 160  $\mu\text{L}/\text{mL}$ ) and DDP (10  $\mu\text{g}/\text{mL}$ ) function on BEL-7404 cells for 24, 48, 72 and 96 h, the inhibitory effect on cell proliferation is significant. The inhibitory effect of KLT injection at 80  $\mu\text{L}/\text{mL}$  is the best. The inhibitory rates are (29.94 $\pm$ 0.87)%, (36.73 $\pm$ 0.63)%, (34.36 $\pm$ 0.91)% and (10.11 $\pm$ 0.57)% respectively, while that of 60  $\mu\text{L}/\text{mL}$  group are (33.21 $\pm$ 0.94)%, (30.41 $\pm$ 1.01)%, (21.57 $\pm$ 0.81)% and (16.45 $\pm$ 0.68)%. Therefore, later research is based on the experimental condition that KLT injection of 80  $\mu\text{L}/\text{mL}$  functions for 48 h (Fig. 1). Similarly, the positive control DDP 10  $\mu\text{g}/\text{mL}$  exerts significant inhibitory effect on BEL-7404 cells. The inhibitory rates of DDP 10  $\mu\text{g}/\text{mL}$  group at 24, 48, 72 and 96 h are (35.64 $\pm$ 0.13)%, (58.63 $\pm$ 1.02)%, (81.02 $\pm$ 1.61)% and (85.93 $\pm$ 2.31)% respectively. Therefore, DDP 10  $\mu\text{g}/\text{mL}$  functioning for 48 h is adopted as the experimental condition in the research.

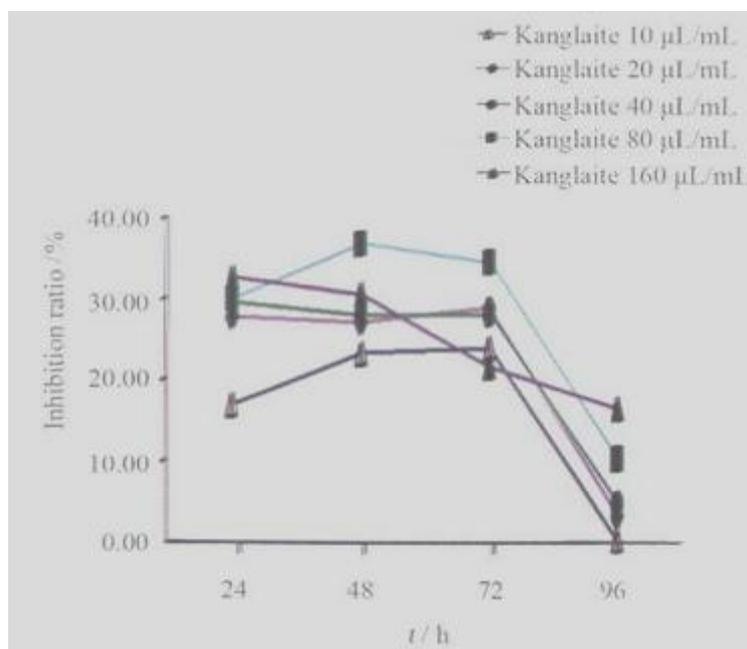


Fig. 1 Inhibitory Effects of Different Concentrations of Kanglaite Injection on BEL-7404 Cell Proliferation

### 2.2. Observe apoptosis with Hoechst33258 fluorescent staining

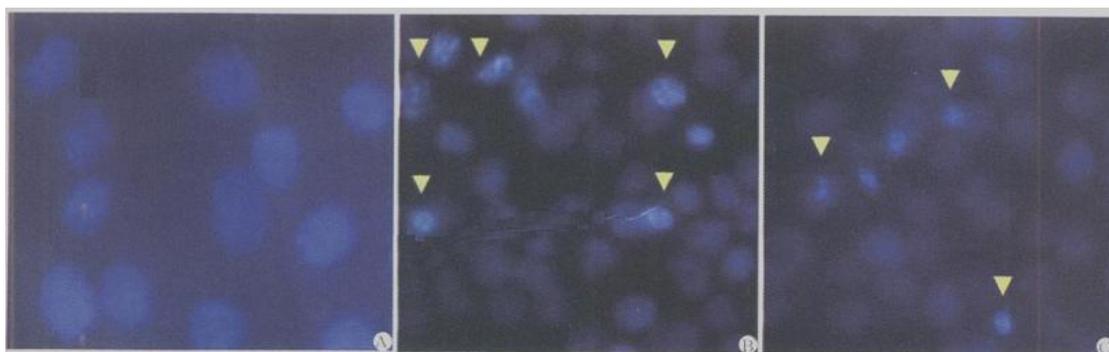
After DDP 10  $\mu\text{g}/\text{mL}$  and KLT injection of 80  $\mu\text{L}/\text{mL}$  function for 48 h, it can be observed under fluorescence microscope that the nucleus volume of partial BEL-7404 cells has been lessened and shrunk (see as arrows show), and has apoptosis. However, the nucleus fluorescence of blank control group is weaker, and the color is even.

### 2.3 Detect apoptosis with FCM

After DDP 10  $\mu\text{g}/\text{mL}$  and KLT injection of 80  $\mu\text{L}/\text{mL}$  function for 48 h, the apoptosis rate of BEL-7404 cells is apparently higher than that of negative control group, which are (1.23 $\pm$ 0.40)%, (32.53 $\pm$ 0.65)% and (3.13 $\pm$ 0.32)%. The  $F$  value is 4016.65. By comparing the three groups, the difference is significant statistically ( $P < 0.01$ ) (Fig. 3).

### 2.4 Detect expression of procaspase-3 and caspase-9 with Western blot analysis

After DDP 10 $\mu$ g/ mL and KLT injection of 80 $\mu$ L/ mL function for 48 h, The detection result through Western blot analysis indicates that the expression level of procaspase-3 in blank control group, DDP positive control group and KLT injection treatment group are 0.75 $\pm$ 0.01, 0.54 $\pm$ 0.03 and 0.65 $\pm$ 0.04 respectively. Compared with blank control group, the expression level of procaspase-3 in two medicine treatment groups have all decreased ( $F=4.568$ ,  $P<0.01$ ). By comparing the apoptosis rates between the positive control DDP 10 $\mu$ g/ mL group and KLT injection of 80 $\mu$ L/ mL group, the difference is significant statistically ( $P<0.01$ , Fig. 4).



A: BEL-7404 cells untreated; B: Treated with DDP (10 $\mu$ g/ mL); C: Treated with Kanglaite injection (80 $\mu$ L/ mL)

Fig. 2 Effects of Kanglaite Injection and DDP on BEL-7404 Cell Apoptosis Detected by Hoechst 33258 ( $\times 200$ )

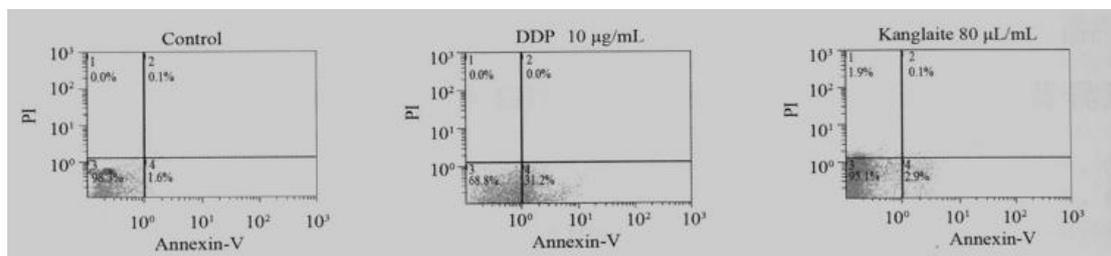


Fig. 3 Effects of Kanglaite Injection and DDP on BEL-7404 Cell Apoptosis Detected by FCM

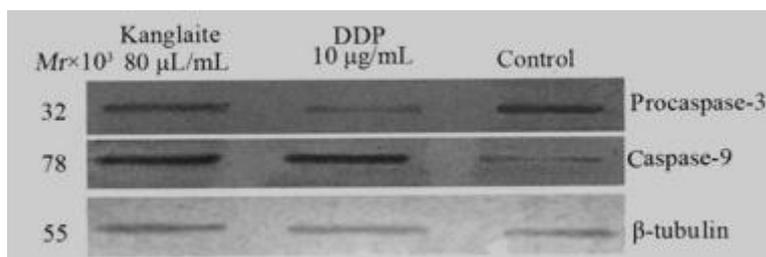


Fig. 4 The Protein Expression of Procaspase-3 and Caspase-9 in BEL-7404 Cells Detected by Western Blotting

### 3. Discussion

KLT injection is a fat emulsion extracted from traditional Chinese medicine coix seed, which boasts wide bioactivity, and can enhance cellular immune function and inhibit proliferation of cancer cells as well as induce apoptosis of cancer cells [1, 2]. Clinical application shows that KLT injection exerts significant curative effect on multiple primary malignancies such as lung cancer,

gastric cancer and breast cancer<sup>[3-5]</sup>. The research shows that it has significant inhibitory effect and inducing apoptosis on human hepatic carcinoma cell line BEL-7404. The detection result through MTT indicates that after treat with KLT injection of 80 $\mu$ L/ mL for 48 h, the inhibitory rate on proliferation of BEL-7404 cells is significantly higher than that of blank control group; Hoechst33258 fluorescent staining reveals typical apoptotic morphological changes, i.e. the nucleus concentrates; the cell volume apparently lessened; and apoptotic body appears; FCM detection results indicate that the apoptosis rate of BEL-7404 cells after being treated with KLT injection is apparently higher than that of blank control group.

Caspase-3 is the key enzyme for apoptosis; procaspase-3 is its original form, which is a key effect enzyme molecule in apoptosis signal transduction pathway. It can activate endonuclease to degrade DNA, and thus lead to apoptosis<sup>[6]</sup>. After treating gastric cancer BGC-823 cell through ursolic acid, Zhang Jing et al<sup>[7]</sup> have discovered that with the increase of ursolic acid, caspase-3 activity will be strengthened to induce apoptosis of BGC-823 cells. Caspase-9 plays a critical role in inducing apoptosis of death receptor signal pathway. When the death receptor on the surface of cell membrane combines with corresponding ligand, caspase-9 as the first apoptosis protein is activated. Caspase-3 protein at downstream will be activated next to finally induce apoptosis<sup>[8]</sup>. Tan Jie et al<sup>[9]</sup> have found that under the function of ursolic acid, the expression of caspase-9 protein in colon cancer HT-29 cell will be enhanced to further activate the apoptosis at downstream. Finally the apoptosis rate will be increased. In the research, it can be discovered by Western blot analysis that the expression level of procaspase-3 in BEL-7404 cells has been significantly reduced after being functioned by KLT injection of 80 $\mu$ L/ mL while the expression level of caspase-9 has been increased. Therefore, it can be inferred that KLT injection has the effect of inducing apoptosis of human hepatic carcinoma cell line BEL-7404 by increasing the expression level of caspase-9; further activating caspase-3 protein at downstream; while the decrease of procaspase-3 protein may be caused by the activation from caspase-9 to procaspase-3 in caspase at upstream so that the expression of caspase-3 has been increased, and further led to apoptosis of BEL-7404 cells.

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