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Effect of Quercetin on Breeding and Apoptosis of Cervical Cancer HeLa Cell and on Growth of Transplanted Tumor in Nude Mice

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Abstract: Effect of quercetin on HeLa cell system of cervical cancer was studied by methods of MTT and Annexin V-FITC/PI. The results show that quercetin has functions of inhibiting breeding of HeLa cells and inducing apoptosis of the cells. The total apoptosis rate is positively proportional to reaction duration and concentration of quercetin used. The maximum apoptosis rate being (88.76±2.35)% was obtained when the concentration was 50.0 μmol/L and the cells were treated with quercetin for 72 hours. Based on establishing a model of tumor of cervical cancer transplanted into nude mice, quercetin of different concentrations was injected into abdominal cavity of nude mice and situation of tumor growth was reviewed. The result showed that with quercetin concentration increasing from 0 to 100.0 μmol/L, the transplantation volume and weight of the tumors decreased from (279.59±70.58) mm^3 and (0.145±0.019) g to (128.72±36.12) mm^3 and (0.089± 0.019) g respectively, while apoptosis rate of the transplanted tumor increased from (9.63±1.85)% to (34.98±0.47)%, which proved that quercetin inhibited increment of volume and weight of transplanted tumor in nude mice bodies.

Key words: quercetin; HeLa cells; nude mice
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0 Introduction

Being a common flavonoid material, quercetin has many functions such as to dilate coronary artery, reduce blood fat, anti-platelet agglutination, prevent complication of diabetes, etc. It has been found in recent years that quercetin has a function to inhibit growth of various malignant tumors[1] such as human prostate cancer cells[2], various carcinoma gastric cancer cells, colon carcinoma cells[3], pheochromocytoma[4], mammary cancer cells[5] and leucocythemia cells[6], etc.Quercetin is capable of reducing duplication of DNA to lead to postpone of cell splitting and to retard various tumor cells within different cell cycles. Growth of human gastric cancer cells and colon carcinoma cells are retarded at the border between G1/S, and human leucocythemia HL60 cell system[7] and human lung cancer cell system (MCF-7 cells) are retarded within the G2/M cycle[8]. Inhibition rate (IR) is positively proportional to dosage of quercetin. Quercetin can also extend survival period of mice in the group, in which each mouse has been inoculated with NK/LY physical tumor at ear portion, and inhibit the tumor growing in mice due to hypodermic inoculation of S180 cells[9]. An *in vitro* test was adopted in this study, which investigated and found that quercetin inhibited breeding process of cervical cancer HeLa cells and induced their apoptosis. A model of transplanted tumor of nude mice was set up, with which the inhibitive function of quercetin upon growth of transplanted tumor in nude mice suffering cervical cancer.

1 Materials and Methods

1.1 Materials for Experiment

Human Cervical Cancer HeLa strain was granted by Wu Xinxing, professor of School of Medicine of Wuhan University. 26 female nude mice classified as BALB/C nu/nu SPF and never experienced mating with average body weight 15-17 g (permission certificate number SCXK(Hubei)2003-0005) was adopted for this experiment; culture medium RPMI-1640 was purchased from Gibco Company; calf serum was purchased from Sijiqing Company of Hangzhou;Quercetin powder was purchased from Chaoren Zhihua Company of Chengdu (lot number 05062). Dimethyl sulfoxide (DMSO) of 0.1% concentration was formulated to be 20 mmol/L solution to be preserved for later use. MTT was purchased from Zhongshan Company of Beijing. A reagent kit for detection of dying Annexin V-FITC/PI was purchased from Bender Medsystem Company. An inverted microscope (Olympus IX51-A11PH) was equipped for this experiment.

1.2 Methods

1.2.1 Cytological experiment

① Culture of cells

HeLa cells were cultured with a RPMI-1640 culture medium that contains 10% calf serum at 37 ℃ in an atmosphere of 5% CO_2. The cells reproduced every generation within 2 or 3 days and were divided into the following 5 groups:

Group A: RPMI-1640 culture solution (for contrast); Group B: RPMI-1640 culture solution plus 0.1% DMSO (DMSO Group); Group C: RPMI-1640 culture solution plus 12.5 μmol/L Quercetin (to be dissolved in 0.1% DMSO); Group D: RPMI-1640 culture solution plus 25.0 μmol/L Quercetin (to be dissolved in 0.1% DMSO); Group E: RPMI-1640 culture solution plus 50.0 μmol/L Quercetin (to be dissolved in 0.1% DMSO).

② Morphological observation

The above-mentioned groups, after being cultured for 24, 48, 72 hours respectively, were observed for their cell morphology under a microscope.

③ Detection of cell activity

Adjust the HeLa cells to have a concentration of 5×10^5 pieces/mL, with each hole capacity being 200 μL and then inoculate them onto 96 pore plates. Each group was added in with quercetin of different concentrations and to be cultured for 24, 48, and 72 hours respectively. The culture solution was then abandoned and 20 μL MTT was added into each pore to incubate. for 4 hours, again 150 μL 0.1%DMSO was added in, then DMSO contrast group and blank contrast group were established. An instrument named enzyme-linked immunization assay (ELISA) was used to detect the value of absorbency (A) of each group. This experiment was performed repeatedly for three times.

Cell inhibition rate (IR_1) was calculated according to the following formula:

$$IR_1 = (1 - A/A_0) \times 100\%$$

Where A_0 is absorbency of group for comparing (DMSO Group).

④ Detecting cell apoptosis rate with annxin V-FITC/PI Technology

Quercetin of various concentrations was added into each experimental group, which was then cultured for 24, 48 and 72 hours and then digested with 0.25％trypsinase Use Annexin V-FITC/PI to detect the dying rate of the cells, from which the result was analyzed with Cell Quest software.

1.2.2 Animal experiment

① Establishment of transplantation tumor model in nude mice

Take HeLa cells that were kept in logarithm growing period (adjust the concentration to 0.8×10^8-1.0×10^8 pieces per mL), then inoculate them hypodermic into mice on the back.

② Groups of experiment

When the tumors grew to 40-80 mm^3 of size, a tumor of one of the tumor-bearing nude mice was taken to be verified pathologically. 25 matured nude mice were divided into 5 groups, 5 for each, among which 4 groups (for curing) was injected with quercetin into abdominal cavity with different dosages of 12.5, 25, 50 and 100 $mg.kg^{-1}.d^{-1}$, for the contrast group, 0.1%DMSO was injected for 15 days continuously. Tumor size was measured every three days for maximum diameter (a), minimum diameter (b) and body weight of he nude mice body. Volume of tumor was calculated according to the formula: $V=0.5ab^2$[10]

③ Calculation of tumor inhibiting rate

The nude mice were killed three days after the final dosage was given. The transplanted tumors were peeled off and weighed. The tumor-inhibition rate (IR_2) was calculated according to the following formula:

$$IR_2 = (1 - m/m_0) \times 100\%$$

Where m, m_0 are average tumor weight of group for curing and group for comparing.

④ Calculation for rate of cell apoptosis

Shear the tumor of tumor-bearing mice into pieces and flush with PBS solution repeatedly, and then separate the cervical cancer cells by rubbing with nets. The cell apoptosis rate was detected by Annexin V-FITC/PI method.

⑤ Pathological inspection

Slices for pathological inspection were cut from liver, kidney, spleen, groin and axillary lymph node of mice.

1.2.3 Statistics analysis

The experiment result is expressed with the formula, $\bar{x} \pm s$, which is then processed by SPSS11.5 software.

2 Results and Discussion

2.1 Effect of Quercetin on Breeding Activity and Apoptosis of HeLa Cells

2.1.1 Morphology observation of heLa cells

① The normal HeLa cells (group A) appear to be polygons, elliptical, closely aligned and grow well(Fig.1).

② Group B (DMSO), having been cultured for 24, 48 and 72 hours respectively, no significant morphological change of cells was found.

③ Having been treated with quercetin for 24 hours, Group C and group D don't seem significant morphological change either; while in group E a little quantity of cells contracted and became round-shaped to float on cell surface and a 'plasma membrane blebbing' phenomenon appeared; the same phenomenon appeared in groups C and D as well, various groups treated with quercetin of different concentrations (from group C to group D) all had the phenomena that cells contract and became round-shaped, part of the cells floated and a small quantity of cells peeled off the cell wall and floated upward, of which the most significant was group E. Having been treated with quercetin for 72 hours, group C and group D had more cells in floating state, while in group E you could see that most of the cells were not sticking on cell wall with the cell quantity significantly reduced, even a lot of cell fragments might be seen (Fig.2).

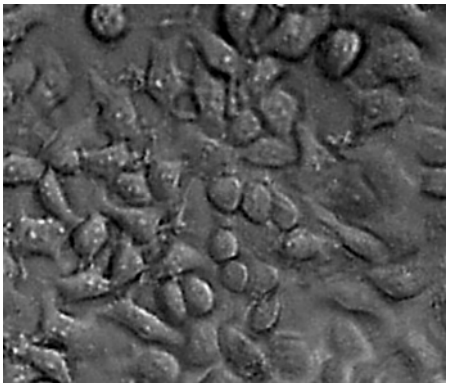

Fig.1 The normal HeLa cells

2.1.2 Effect of quercetin on breeding activity of heLa cells

Table 1 shows that HeLa cells in group B (DMSO group), after being cultured for 24,48 and 72 hours, breeding of cells had no significant change ($p > 0.05$), of which the concentration was between 12.5-50.0 μmol/L, while quercetin had a significant higher inhibition upon HeLa cells; on the other hand, the inhibition rate was positively proportional to the reaction time (see Table 1). The experiment result indicated that quercetin had significant inhibition function against the breeding activity of HeLa cells. The 24 hour 50% inhibiting concentration was obtained by means of regression equation analysis, valued 42.7 μmol/L.

2.1.3 Effect of quercetin on apoptosis of HeLa Cells

Figure 3 and Table 2 show that in group B (DMSO group), HeLa cells, having been cultured for 24,48 and 72 hours, gave no significant change of apoptosis rate, which explains DMSO had no effect on apoptosis of cells. In group C and D, whether along with increase of quercetin concentration or along with prolongation of culture, the total apoptosis rates of cells in both groups were significantly increased in either early period or late period. In Group E, along with prolongation of culture,

Table 1 Using MTT to detect the effect of quercetin on HeLa cell activity($\bar{x} \pm s$)

c(Qμer)/μmol • L^{-1}	24 h		48 h		72 h	
	A_{490}	IR_1 / %	A_{490}	IR_1 / %	A_{490}	IR_1 / %
blank	1.389±0.043	—	1.389±0.043	—	1.389±0.043	—
0.0	1.360±0.043	2.09	1.349±0.045	2.88	1.347±0.048	3.02
12.5	1.160±0.053**	16.49	0.873±0.030**	37.15	0.596±0.045**	57.09
25.0	0.978±0.033**	29.59	0.686±0.028**	50.61	0.340±0.036**	75.52
50.0	0.578±0.023**	58.39	0.240±0.022**	82.72	0.188±0.021**	86.47

Compared with DMSO group, * $p<0.05$ ** $p<0.01$ (F detection, SNK-q detection)

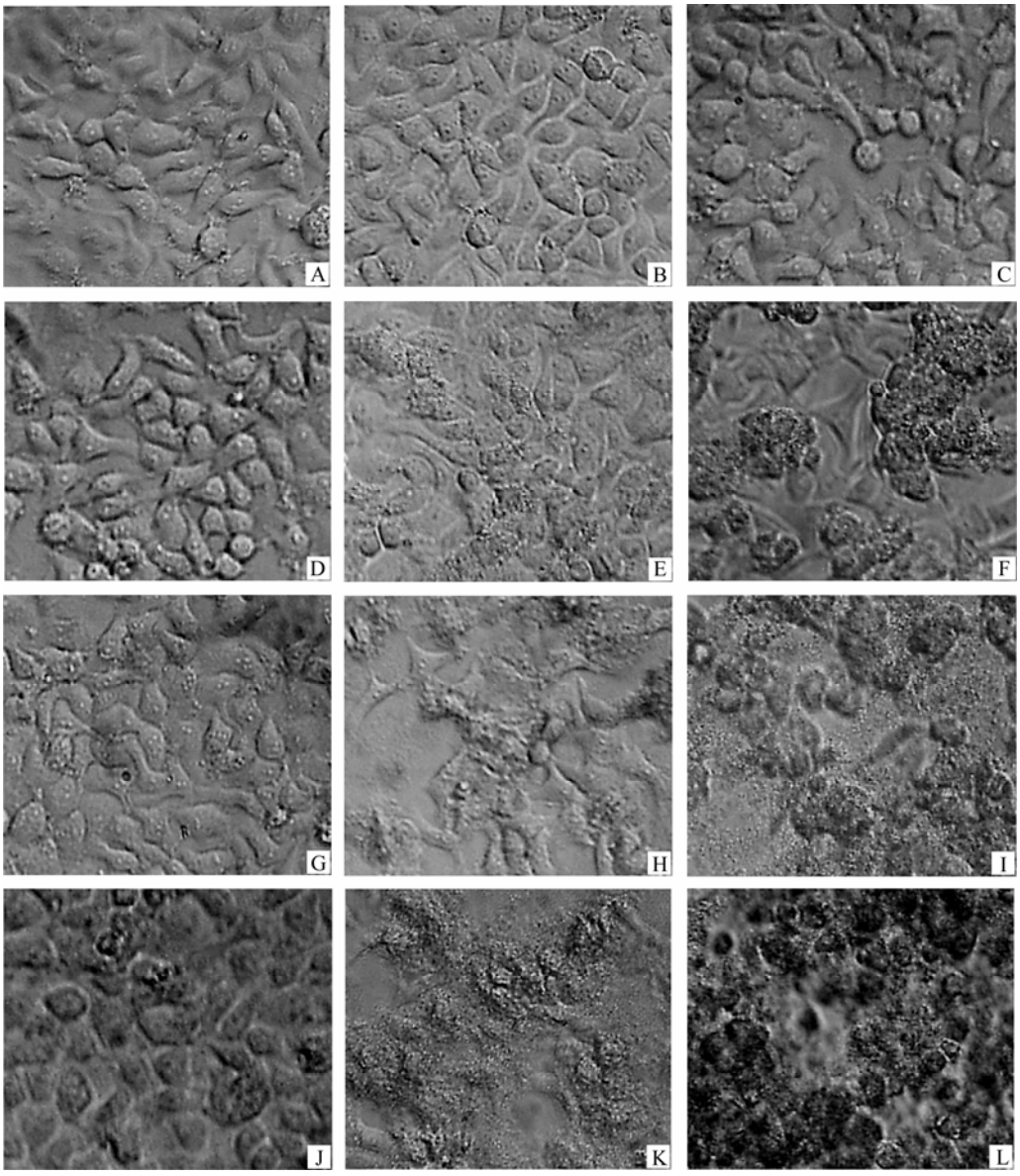

Fig.2 Morphology of cells in each group at different time

A.DMSO, 24 h; B.DMSO, 48 h; C.DMSO, 72 h; D. *c*(Qμer)=12.5 μmol/L, 24 h; E. *c*(Qμer)=12.5 μmol/L, 48 h; F. *c*(Qμer)=12.5 μmol/L, 72 h; G. *c*(Qμer)=25.0 μmol/L, 24 h; H. *c*(Qμer)=25.0 μmol/L, 48 h; I. *c*(Qμer)=25.0 μmol/L,72 h; J. *c*(Qμer)=50.0 μmol/L, 24 h; K. *c*(Qμer)=50.0 μmol/L, 48 h; L. *c*(Qμer)= 25.0 μmol/L, 24 h

the apoptosis rate decreases in early period but significantly increased in late period. This indicated that the cells already had a tendency of secondary necrosis when quercetin concentration is 50.0 μmol/L. This experiment shows that quercetin has an obvious in inductive function on apoptosis of HeLa cells.

2.2 Effect of Quercetin on Growth of Transplanted Tumor in Nude Mice

2.2.1 Circumstance of tumor-formation in nude mice and the morphological features

6-8 days after inoculation tumors began to grow one after another at the location where tumor cells had been inoculated. Tumor developed in all the 26 mice. The transplanted tumor appeared to be hemispheric, elliptical in shape or like lobulated leaves; expanding and building up and grew rapidly. The skin covered the transplanted tumor became red, inactive, on which fresh vascular can be seen obviously. The tumor reached up to 40-80 mm^3 in volume 22 days after inoculation. Took the tumor organ from a tumor-grown mouse to observe, it looked like fish flesh, hard, with cross section gray in color, around which gemmation-shaped tumor nodules could be seen (see Fig.4). The transplanted tumor of the nude mice appeared to adenocarcinoma with low differentiation (see

Fig.5). There was fibrous tissue covering around outside the tumor, which was extending into the tumor body. The tumor cells formed a glandular nest structure, which, compared with normal cells, appeared to be of polytypism with quite different sizes and disorderly layers, big cell kernel, deep coloration, obvious heteromorphism,

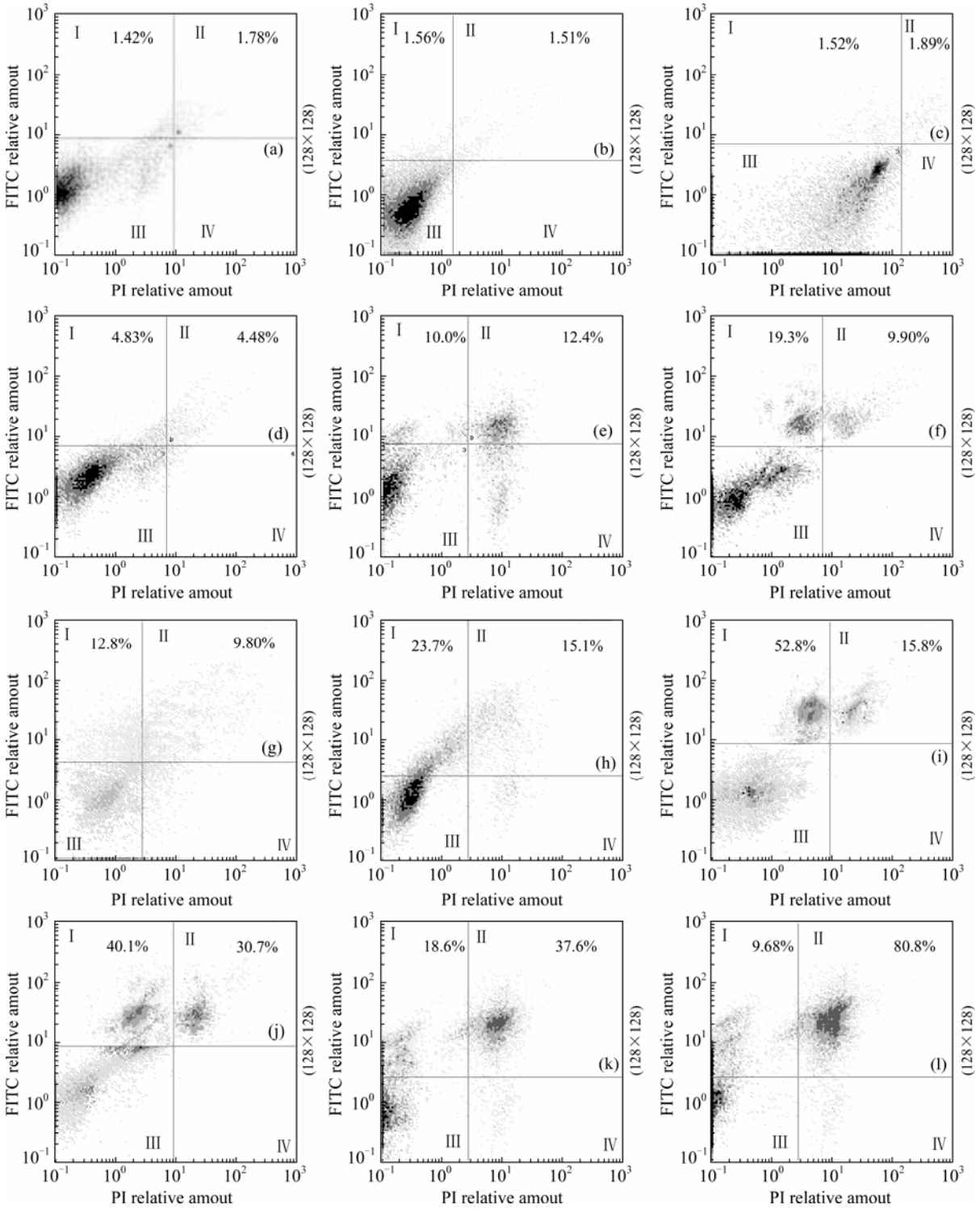

Fig.3 Apoptosis rate of HeLa cells detected with Annexin V-FITC/PI after being treated with quercetin of different concentrations and for different durations

Ⅰ.Annexin V-FITC+,PI−(apototisis cell in early period); Ⅱ.Annexin V-FITC+,PI+(apototisis cell in early period and cell of secondary necrosis); Ⅲ.Annexin V-FITC−,PI−(living cell); Ⅳ.Annexin V-FITC−,PI+(cell of mechanical injury);

a. DMSO,24 h; b. DMSO, 48 h; c. DMSO,72 h; d. *c*(Qμer)=12.5 μmol/L,24 h; e. *c*(Qμer)=12.5 μmol/L, 48 h; f. *c*(Qμer)=12.5 μmol/L,72 h; g. *c*(Qμer)=25.0 μmol/L, 24 h; h. *c*(Qμer)=25.0 μmol/L, 48 h; i. *c*(Qμer)=25.0 μmol/L,72 h; j. *c*(Qμer)=50.0 μmol/L,24 h; k. *c*(Qμer)=50.0 μmol/L,48 h; l. *c*(Qμer)=50.0 μmol/L,72 h

Table 2 Annexin V-FITC/PI double-index method for detecting HeLa cell apoptosis induced by quercetin of different concentrations

c(Qμer)/ μmol • L^{-1}	24 h			48 h			72 h		
	Early period	Late period	Total	Early period	Late period	Total	Early period	Late period	Total
0.0	1.89±0.63	1.75±0.65	3.63±1.22	1.66±0.83	2.00±0.92	3.66±1.63	1.98±0.36	2.18±1.03	4.15±1.02
12.5	4.82±1.32	5.01±1.63	9.84±2.91	7.81±1.61	10.90±1.09	18.71±2.61	18.45±2.30	11.00±1.02	29.45±2.43
25.0	11.18±1.28	10.35±1.43	21.53±1.14	20.90±2.28	15.93±1.08	36.83±2.57	51.15±1.88	18.87±3.22	70.02±4.01
50.0	37.35±2.50	27.55±2.44	64.90±4.90	18.22±1.84	51.78±3.76	70.00±4.05	9.39±2.69	9.33±3.77	88.76±2.35

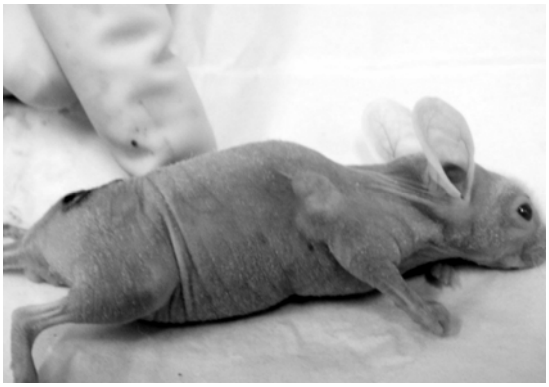

Fig.4 Tumor-developed mice and tumor body

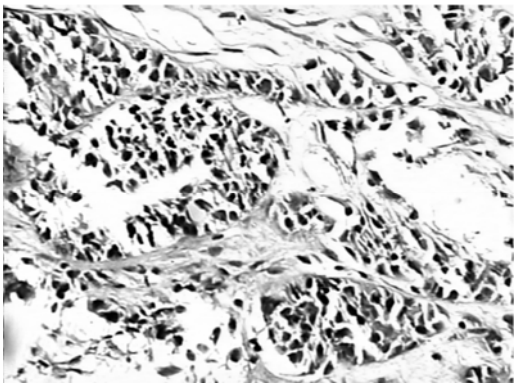

Fig.5 Histomorphology of transplanted tumor (lowly differentiated adenocarcinoma) HE×400

also it was found that the pathological karyokinesis and nucleo-cytoplasmic ratio increased significantly.

During the period when dose was given continuously no tumor-bearing mouse was found dead. Volume of the transplanted tumor was negatively proportional to the dosage amount of quercetin.

2.2.2 Effect of quercetin on volume and weight of transplanted tumor in nude mice

Table 3 and Fig.6 show that 15 days after treated with quercetin, along with the dosage of quercetin increasing, volume(V)of the transplanted tumor decreased. Table 4 shows the same situation as for the weight of the transplanted tumor ($p < 0.01$).

Table 3 Effect of quercetin on volume (V) of transplanted tumor in nude mice $\bar{x} \pm s$ (n=10)

Dosage of guercetin / mg • kg^{-1} • d^{-1}	V/mm^3
DMSO	279.59±70.58
12.5	254.73±102.56
25.0	244.74±83.95
50.0	151.37±57.52
100.0	128.72±36.12

p(inter group)=0.036

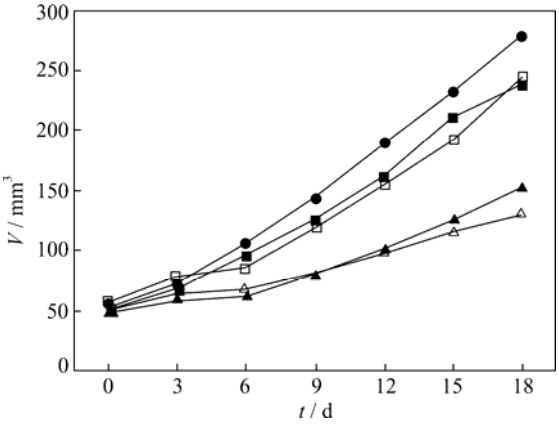

Fig.6 Effect of quercetin on growing up of subcutaneous transplanted tumor in nude Mice

DMSO; 12.5 mg • kg • d^{-1}; 25 mg • kg • d^{-1}; 50 mg • kg • d^{-1}; 100 mg • kg • d^{-1}

Table 4 Effect of quercetin on weight(m) of transplanted tumor in nude mice $\bar{x} \pm s$ (n=10)

Dosage of quercetin /mg • kg^{-1} • d^{-1}	m/g	IR_2/%
0.0	0.145±0.019	—
12.5	0.132±0.025	8.97
25.0	0.131±0.019	9.66
50.0	0.102±0.024	29.66
100.0	0.089±0.019	38.62

p(inter group)<0.01

2.2.3 Effect of quercetin on apoptosis rate of transplanted tumor cells of nude mice

Table 5 and Fig.7 show that having been reacted by quercetin for 15 days, with quercetin concentration increasing, the total apoptosis rate in both early and late periods increased, of which there were significant dis-

Table 5 Apoptosis rate of translated tumor in mice of various groups treated with quercetin of different concentrations ($\bar{x} \pm s$)

Dosage of quercetin/mg • kg^{-1} • d^{-1}	Early period apoptosis rate/%	Late period apoptosis rate/%	Total apoptosis rate/%
0	2.00±0.52	7.63±1.46	9.63±1.85
12.5	6.32±0.71	11.15±0.99	17.47±1.45
25.0	9.94±0.73	14.10±0.77	24.03±1.19
50.0	13.68±0.54	16.00±0.60	29.68±0.95
100.0	17.14±0.70	17.84±0.68	34.98±0.47

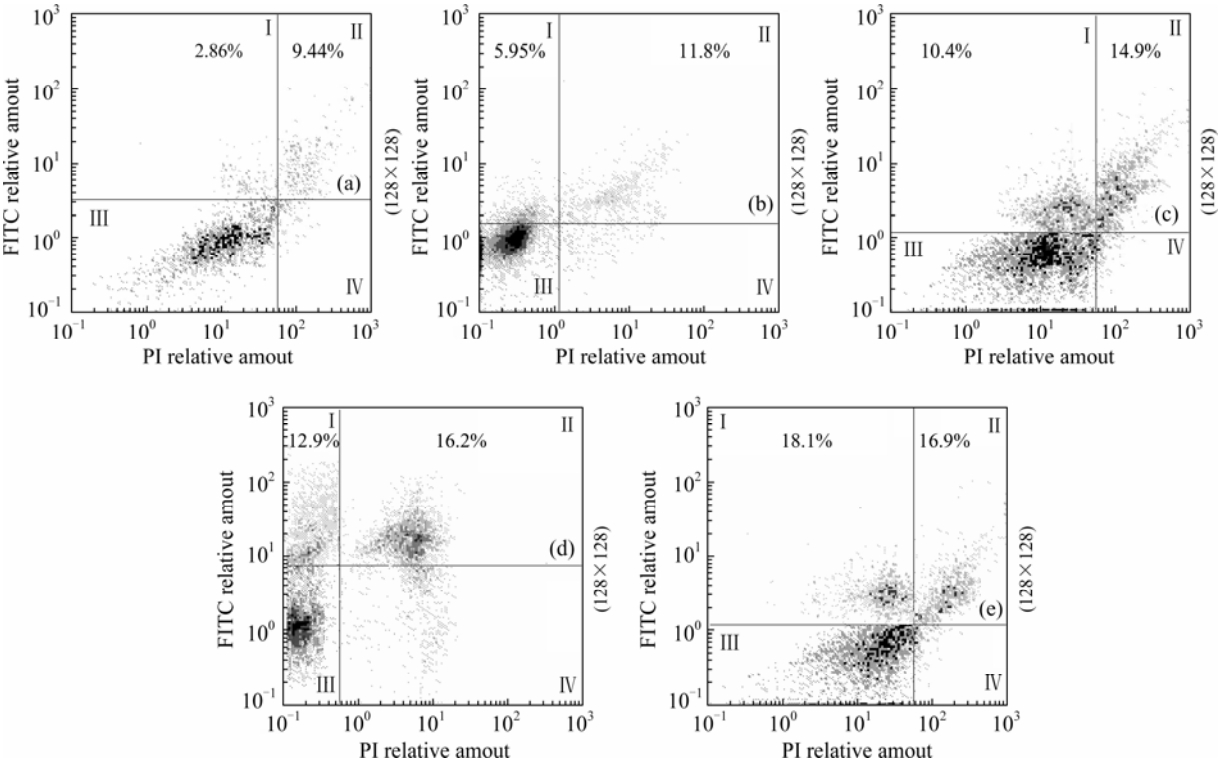

Fig.7 Apoptosis rate of transplanted cell tumor having been treated with quercetin of different concentrations that was injected into mice abdominal cavity

Ⅰ.Annexin V-FITC+,PI−(apototisis cell in early period); Ⅱ.Annexin V-FITC+,PI+(apototisis cell in early period and cell of secondary necrosis); Ⅲ.Annexin V-FITC−,PI−(living cell); Ⅳ.Annexin V-FITC−,PI+(cell of mechanical injury)

a.DMSO; b.Dosage of quercetin=12.5 mg • kg^{-1} • d^{-1}; c. Dosage of quercetin =25.0 mg • kg^{-1} • d^{-1}; d.Dosage of quercetin =50.0 mg • kg^{-1} • d^{-1}; e.Dosage of quercetin =100.0mg • kg^{-1} • d^{-1}

crepancies between the groups with different quercetin concentrations and the group for comparison (all p values < 0.01).

Compared with those HeLa cell that experienced *in vitro* culture, apoptosis of transplanted cell of transplanted tumor was not so obvious as the former but had a low apoptosis rate. This may be because that the culture solution for *in vitro* culture had a higher quercetin. concentration, in which the cells contacted quercetin directly. Also may be because that, after *in vitro* metabolism of the quercetin, internal fluid of tumor tissues had a blood drug concentration that was lower than that of quercetin in the culture solution to affect apoptosis of cells.

2.2.4 Comparison between various groups for body weight of nude mice treated with quercetin

Table 6 shows that 15 days after being injected into abdominal cavity with quercetin, compared with that without quercetin treatment, all the nude mice body weights of the 5 groups for experiments with different quercetin dosages increased, but no discrepancy of increment was found($p > 0.05$).

Table 6 Effect of quercetin on nude mice body weight ($\bar{x} \pm s$)

Dosage of quercetin /mg • kg^{-1} • d^{-1}	Number of nude mice	Body weight of nude mice/g	
		Before drug given	After drug given
0	5	16.80±0.91	18.50±1.32
12.5	5	17.00±1.00	18.70±1.60
25. 0	5	17.30±0.57	19.00±0.79
50.0	5	17.40±1.08	19.40±1.52
100. 0	5	17.5±0.935	20.30±1.15

p(inter group)=0.246

2.2.5 Effect of quercetin treatment on lymphatic metastasis in nude mice

15 days after being treated with quercetin, the lymph nodes in nude mice were taken to be pathologically inspected with the results as shown in Table 7. With quercetin dosage increasing, number of nude mice having lymphatic metastasis decreased. However, inspection with Fisher's Exact Method showed no statistic discrepancy ($p > 0.05$). By inference, such phenomenon may be due to the number of nude mice being comparatively small. The transplanted tumor in nude mice transferred into the lymph nodes to form metastasectomies, in which a glandular cavity structure could be seen (see Fig.8).

Table 7 Effect of quercetin on lymphatic metastasis from cervical cancer of nude mice

Dosage of quercetin / mg • kg^{-1} • d^{-1}	Number of nude mice		Total
	with lymphatic metastasis	with out lymphatic metastasis	
0	3	2	5
12.5	2	3	5
25.0	1	4	5
50.0	1	4	5
100.0	0	5	5
Total	7	18	25

Fisher's Exact Method, $p = 0.467$

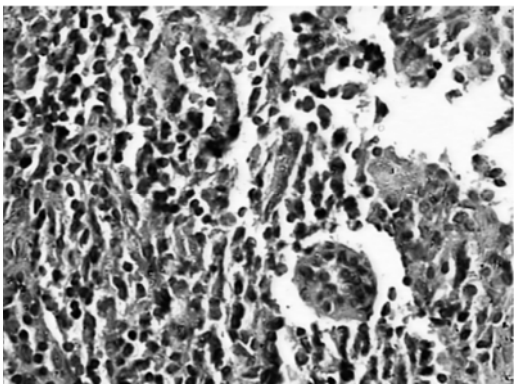

Fig.8 Lymph node transferred of transplanted tumor in nude mice the arrow shows a metastastic nidus

3 Conclusion

(1) Quercetin is capable of inhibiting HeLa cell breeding, inducing them to apoptosis and preventing the transplanted tumor of HeLa cell from growing *in vivo* as well as capable of inducing apoptosis of cells of transplanted tumors. (2) Quercetin doesn't have obvious effect on body weight of and lymphatic metastasis in nude mice so that it has less toxic side effects and is expected to become an effective medicine for assistance of chemical-radiation treatment for cervical cancer. (3) In this experiment, with quercetin dosage increasing, quantity of nude mice bearing lymphatic metastasis decreased, which proves that quercetin has the function of preventing the tendency of cervical cancer metastasis. However, it has to be confirmed by further theory.

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