

Studies of traditional Chinese medicine monomer on HeLa cell of cervical cancer

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Abstract: This paper is to study the effect of traditional Chinese medicine monomer including quercetin, curcumin and Glaucoalyxin A on HeLa cell of cervical cancer. The inhibiting effect of quercetin, curcumin and Glaucoalyxin A on HeLa cells' proliferation is detected through using MTT method. Analysis for the effect of quercetin, curcumin and Glaucoalyxin A on proliferation cycle of HeLa cell is performed through adopting flow cytometry. Three kinds of traditional Chinese medicine monomer can inhibit the growth of HeLa cell, and they show dependent relationship between time and dose. Quercetin, curcumin and Glaucoalyxin A could inhibit cell proliferation, probably through making HeLa cell be in stagnation and inducing its apoptosis.

Keywords: Quercetin, curcumin, Glaucoalyxin A, HeLa cell of cervical cancer, proliferation, inhibition.

INTRODUCTOIN

Cervical cancer is the most common malignant tumors in female reproductive tract. In recent years, there is an upward trend in the incidence and mortality of cervical cancer, and even the patient's age is turning to the young, which forces us to pay more attention to the treatment of cervical cancer (Kim *et al.*, 2005). Tumor treatments include surgery, chemotherapy, radiation therapy, biological treatment, heat treatment, minimally invasive interventional therapy and Chinese medicine treatment (Chen *et al.*, 2004). Traditional Chinese medicine treatment has gotten more and more attention of people among them, the traditional Chinese medicine (TCM) takes the irreplaceable role as an auxiliary treatment in the process of treatment (Ni *et al.*, 2008). This article is to analyze the inhibitory effect of quercetin, curcumin and Glaucoalyxin A of cervical cancer on HeLa cell of Cervical cancer through Flow cytometry.

The concept of chinese medicine monomer

Quercetin

Quercetin is the most common form of Flavonoid material, which widely exists in vegetables, fruits and Chinese herbal medicine, and it is easy to extract, separate and detect. Pharmacological effects of Quercetin is very broad and has various function of expanding coronary artery, reducing blood fat, resisting platelet aggregation, fighting diabetes complications and so on. In recent years, We have found that quercetin has inhibitory effect on many kinds of cancerogen, promoting carcinogen and mutagenicity, but also has effect of growth inhibition on a wide variety of tumor cell (Bar-Sela *et al.*, 2010; Zhongmin *et al.*, 2007). The study of quercetin on

antitumor activity has drawn more and more widely attention.

Curcumin

Curcumin is the polyphenol extracted from the rhizome of curcuma plants like turmeric, curcuma aromatica, curcuma zedoary etc. These three drugs of turmeric, curcuma aromatica, curcuma zedoary belong to the same family of plants, the medicinal property of turmeric is the most warm, rhizoma zedoariae's medicinal property is the second warm, curcuma aromatica is neutral in medical nature. Three drugs have something in common, which is the effect of regulating vital energy and breaking blood, both could cure the syndrome of Qi and blood stasis. There are a lot of literatures, which have recorded that curcumin has a certain role in preventing and controlling gynecological tumors (Baoqiang *et al.*, 2010; Zhanqiang *et al.*, 2011). The America NCI (the national cancer institute) has listed curcumin as the third generation of anti-cancer drugs for research. The main mechanisms of curcumin's role in preventing and treating tumor is applying the method of "promoting blood circulation to remove blood stasis and softening hardness to dissipate stagnation". In recent years, many studies related with curcumin have found that it has lots of pharmacological activities: resistance to the hardening of the arteries, anti-inflammatory, antioxidant, anti-coagulation, antimicrobial and so on, especially explicit anti-tumor effect (Wei *et al.*, 2011; Fengyun *et al.*, 2012; Guohui *et al.*, 2008; Hongsheng *et al.*, 2009; Sujie, 2007).

Glaucoalyxin A

Glaucoalyxin A is the two terpene compounds of antipodal - kauri alkene extracted from labiatae plants of

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the genus spiced tea dishes (Ping *et al.*, 2009; Jin *et al.*, 2004). Antibacterial effect of Glaucoalyxin A is related with α -methylene cyclopentanone structure in its construction (WU *et al.*, 2006), whose acting mechanism is to make active center combine with hydrosulphonyl in germ and interact through reaction similar to the Michael addition, thus to militate. The antitumor activity of Glaucoalyxin A is related with lactone structure, Alpha methylene cyclopentanone, Alpha, beta unsaturated ketones, aldehyde group and epoxy ketone structure within molecule, these active structure could enhance the antitumor activity of Glaucoalyxin A. The antitumor activity of Glaucoalyxin A has also drawn the attention of all parties (YA, 2007; Xiang *et al.*, 2008; Liwen, 2010).

MATERIALS AND METHODS

The major experimental material (table 1)

Experimental method

(1) Cultivating Hela cell of cervical cancer

1. The Hela cell of cervical cancer in the period of logarithmic growth is extracted, nutrient solution in culture bottle is sucked out, and then it is discarded, suitable amount of PBS is added for cleaning suspension cells, PBS with graduated pipette is sucked out and rejected.

2. Suitable amount of 0.25% of the pancreatic enzyme is added for digestion, the morphological changes of cells in microscope is observed, when soma of Hela cell in cervical cancer gradually gets round and intercellular space greatens, pancreatic enzymes is immediately sucked out, and suitable amount of 10% RPMI-1640 nutrient solution is added for ending digestion; cells are blown and beaten carefully through using sterile elbow straw, so that cells will be blow down from the surface of culture bottle, and they are conformed into single-cell suspension. According to the experimental requirements, appropriate amount of single-cell suspension is applied for being taken into inoculating in another culture bottle or culture plate.

(2) The method of quercetin experiment

1. Cell culture: the Hela cell of cervical cancer is putted into 10% RPMI-1640 nutrient solution of newborn bovine serum and it is replaced with new suspension every day, which makes cell breed itself 2~3 days under the condition of 37°.

2. Detection of cell inhibition rate: Hela cell should be exchanged to 96 - well culture plate in the period of logarithmic phase with each hole having 100 U1cells (about 3×10^4 cells), then they will be added into quercetin of different concentration after 24 hours' training. The control group is complete medium, each control group has 5 holes. Every hole will be added into MTT (5mg/ml) 100 U14 hours before the order in each time stage after 6, 12, 24, 48, 72 hours' training respectively, each hole will be

added into DMSO100 U1 upon computer test, then the absorbancy will be measured at 570 mm wavelength on DG-3022A microplate reader. Repeat the experiment 3 times.

3. Test cell cycle: Hela cell in logarithmic phase will be taken out and inoculated with culture plate of 12 holes, quercetin in groups with different concentration will be added into it for 48 hours' constant culture after 24 hours' cultivating, complete medium will be added into control group. Then testers perform centrifuge (1000rps/min, 5min), PBS rinse and suspension with 0.25% trypsin digestion cells, then they are fixed with 70% cold ethanol at 4° overnight, they should avoid light and be dyed for 1 hours at 4° with 400 μ g/ml propidium iodide, cell cycle analysis is made with flow cytometry instrument.

(3) The method of curcumin experiment

1. Perform conventional culture to Hela cell of cervical cancer according to above operation.

2. Divide the concentration of curcumin into 6 groups including 50, 25, 12.5, 6.25, 3.125, 0 μ mol/L in total of 6 groups according to references, and each group is set with 5 deputy holes.

3. The Hela cell in logarithmic phase will be taken out and conformed into single-cell suspension.

4. Inoculate them in 96-well plates with density of 2×10^4 cells in each hole respectively, after the cells attach the surface, they are given curcumin of different concentration respectively according to above grouping.

5. After perform irradiation to them for 24, 48, 72 hours respectively, each hole will be added with 20 μ L MTT (5g/L) and placed into CO2 incubator at 37° for continuous 4 hours' incubation, then the tester ends culture, the suspension in each hole will be sucked out with pipetting gun and discarded, and 150 μ l dimethyl sulfoxide will be added into each hole, which will be placed in the WD - 9405 B horizontal shaking bed for shaking 10 minutes at low speed, thus make the crystal violet formazan completely dissolved. Detect photometric value of light absorption for each hole when it is at 490nm wavelength with micro plate reader of SUNRISE type (OD value).

6. Repeat experiments three times, and take the average value.

(4) Experimental method of Glaucoalyxin A

Morphological observation for the inhibiting effect of Glaucoalyxin A on proliferation of Hela cell: The cells in logarithmic phase will be taken out and digested with trypsin, which are to be blown and beaten into single cell suspension with DMEM nutrient solution which contains 10% fetal calf serum, the cell concentration would be adjusted to be 10×10^5 /ml and inoculated on six orifice, which has 2 ml/hole, after regular training for 24 hours, testers replace the nutrient solution and add it with different concentration;

Glucocalyxin A (Hela cell 5, 10, 20 $\mu\text{mol}\cdot\text{L}^{-1}$) will be cultivated for 12 hours in incubator with 5% CO₂ at 37°, culture medium will be discarded, and it will be washed with PBS twice, 300 μL reagent 1 will be added into it, which will be dyed for 1 minute or so, then 600 μL reagent 2 will be added into it. The testers gently mix them, and then dye them for 5 minutes, the dye liquor should be discarded, the incubator will be washed twice and get microscopic examination after drying. Morphology change of cell, which is affected by Glucocalyxin A will be observed with inverted microscope.

Detect the change of cell cycle with flow cytometry instrument: cells of logarithmic growth phase is to be taken out and digested with trypsin, they will be blown and beaten into single cell suspension with DMEM nutrient solution which contains 10 % fetal calf serum. The cell concentration will be adjusted to be 10 x 10⁵ / ml and then inoculated into plastic culture bottle, each is 5 ml/bottle, after regular training for 24 hours, the tester should replace the nutrient solution and choose GLA (Hela cell 5, 10, 20 $\mu\text{mol}\cdot\text{L}^{-1}$ of different concentration). The tester should give them water bath for 30 minutes at 37°, and PI will be added into it until the concentration is up to 50 $\mu\text{g}/\text{ml}$. Perform dyeing and light avoiding at 4° for 30 minutes. The change of cell cycle would be detected with flow cytometry.

RESULTS

Inhibiting effect of quercetin on proliferation of hela cell

(1) The influence of quercetin on HeLa cell's proliferation

The group whose quercetin's concentration is 80 $\mu\text{mol}/\text{L}$ and HeLa cell of cervical cancer combine into incubation for 0 ~ 72 hours outside the body, significant inhibitory effect will occur after 12 hours. With extended time, the inhibition effect enhances obviously, its role is in positive correlation with time. It is shown as the Table 2:

(2) The influence of quercetin on HeLa cell's proliferation

As it is shown in the table3, 0~160 $\mu\text{mol}/\text{L}$ quercetin and HeLa cell of cervical cancer combine into vitro incubation for 48 hours, according to the results more than 20 $\mu\text{mol}/\text{L}$ quercetin can significantly inhibit proliferation of HeLa cell. For the group whose concentration is 80 $\mu\text{mol}/\text{L}$, its inhibition rate is up to 43.88%, half of the

Table 1: Experimental material

Quercetin experiment	Curcumin experiment	Experiment of Glucocalyxin A
Hela cell strain of human cervical carcinoma MTT instrument, propidium iodide (PI), DMEM culture medium, 10% RPMI-1640 nutrient solution new-born calf serum		
Quercetin, NaOH assisted solution, 1% diluted hydrochloric acid (pH:7~7.4)	Curcumin powder, Dimethyl sulfoxide solution,	Glucocalyxin A medicine, thiazolyl blue, dimethyl sulfoxide

inhibition amount (IC₅₀) is in concentration group of 160 $\mu\text{mol}/\text{L}$. Inhibition effect is in high correlation with the dose.

(3) The influence of quercetin in different concentration on HeLa cell's cycle

HeLa cell of cervical cancer is processed with different dose of quercetin for 48 h respectively, and the equal complete medium is taken as blank contrast, the cell cycle analysis is performed with flow cytometry instrument. Results show Table 4, as the dose of quercetin that act on HeLa cell increases, the percentage that HeLa cell in G1 phase increases significantly, which presents the concentration-response relationship, the rest cells of each phase decreases relatively. We can see it from the graph:

The proliferation inhibition effect of curcumin on hela cell

(1) The influence of curcumin on the proliferation of HeLa cell

It could be seen from the fig. 1, the cell in blank group without adding medication shows active proliferation, and through the processing of curcumin at different concentrations for 24, 48, 72 hours respectively, the proliferation of HeLa cell has been inhibited at different extent, and it represents the time and the concentration dependence. With the processing of curcumin at different concentrations for 48 hours, the inhibition rate of cell proliferation is between 7% and 81%, compared with the blank group without adding medication, 12.5 $\mu\text{mol}/\text{L}$ curcumin can produce inhibiting effect which is statistically significant ($p < 0.05$), while curcumin whose concentration is 25 $\mu\text{mol}/\text{L}$ or higher than that could inhibit more intensely on HeLa cell ($p < 0.01$).

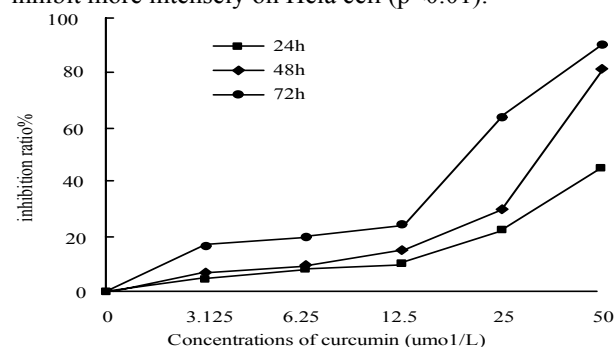


Fig. 1: The influence of curcumin on HeLa cell proliferation of cervical cancer

(2) The cell cycle analysis and apoptosis detection

As it is shown in the table 5, the results of the experiments that detect the cell cycle and apoptosis through using flow cytometry instrument show that compared with the blank group, the role of curcumin can improve the proportion of cells in G2 / M phase, and induce cell apoptosis ($p < 0.05$).

The Proliferation Inhibition Effect of Glaucocalyxin A on HeLa Cell

(1) The results of MTT test

As it is shown in the table 6, the respective inhibition ratio after exerting $5\mu\text{mol}\cdot\text{L}^{-1}$ Glaucocalyxin A on HeLa cell for 12, 24, 48, 72 hours are 20.08%, 21.51%, 24.77%, 72.22%. The respective inhibition ratio after exerting $10\mu\text{mol}\cdot\text{L}^{-1}$ Glaucocalyxin A on HeLa cell for 12, 24, 48, 72 hours are 27.24%, 34.91%, 38.14%, 85.07%. The respective inhibition ratio after exerting $20\mu\text{mol}\cdot\text{L}^{-1}$ Glaucocalyxin A on HeLa cell for 12, 24, 48, 72 hours are 50.32%, 65.83%, 73.91%, 92.78%. The above results suggest: Glaucocalyxin A has obvious inhibitory effect on HeLa cell proliferation, and it shows dependence trend of dose effect and time effect. Compared with control group, the inhibition ratio of each Glaucocalyxin A group increases obviously ($P < 0.01$). Results show that at different time points of effecting, the inhibition effect of Glaucocalyxin A on HeLa cell was enhanced with the increase of drug concentration, when compare different concentration group with normal control group, the otherness is significant ($P < 0.01$). For Glaucocalyxin A group with different concentration, its inhibiting effect will increase with the extension of time, the comparison among different groups shows relatively obvious difference ($P < 0.01$). The above results suggest: Glaucocalyxin A has obvious inhibitory effect on proliferation of HeLa cell, and it represents dependence trend of dose effect and time effect. Half the statistical result of software for calculation which inhibits concentration IC_{50} indicates: the IC_{50} of HeLa cell after effecting with Glaucocalyxin A for 12, 24, 48, 72 hours are: (21.78 ± 1.31), (9.90 ± 0.44), (3.84 ± 1.91), (2.44 ± 0.26) $\mu\text{mol}/\text{L}$ respectively.

(2) The morphological change existing in the proliferation inhibition effect of Glaucocalyxin A on HeLa cell Observed under inverted microscope, HeLa cell in normal control group grow well, most cells are polygonal and merge into colony. After processing with 5, 10 and 20 $\mu\text{mol}\cdot\text{L}^{-1}$ GLA, we could observe that cells grow slowly and become round, its dye deepens, and with the increase of drug concentration, cell density decreases as shown in fig. 2.

The graph is the observed morphological variation fig that represents light microscope of HeLa cell and treatment of Glaucocalyxin A, A is blank group; B is $5\mu\text{mol}\cdot\text{L}^{-1}$ Glaucocalyxin A; C is $10\mu\text{mol}\cdot\text{L}^{-1}$ Glaucocalyxin A; D is $20\mu\text{mol}\cdot\text{L}^{-1}$ Glaucocalyxin A. You can see the

inhibitory effect of Glaucocalyxin A on HeLa cell is very big.

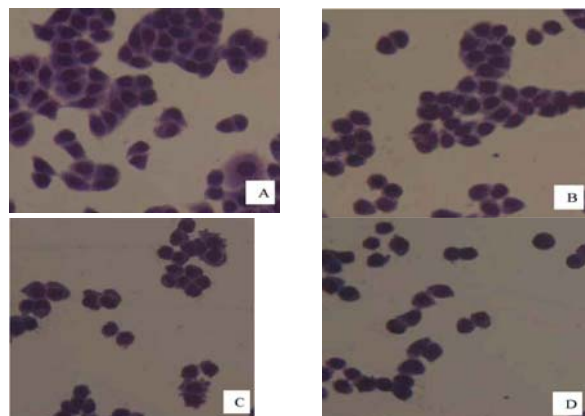


Fig. 2: Cell's light microscope and morphological variation of observations in treatment with Glaucocalyxin A.

Table 2: The effect of quercetin on the proliferation of HeLa cell (aging relation) figure

Time (h)	MIT-OD value ($X \pm SD$)	
	Contrast group	Que ($80\mu\text{mol}/\text{L}$)
0	0.24 ± 0.05	0.22 ± 0.03
6	0.43 ± 0.06	0.38 ± 0.02
12	0.65 ± 0.08	$0.49 \pm 0.03^*$
24	0.87 ± 0.11	$0.51 \pm 0.05^{**}$
48	0.98 ± 0.07	$0.55 \pm 0.06^{**}$
72	0.90 ± 0.11	$0.45 \pm 0.10^{**}$

Compared with contrast group

Table 3: The influence of quercetin on the proliferation of HeLa cell (dose-effect relationship)

Que ($\mu\text{mol}/\text{L}$)	OD value ($X \pm SD$)	Inhibition rate (%)
0	0.98 ± 0.07	—
5	0.85 ± 0.10	13.27
10	0.72 ± 0.12	26.53
20	$0.66 \pm 0.03^*$	32.65
40	$0.63 \pm 0.07^*$	35.71
80	$0.55 \pm 0.06^{**}$	43.88
160	$0.48 \pm 0.08^{**}$	51.02

$n=5$, compared with contrast group, $*P < 0.05$, $**P < 0.01$

Table 4: The effect of quercetin at different concentrations on cell cycle of HeLa

Drug dose ($\mu\text{mol}/\text{L}$)	Cell cycle		
	G1	S	G2/M
0	19.5	75.3	5.2
20	37.0	63.0	0.0
40	49.5	28.1	22.4
80	68.3	5.2	26.5

Table 5: The comparison of cell cycle and apoptosis results

Group	G0/G1	S	G2/M	Apoptosis rate(%)
Blank group	47.3 ± 8.77	41.9 ± 2.62	11.4 ± 2.14	2.06
Single drug group	36.4 ± 1.98	37.7 ± 3.16	24.3 ± 3.01*	39.6%

Note: Compared with blank group, *p<0.05, **p<0.01

Table 6: The influence of the Glaucoalyxin A on activity of Hela cell ($\bar{x} \pm s$ n=6)

Group	OD Value	Dose ($\mu\text{mol}\cdot\text{L}^{-1}$)	12h	24h	48h	72h
Control			0.568±0.08	1.027±0.038	1.071±0.009	1.361±0.018
GLA		2.5	0.498±0.05*	1.019±0.032*	0.915±0.004*	0.779±0.163*
		5	0.452±0.057**	0.988±0.029**	0.806±0.027*	0.378±0.051**
		10	0.382±0.031**	0.702±0.010**	0.662±0.012*	0.203±0.024**
		20	0.305±0.016**	0.369±0.012**	0.230±0.008**	0.098±0.006**
		40	0.266±0.009**	0.109±0.006**	0.089±0.002**	0.091±0.005**

CONCLUSION

- (1) The proliferation of quercetin on Hela cell of cervical cancer appears obvious inhibiting effect, and it represents time-effect relationship and concentration-response relationship.
- (2) Quercetin induces cell into programmed death through obstructing Hela cell in G1 phase, thus it plays its restraining role in the growth of Hela cell.
- (3) Curcumin has obvious inhibiting effect on the proliferation of Hela cell in cervical cancer.
- (4) Curcumin could hold back Hela cell of cervical cancer in G2/M phase and induce cell apoptosis.
- (5) Glaucoalyxin could inhibit the proliferation of Hela, Siha cell in cervical cancer *in vitro*, and it represents dependence trend of dosage effect or time effect.
- (6) Glaucoalyxin A could induce Hela cell apoptosis of cervical cancer, and make cell be retardant in S phase, and it represents dependence trend of dosage effect.

Cervical cancer is one of the most common malignant tumor in women. In recent years, research about the cause of cervical cancer at home and abroad has found that infecting with certain viruses such as herpes simplex virus type α and human papillomavirus (HPV) etc can cause cervical cancer. This paper performs a series of investigation through pharmacologic action of Chinese traditional herbs monomer including quercetin, curcumin and Glaucoalyxin A, especially on basis of its significant inhibiting effect on tumor cell growth and antiviral proliferation. The results confirm that three kinds of traditional Chinese medicine monomer have obvious inhibitory effect on HeLa cell proliferation of cervical cancer, which has a certain foundation effect on treatment of cervical cancer's medical research, and it has guiding significance.

FUNDING PROJECT

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