• Basic Research •

Effects of curcumin nanoparticles on proliferation and VEGF expression of human retinal pigment epithelial cells

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Abstract

● **AIM:** To investigate the effects of curcumin (Cur) nanoparticles loaded with chitosan derivatives grafted by deoxycholic acid (Chit-DC) on human retinal pigment epithelial (hRPE) cell proliferation and vascular endothelial growth factor (VEGF) mRNA expression.

● **METHODS:** Cur nanoparticles were synthesized with Chit-DC as the carrier and Cur as the supported drug. Cell counting kit-8 (CCK-8) method was used to detect the effects of different concentrations of Cur/Chit-DC, Chit-DC, and Cur on the proliferation of hRPE cells for different times. The changes of Cur/Chit-DC and Cur on hRPE cell cycle were determined by flow cytometry. Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) was used to detect the mRNA expression levels of VEGF in hRPE cells treated with Cur, Chit-DC and Cur/Chit-DC at 10 μg/mL for 24h.

● **RESULTS:** Different concentrations of Chit-DC nanoparticle treated hRPE cells had no significant difference in terms of optical density (OD) values compared with the control group at 24h and 48h. Moreover, there was no change in the cell morphology under a light microscope. After 24h treatment with Cur/Chit-DC and Cur, the percentage of G0-G1 phase cells increased and the percentage of S phase cells decreased in all concentration groups. Cur/Chit-DC and Cur in all concentration groups inhibited the proliferation of hRPE cells in a time and dose dependent manner, and reduced the expression level of VEGF mRNA.

● **CONCLUSION:** The Cur/Chit-DC nanoparticles can release Cur continuously and have sustained release function. Both Cur/Chit-DC nanoparticles and Cur could inhibit hRPE cells cultured *in vitro*, and could reduce the expression level of VEGF mRNA in hRPE cells.

● **KEYWORDS:** curcumin; retinal pigment epithelial cells; vascular endothelial growth factor; cell counting kit-8; polymerase chain reaction

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INTRODUCTION

Retinal hypoxia in fundus neovascular disease can lead to the release of various cytokines, and vascular endothelial growth factor (VEGF) is considered to be the most closely related factor to neovascular disease. Hypoxia can induce VEGF expression in retinal pigment epithelial (RPE) cells both *in vivo* and *in vitro*, and VEGF can promote intraocular angiogenesis[1]. It is important to find a drug that can effectively inhibit the proliferation of RPE cells and reduce the level of VEGF. Curcumin (Cur) has a variety of curative effects[2-4]. Studies on the application of Cur in ophthalmology have shown that Cur can inhibit the proliferation of human RPE (hRPE) cells and the expression of VEGF in the retina of diabetic rats[5-6], so it can play its pharmacological role in the prevention and treatment of fundus neovascular lesions. However, Cur is difficult to dissolve in water, is rapidly metabolized in the body, has a short half-life in the body, and requires long-term and repeated use. This brings a lot of inconvenience and pain to patients, so it is of great clinical significance to develop a new dosage form of Cur. Nano Chinese medicine refers to the active components, active parts,

active drugs and compound preparations of Chinese medicine with particle size less than 100 nm that are manufactured by nanotechnology[7]. Compared with traditional Chinese medicine, it has its advantages[8-11]: It increases the solubility of the drug; More likely to target the retina through the blood-retina barrier; With sustained release; Reduce side effects and so on. In this topic, Cur is mainly encapsulated into chitosan nanoparticles grafted by deoxycholic acid (Chit-DC) through nanotechnology to prepare drug-carrying nanoparticles. It is hoped that the nanoparticles have the function of slow release of drugs, improve the efficacy of drugs, maintain the drug concentration for a long time, and reduce the pain caused by repeated administration of drugs to patients. At the same time, the effects of Cur/Chit-DC and Cur on the proliferation and VEGF mRNA expression levels of hRPE cells cultured *in vitro* were observed and compared, providing a preliminary theoretical basis for finding a new, sustained-release, safe and effective drug for the prevention and treatment of fundus neovascular diseases.

MATERIALS AND METHODS

Materials and Main Instruments and Equipment

Reagent Cur (purchased from SIGMA Company), fetal bovine serum (FBS; Biological Industries Company), Vimentin monoclonal antibody (Boshide Company), cell counting kit-8 (CCK-8; Dojindo Company), Trizol (TaKaRa Company), reverse transcription-polymerase chain reaction (RT-PCR) kit (TaKaRa Company) and hRPE cells (the 3rd to 6th generations of cells were selected for the experiment provided by the Affiliated Eye Hospital of Sun Yat-sen University).

Main instruments and equipment Surgical microscope (Zeiss, Germany), optical inverted microscope (Leica MPS-30, Germany), fluorescence microscope Axioplan2 Imaging (Zeiss, Germany), flow cytometry (BD, USA), Wellscan MK3 Microplate meter (USA) Labsystem Dragon, Gene Amp2700 PCR instrument (US Syn Gene), Constant voltage constant current Electrophoresis instrument (US Bio-RAD), Gene GEN IUS gel Imager (US Syn Gene).

Experimental Method

Material synthesis Synthesis technique of Cur/CHit-DC nanoparticles was performed mainly as described by Zheng *et al*[12].

The drug reached equilibrium after 96h of release from the nanoparticles, and the cumulative drug release amount was 31.6%[12].

Determination of loading capacity and loading efficiency After centrifugation, the lower layer of the liquid precipitated into the unloaded drugs, which were dissolved in 20% ethanol phosphate buffer (pH=6.2), and their content was determined *via* ultraviolet (UV) spectrophotometry (λ=433 nm). The formula for calculating the drug loading capacity and loading efficiency of the drug-loaded nanoparticles was as follows:

$$\text{Drug loading}=(A-B)/C\times 100\% \quad (1)$$

$$\text{Load efficiency}=(A-B)/A\times 100\% \quad (2)$$

A: Total amount of Cur added (mg); B: Amount of unloaded Cur drug (mg); obtained after centrifugation, precipitation, and drying; C: Total input of chitosan derivatives (mg).

Cytological experiment

Proliferation effect of Chit-DC nanoparticles on hRPE cells hRPE cells in the logarithmic growth phase were prepared in 2500/mL cell suspensions in a Dulbecco's modified Eagle's medium/nutrient mixture F12 (DMEM-F12) with 10% FBS. Then, 100 μL cell suspensions were added to each well and inoculated into four culture plates for 24h, each plate with 96 holes. After the cells grew a monolayer, 100 μL of Chit-DC nanoparticles with final concentrations of 5, 10, 20, and 40 μg/mL were added to the cells. At the same time, the negative control (without drug addition) and blank control (with only the culture medium) were set up, with four multiple wells set up in each group. Then, the 96-well plates were cultured for 24 and 48h, and 100 μL 10% FBS, DMEM-F12 medium, and 10 μL CCK-8 were added to each well for 3h. The optical density (OD) value of each well at 450 nm was detected using a microplate tester. The experiment was repeated three times for each group. At the same time, the morphological changes in the cells were observed under a light microscope.

Effects of Cur/Chit-DC nanoparticles and Cur on the proliferation of hRPE cells

Experimental methods and procedures hRPE cells in logarithmic growth phase were prepared into 2500/mL cell suspensions using 10% FBS DMEM-F12. The 100 μL cell suspensions were added to each well and inoculated into 4 culture plates for 24h, and each plate had 96 holes. After the cells grew to monolayer, 100 μL of Cur and Cur/Chit-DC nanoparticles with final concentration of 5, 10, 20, 40 μg/mL were added into the cells respectively (the corresponding dose of Cur in Cur/Chit-DC nanoparticles was calculated based on drug loading rate of 27.5%). At the same time, a blank control with only culture medium was set, and each group had 4 repeating wells. Then the 96-well plates were cultured for 1, 2, 3, 4, 5, and 6d, and then add 100 μL DMEM-F12 medium containing 10% FBS and 10 μL CCK-8 to each well for 3h. OD value of each well was detected by microplate tester (450 nm). The experiment was repeated three times for each group.

Calculation of inhibition rate of cell proliferation The inhibition rate of cell proliferation was calculated according to CCK-8 OD value, and the time and dose effect curves were plotted. Proliferation inhibition rate (%)=(control group OD valu−experimental group OD value)/control group OD value ×100%.

Effect of Cur/Chit-DC nanoparticles and Cur on hRPE cell cycle The 3rd to 6th generations of cells at logarithmic growth stage were inoculated in 25 mL flask at a density of 5×10^5/mL, and 24h later, 100 μL DMEM-F12 medium containing 10% FBS was for further culture for 24h. The final concentration of 5, 10, 20, 40 μg/mL Cur and Cur/Chit-DC nanoparticles were treated for 24h. The control group only added culture medium. Adherent cells were collected for flow cytometry analysis and cell cycle analysis. The experiment was repeated three times for each group.

Effects of Cur, Chit-DC nanoparticles and Cur/Chit-DC nanoparticles on VEGF mRNA in hRPE cells

Cell grouping and sample addition The experiment was divided into four groups: 1) Control group: DMEM-F12 culture medium; 2) Cur group: the concentration of Cur is 10 μg/mL; 3) Cur/Chit-DC nanoparticle group: the concentration of Cur in Cur/Chit-DC nanoparticles is 10 μg/mL; 4) Chit-DC nanoparticle group: the final concentration of Chit-DC was the same as that of group 3.

PCR primers Primer sequence VEGF RT sense: 5'GACA AGAAAATCCCTGTGGGC3', RT anti-sense: 5'AACGCG AGTCTGTGTTTGC3', theoretical length of amplified fragment was 102 bp; Internal reference β-actin RT sense: 5'CTCAAGTTGGGGGACAAAAA3', RT anti-sense: 5'GATGAGATTGGCATGGCTTT3', theoretical length of amplified fragment was 428 bp.

Extraction of total RNA The total RNA of each treatment group was extracted by Trizol one-step method. Four specimens were taken from each group. According to the instructions of the RT-PCR kit, the target gene was amplified by polymerase chain reaction using cDNA obtained after reverse transcription as the template. PCR reaction conditions: 94℃ pre-denaturation for 3min; denaturation at 94℃ for 30s; annealing at 55℃ for 30s; elongation at 72℃ for 1min; a total of 33 cycles were carried out, and the last cycle was extended at 72℃ for 8min.

Agarose gel electrophoresis and photography were carried out for the products Gel image scanning analysis software was used to analyze the electrophoresis results and determine the OD value of the electrophoresis bands. The relative VEGF content is expressed as the ratio of VEGF to the PCR product content of β-actin from the same specimen (OD value of VEGF/OD value of β-actin).

Table 1 Effect of different concentrations of Chit-DC nanoparticles on hRPE cells 24 and 48h n=15, mean±SD

Concentrations, μg/mL	OD values	
	24h	48h
0 (control group)	0.782±0.008	1.063±0.009
5	0.785±0.018	1.066±0.020
10	0.783±0.026	1.065±0.013
20	0.783±0.015	1.061±0.015
40	0.780±0.013	1.063±0.018
P	0.272	0.324

Chit-DC: Chitosan derivatives grafted by deoxycholic acid; hRPE: Human retinal pigment epithelial; OD: Optical density.

Statistical Analysis SPSS18.0 software package was used for statistical processing. One-way analysis of variance of multiple groups of quantitative data was used for statistical analysis. P<0.05 was considered statistically significant.

RESULTS

Determination of Drug Loading and Loading Efficiency The drug loading capacity and loading efficiency of the synthesized Cur/Chit-DC nanoparticles were determined *via* UV spectrophotometry, as follows: drug load (Cur)=27.5%, load rate (Cur)=55%.

Proliferation of hRPE Cells OD values of hRPE cells treated with Chit-DC nanoparticles of different concentrations for 24 and 48h were shown in Table 1. There was no significant difference in OD value between the control group (drug concentration of 0 mg/L) and the experimental group (F=0.381, F=0.281; P>0.05). No changes in the cell morphology were observed under a light microscope (Figure 1), suggesting that Chit-DC nanoparticles had no toxicity on hRPE cells.

Effects of Cur and Cur/Chit-DC Nanoparticles on the Proliferation of hRPE Cells CCK-8 method was used to detect the effects of Cur and Cur/Chit-DC nanoparticles at different concentrations and time points on the proliferation of hRPE cells (Table 2).

In the treatment of hRPE cells, both Cur/Chit-DC nanoparticles and Cur had inhibitory effects on hRPE cells (P<0.01), and the inhibition rate increased with the increase of drug concentration and the prolongation of action time. At the initial stage (1-4d), the inhibition rate of the Cur/Chit-DC nanoparticles group on hRPE cells was lower than that of the corresponding concentration of Cur group, and the difference was statistically significant (P<0.05). With the further prolonging of the treatment time (5-6d), the inhibition rate of hRPE cells in Cur/Chit-DC group was similar to that in Cur group, and there was no significant difference in statistical analysis (P>0.05).

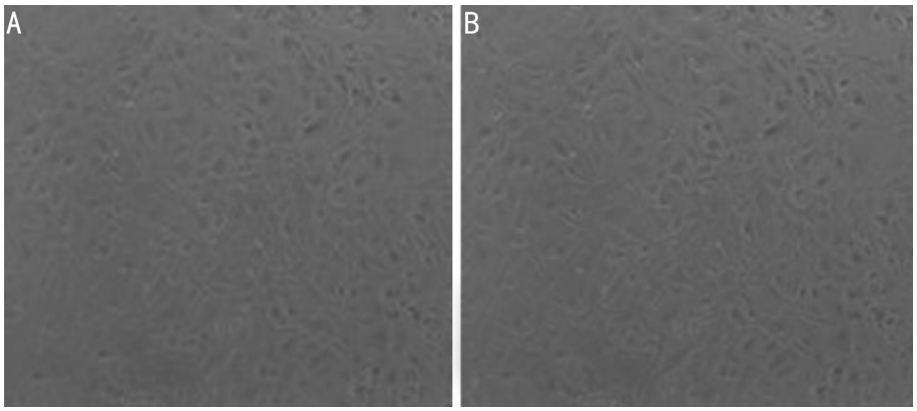

Figure 1 hRPE cells A: hRPE cells treated with Chit-DC nanoparticles for 24h. ×100; B: hRPE cells treated with Chit-DC nanoparticles for 48h. ×100. hRPE: Human retinal pigment epithelial; Chit-DC: Chitosan derivatives grafted by deoxycholic acid.

Cell Cycle Changes of hRPE Cells Flow cytometry results (Table 3) showed that in the range of 5-40 μg/mL, the percentage of cells in G0-G1 phase increased in both Cur and Cur/Chit-DC nanoparticles, while the percentage of cells in S phase decreased. It indicated that the proliferation period of hRPE cells was reduced.

Effects of Cur, Cur/Chit-DC Nanoparticles and Chit-DC Nanoparticles on VEGF mRNA Expression in hRPE Cells The mRNA integrity test of extracted VEGF showed three bands of 5S, 18S, and 28S (Figure 2). Semi-quantitative RT-PCR gel diagram (Figure 3) image analysis to obtain the relative OD of VEGF in each group (Table 4, Figure 4) showed that there was no difference in VEGF mRNA expression between Chit-DC nanoparticles and control group after 24h treatment (P>0.05). The mRNA expression of VEGF in Cur/Chit-DC and Cur group was lower than that in control group (P<0.05).

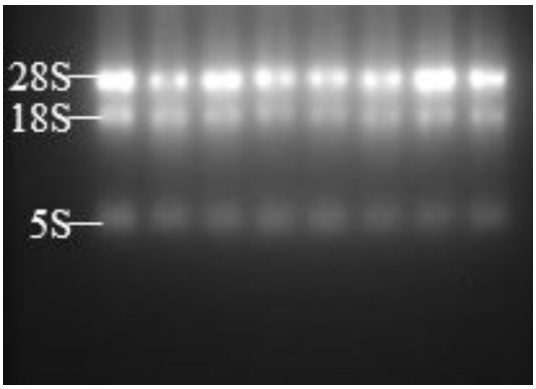

Figure 2 RNA integrity test: 5S, 18S, and 28S bands were visible.

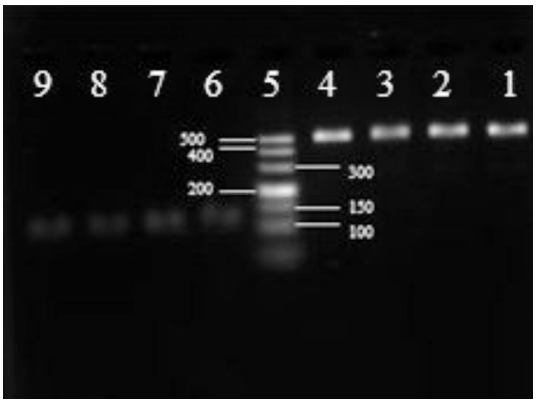

Figure 3 RT-PCR gel diagram was used to detect VEGF mRNA expression levels in hRPE cells of each experimental group for 24h The 1, 2, 3, and 4 were internal reference β-actin of control group, Chit-DC nanoparticle group, Cur-active drug group and Cur/Chit-DC nanoparticle group, respectively. The 5 for Marker; 6, 7, 8, and 9 were control group, Chit-DC nanoparticles group, Cur-crude drug group and Cur/Chit-DC nanoparticles group, respectively. RT-PCR: Reverse transcription-polymerase chain reaction; VEGF: Vascular endothelial growth factor; Cur: Curcumin; Chit-DC: Chitosan derivatives grafted by deoxycholic acid.

DISCUSSION

Chitosan is the only cationic basic aminopolysaccharide discovered. It is a kind of natural polymer and suitable for preparation of nano-controlled release system as carrier material. In nature, chitosan is widely found in lower organisms such as fungi, shells of shrimps, crabs and insects. It is widely used in food, medicine, bioengineering and so on[13]. In this experiment, the Cur/Chit-DC nanoparticles synthesized used Chit-DC as the drug carrier, and Chit-DC used EDC as the coupling agent to graft deoxycholic acid onto chitosan to synthesize amphiphilic modified chitosan. The polymer can form nanoparticles in aqueous solution. CCK-8 method was used to compare the inhibition rate of proliferation of hRPE cells with different concentrations of Chit-DC nanoparticles group and the control group. The results showed that there was no significant difference between the groups with different concentrations of Chit-DC nanoparticles and the control group, and no change in cell morphology was observed under inverted microscope. These results indicated that Chit-DC nanoparticles

Table 2 Effects of Cur and Cur/Chit-DC nanoparticles on proliferation of hRPE cells from day 1 to day 6 *n*=10, mean±SD

Drug concentration (μg/mL)	Cur		Cur/Chit-DC	
	OD values	Inhibition rate (%)	OD values	Inhibition rate (%)
1d				
0	0.782±0.008			
5	0.647±0.005[a]	17.2	0.732±0.007[a,c]	6.3
10	0.523±0.008[a,b]	33.1	0.685±0.003[a,b,c]	12.4
20	0.464±0.009[a,b]	40.7	0.620±0.012[a,b,c]	20.7
40	0.375±0.006[a,b]	52.1	0.536±0.006[a,b,c]	31.5
2d				
0	1.063±0.009			
5	0.739±0.007[a]	30.5	0.955±0.006[a,c]	10.2
10	0.619±0.006[a,b]	41.8	0.856±0.012[a,b,c]	19.5
20	0.433±0.006[a,b]	59.3	0.809±0.005[a,b,c]	23.9
40	0.336±0.009[a,b]	68.4	0.672±0.006[a,b,c]	36.8
3d				
0	1.356±0.013			
5	0.602±0.015[a]	55.6	1.073±0.006[a,c]	20.9
10	0.495±0.004[a,b]	63.5	0.925±0.010[a,b,c]	31.8
20	0.244±0.017[a,b]	82.0	0.764±0.009[a,b,c]	43.7
40	0.072±0.008[a,b]	94.7	0.500±0.006[a,b,c]	63.1
4d				
0	1.540±0.006			
5	0.554±0.010[a]	64.0	0.875±0.016[a,c]	43.2
10	0.510±0.014[a,b]	66.9	0.799±0.014[a,b,c]	48.1
20	0.114±0.009[a,b]	92.6	0.536±0.008[a,b,c]	65.2
40	0.103±0.012[a]	93.3	0.109±0.013[a,b]	92.9
5d				
0	1.615±0.004			
5	0.602±0.015[a]	62.7	0.686±0.009[a,c]	57.5
10	0.564±0.023[a,b]	65.1	0.555±0.012[a,b]	65.6
20	0.096±0.016[a,b]	94.1	0.108±0.016[a,b]	93.3
40	0.089±0.012[a]	94.5	0.122±0.020[a]	92.4
6d				
0	1.623±0.015			
5	0.591±0.022[a]	63.6	0.605±0.013[a]	62.7
10	0.518±0.016[a,b]	68.1	0.537±0.014[a,b]	66.9
20	0.036±0.012[a,b]	97.8	0.055±0.017[a,b]	96.6
40	0.030±0.008[a]	98.2	0.045±0.015[a,b]	97.2

One-way analysis of variance showed statistically significant differences among each group (P<0.05), and pairwise comparison was performed among each group. [a]Compared with the control group, P<0.01; [b]Compared with the adjacent concentration group, P<0.01; [c]Compared with the Cur group, P<0.01. Cur: Curcumin; Chit-DC: Chitosan derivatives grafted by deoxycholic acid; OD: Optical density.

Table 3 Effect of Cur and Cur/Chit-DC on hRPE cell cycle detected by flow cytometry

Concentration (μg/mL)	Cell cycle phase (Cur)			Cell cycle phase (Cur/Chit-DC)		
	G0-G1	S	G2-M	G0-G1	S	G2-M
0	53.02±1.03	33.82±0.89	13.16±0.96			
5	77.61±0.96[a]	11.86±1.01[a]	10.53±1.58	74.29±1.20[a]	21.78±1.01[a]	4.53±0.76[a]
10	83.83±1.31[a]	7.08±0.53[a]	9.09±0.99[a]	69.84±0.97[a]	19.80±1.12[a]	10.36±1.42
20	86.97±1.23[a]	6.32±0.72[a]	6.71±0.81[a]	80.60±1.36[a]	11.56±1.23[a]	7.85±0.63[a]
40	89.47±1.16[a]	4.14±0.55[a]	6.39±0.36[a]	78.57±0.93[a]	7.51±0.69[a]	13.92±1.06

The experimental group was compared with the control group (drug concentration 0 μg/mL) [a]P<0.05. Cur: Curcumin; Chit-DC: Chitosan derivatives grafted by deoxycholic acid; hRPE: Human retinal pigment epithelial.

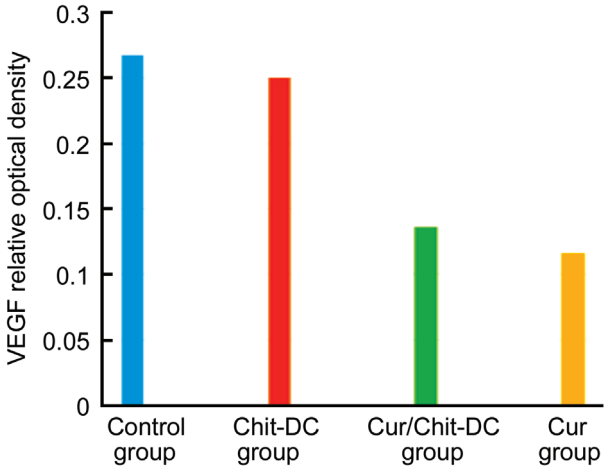

Figure 4 The relative optical density of VEGF in hRPE cells after 24h treatment in each experimental group Cur: Curcumin; Chit-DC: Chitosan derivatives grafted by deoxycholic acid; VEGF: Vascular endothelial growth factor; hRPE: Human retinal pigment epithelial.

Table 4 Relative OD of VEGF in hRPE cells after 24h treatment in each experimental group n=4

Groups	Relative OD
Control group	0.267±0.017
Chit-DC group	0.250±0.013
Cur/Chit-DC group	0.136±0.011[a]
Cur group	0.116±0.005[a]

One-way ANOVA showed statistically significant differences among each group (P<0.05), and pairwise comparison was performed among each group. Cur/Chit-DC group, Cur group compared with control group, [a]P<0.05. Cur: Curcumin; Chit-DC: Chitosan derivatives grafted by deoxycholic acid; VEGF: Vascular endothelial growth factor; hRPE: Human retinal pigment epithelial; OD: Optical density.

as a drug carrier did not affect the growth of hRPE cells, suggested that Chit-DC nanoparticles had no toxicity on hRPE cells and can be used as a drug nanocapsule in this study. Liu *et al*[14] found that there was no significant difference between cytotoxicity and free Cur-coated chitosan/polycaprolactone nanoparticles in human cervical cancer cells, Hela cells, human choroidal melanoma cells, and OCM-1 cells.

In this paper, we synthesized Cur/Chit-DC nanoparticles, and analyzed their properties. It was found that the drug-loading capacity of the Cur/Chit-DC nanoparticles was 27.5%, which was close to the drug-loading capacity (24%) of the Cur albumin nanoparticles prepared by Zhang *et al*[15] by using defusing method. The release of the drugs from nanoparticles revealed two stages[12]: burst release and sustained release. Equilibrium was reached after 96h of release, and the cumulative release amount of Cur/Chit-DC nanoparticles was 31.6%. In the first 20h, the drug release in the sudden release stage reached 19.9%. The slow release of the Cur nanoparticles was confirmed by diffusion release experiments *in vitro*. CCK-8 method was used to detect the proliferation effects of different doses of Cur/Chit-DC nanoparticles and Curs on hRPE cells at different time points. It was found that both Cur/Chit-DC nanoparticles and Cur had inhibitory effects on the cells, but there were differences in the inhibition rate of cell proliferation. In the initial period (1-4d), the inhibition rate of hRPE cells in the Cur/Chit-DC group was lower than that in the Cur group. However, with the prolongation of time, the difference in the inhibition rate of hRPE cells between two groups decreased gradually and the last the two groups showed the same inhibition rate of hRPE cells. Cur, as a traditional Chinese medicine with a long history, has a wide range of pharmacological effects, no obvious toxic, wide range of drug sources and low price. At present, the United States Food and Drug Administration (FAD) has regarded it as a new anticancer drug in the 21st century[16]. In addition, clinical trials have been conducted to gradually study the pharmacokinetics of Cur and the bioeffective dose for cancer treatment[17-20]. The research on its application in ophthalmology mainly focuses on its antiangiogenesis effect, which may play an important role in the treatment of pterygium and diabetic retinopathy[5,21]. Gong *et al*[22] studied the effect of Cur on the proliferation of human embryonic retinal pigment epithelial cells cultured *in vitro*,

and found that Cur could significantly inhibit the proliferation of human embryonic retinal pigment epithelial cells. The results obtained in this study were consistent with the above findings, indicating that the Cur/Chit-DC nanoparticles could inhibit the proliferation of hRPE cells. The sustained release mechanism of nanomaterials is generally considered as follows: 1) the drug connected to or adsorbed on the particle surface is separated from the nanoparticles; 2) the drugs in the nanodrug micelles are constantly diffused outward; 3) the nanomicelles themselves are constantly degraded, and the drugs encapsulated in the micelles are constantly released[23]. In the results of this experiment we believed that the Cur/Chit-DC nanoparticles did not enter cells or a small amount entered cells at the initial stage (1-4d). The reason may be that a small amount of drugs adsorbed on the surface of the nanoparticles fall off from the surface, and the drugs encapsulated in the nanoparticles slowly diffuse outward from the inside, or a small amount of drug nanoparticles entering the cells are degraded by intracellular enzymes to release drugs. Therefore, although both Cur/Chit-DC nanoparticles and Cur have inhibitory effects on hRPE cells. However, the inhibition rate of Cur/Chit-DC nanoparticles on hRPE cells was lower than that of Cur. With the prolongation of the action time, more and more Cur/Chit-DC nanoparticles entered the cells, and the nanoparticles were degraded continuously, the drug release and the concentration of effective drugs on the cells gradually increased. The difference of inhibition rate between the two groups on hRPE cells was gradually narrowed. At the later stage (5-6d), the Cur/Chit-DC nanoparticles were almost degraded, and the cumulative drug concentration reached the maximum, approaching and reaching the level of the action of Cur on hRPE cells. At present, there are many studies on the nano sustained release function of different drugs, but there are few reports on the use of Chit-DC as drug carrier to carry Cur. Li *et al*[24] studied the effect of novel Cur nanoparticles on the proliferation and apoptosis of Lewis lung cancer (LLC) cells and observed that Cur nanoparticles could significantly improve the inhibitory effect of free Cur on the proliferation of LLC cells *in vitro*. Huang *et al*[25] applied 5-FU-PLA-NPS and 5-FU active agents to act on gastric cancer cells respectively in the study of 5-FU immunodrug loading preparation and anti-gastric cancer effect, and observed that the total amount of 5-FU-PLA-NPS released continuously increased with the extension of time, and its effect on gastric cancer cells was continuously enhanced. The results obtained in the above experiments are consistent with the findings of our study, which indicates that the nanomaterials synthesized in the experiments are also ideal long-acting sustained-release drugs.

Apoptosis is an important reason for inhibiting cell proliferation[26-28]. In order to understand the changes of Cur and Cur/Chit-DC nanoparticles on hRPE cell cycle, we used flow cytometry to detect the apoptosis of hRPE cells, and the results showed that in the range of 5-40 μg/mL, the percentage of cells in G0-G1 phase increased and the percentage of cells in S phase decreased in both Cur group and Cur/Chit-DC nanoparticles group, indicating that the hRPE cells enter the cell proliferation stage decreased. Cur can block the hRPE cell cycle in S phase. It may be one of the important reasons why Cur inhibits cell proliferation[29]. Li *et al*[24] studied the LLC cells treated with Cur and Cur nanoparticles at the same concentration for 24h, and the apoptosis rates of LLC cells in Ct group, Cur group and Cur-NPS group were 4.94%, 7.43%, and 67.69%. It was also found that Cur could block LLC cell cycle in S phase. Therefore, the results obtained in this experiment are consistent with the above findings.

Hypoxia can induce VEGF expression in RPE cells both *in vivo* and *in vitro*, and VEGF can promote intraocular angiogenesis[30-31]. Vasquez *et al*[5] found that Cur not only inhibited the upregulation of VEGF in hypoxia-induced RPE cells, but also inhibited the angiogenesis of choroid vascular cells. At the same time, Mrudula *et al*[6] also found that Cur could inhibit the expression of VEGF in the retina of diabetic rats, thus inhibiting fundus neovascularization. We proposed a hypothesis: Can Cur inhibit the proliferation of fundus neovascularization by affecting the secretion level of VEGF in hRPE cells? Whether nano drugs have the same effect? We conducted experiments to clarify these questions. The effects of Cur/Chit-DC nanoparticles and Cur on VEGF mRNA expression level of hRPE cells were detected by semi-quantitative RT-PCR. The results showed that both of them could decrease the expression level of VEGF mRNA in hRPE cells. The results are consistent with the above findings. However, whether Cur nanoparticles can reduce VEGF protein expression level of hRPE cells remains to be confirmed by further study. This study proved a research basis for further preparation of Cur/Chit-DC nanoparticles with targeted function by coupling specific VEGF antibody and *in vivo* animal experiments. It provides a preliminary theoretical basis for finding a new, durable, safe and effective drug for fundus neovascular disease.

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