Original Article

The influence of di-acetylation of the hydroxyl groups on the anti-tumor-proliferation activity of lutein and zeaxanthin

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Lutein and zeaxanthin have various activities such as antiage-related macular degeneration, anticataract, anticancer and cardiovascular diseases risk lowing, etc.; however, few studies have been reported on the role of free hydroxyl groups in the antitumor effects of lutein and zeaxanthin. The structure-activity relationship (SAR) of lutein and zeaxanthin di-acetylation derivatives has been investigated. The lutein and zeaxanthin were purified from corn protein residues and structurally modified by di-acetylation, which was characterized with UV/visible and HPLC/MS spectroscopy. The anti-proliferative effects of di-acetylated lutein or zeaxanthin on the human mouth epithelial cancer line KB cell were compared with controls of their original counterparts. Results showed that the anti-tumor activities of the di-acetylation of lutein and di-acetylation of zeaxanthin decreased significantly ($p<0.05$) at 50 µmol/L. Also the related IC50 dose was increased after di-acetylation. It was suggested that the hydroxyl groups contribute to the anti-tumor activity of lutein and zeaxanthin.

Key Words: zeaxanthin, lutein, the hydroxyl groups, di-acetylatation, anti-tumor activity

Introduction

Numerous epidemiological studies suggest that consumption of carotenoids is associated with lower risks for several types of degenerative diseases in human beings.[1] Many reports have indicated that carotenoids quench singlet oxygen and scavenge free radicals. Therefore, intake of carotenoids could be expected to protect humans against certain types of cancer, cardiovascular and other diseases associated with aging.[2] Lutein and its stereo isomer zeaxanthin, found mainly in dark green leafy vegetables [3] and egg yolks [4], belong to the xanthophyl family, a subclass of carotenoids possessing oxygenated groups. Several reports have revealed the inverse correlations between lutein or zeaxanthin intake and cancer occurrence. Lutein could significantly inhibit the growth of both androgen-dependent (LNCaP) and androgen-independent (PC-3) prostate cancer cell lines *in vitro* [5], and prevent colon carcinogenesis *in vivo*. [6] Our previous studies also showed that lutein and zeaxanthin extracted from corn protein residues played important roles in inhibiting the human mouth epithelial cancer line KB cell proliferation.[7]

It has been proved the antitumor proliference activity of carotenoids significant dependence of anti-oxidative activity. The antioxidant effects of carotenoids were shown to be dependent on the number of conjugated double bonds, the chain structure, and functional groups. However, there are some contradictory results obtained from studies of lutein and the esterification of lutein in the antioxidant effects. According to some reports, although esterification of lutein with fatty acid increased its stabilities against heat and light [8], but had no effect on its antioxidant activity [9], which indicated that the polyene chain in carotenoids was attributable to its related activity. It has also been proved that esterified (monoesterified and diesterified) capsanthins were also good radical scavengers.[10] But it has been reported to have slower rate of corn-corn-triacylglycerid oxidation induced by lutein dimyristate than that by lutein, due to the higher stability of lutein dimyristate.[11]

Carotenoids, which are consisted of over 600 types of terpene compounds, derive from C_{40} isoprenoid via modification of the molecular skeleton by cyclization, substitution, elimination, addition and rearrangement. The minute structural differences are responsible for variations in the biological activities of these compounds.[12] Most reports have dealt with the conjugated double bonds with regard to the biological activities of carotenoids, however, few experimental result have been reported on the role of the hydroxyl groups in the carotenoids on their bioactivities. Hence, in this study we investigate the role of the hydroxyl groups by di-acetylation in the anti-tumor activities of lutein or zeaxanthin extracted from corn protein residues.

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Materials and methods

Design

In this study, the -OHs of lutein or zeaxanthin molecular structures were di-acetylated by chemical methods, and the anti-tumor-proliferation effects of lutein or zeaxanthin di-acetylated derivatives were compared with their natural forms.

Samples

Lutein and zeanxanthin were isolated from raw corn protein residues provided by Zhumadian Pharmaceuticals Factory, Henan Province (P.R. China). The raw corn protein residues were extracted with 100% ethyl alcohol. The extract was dried in a rotary evaporator, dissolved in acetone, and then filtered through anhydrous sodium sulfate. The filtrate was applied onto preparative TLC (immobilized phase: magnesium oxide; eluant: petroleum ether/acetone 65:35 (v:v)). The purity of the lutein and zeaxanthin was identified at >90% and 85% by HPLC, respectively. The purified samples were vacuum dried and stored in the dark at -20℃ for use within two weeks.

Preparation of lutein-3, 3'-diacetate and zeaxanthin-3, 3' diacetate

Freshly distilled Ac_2O (2 ml) was added to the zeaxanthin or lutein solution (163 mg in 6ml pyridine) in a three-necked round-bottom flasks with a magnetic stirrer, a septum and an inlet for N_2, and the reaction mixture with the crystals of 4-(dimethylamino) pyridine was stirred under reflux at room temperature for 12 h, then cooled and poured into ice water. The resulting crystalline residue precipitate was further purified by preparative TLC (ethyl acetate/hexane 1:5) to give red crystals. The modified product thus obtained was characterized by HPLC-MASS [13].The reactions flow charts are shown in Fig 1 and Fig 2.

HPLC-ESI-MS

10 μL sample solution, filtered through a 0.22 μm filter, was injected into the HPLC system (ZMD4000, Waters, America) coupled with C_8 column (symmetry® 2.1×150mm). The mobile phase was 15% methanol (A) and 100% methanol (B) solvent with a flow rate 0.9 ml/min at 35℃, with a gradient from 0% to 100% of B in 20 min. And the detection wavelength of UV/Vis detector was 450nm.

Mass spectroscopy was conducted using following conditions: ESI method; capillary tube voltage: 4.3kV; cone voltage: 60V; ion source temperature: 100℃; and solvent removal temperature: 250℃.

Cell culture

The human mouth epithelial cancer line KB cell obtained from the Shanghai Institute of Cell Biology (P. R. China) was maintained at 37℃ in a humidified atmosphere with 5% CO_2. The medium is composed of RPMI 1640 (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/ml streptomycin. Cells were passaged twice weekly and routinely examined for mycoplasma contamination.

Antiproliferation effects of the samples on KB cells (MTT assay)

The inhibition rate of the samples was determined by MTT assay [14] using the microculture tetrazolium method. Briefly, 5000 cells in logarithmic growth-phase were seeded in 100μL medium in each well of 96-well flat-bottomed microtiter plates (Coster). Lutein, zeaxanthin and their modified products were added at various concentrations to each group in sextuple wells respectively. The final concentration of solvent (ethanol) in the culture medium was <1%, and the culture medium supplemented with fresh samples was added at 24h, 48h respectively, to replace the used culture medium. At 48 h, 72 h MTT labeling mixture (SIGMA) was added and cells were further incubated for 4 h. All culture medium supernatant was removed from wells and replaced with 100 μL of DMSO. Following thorough solublization, the absorbance (A-value) of each well was measured at 570 nm using a microplate (ELISA) reader (Multiskan MK3 Thermo Labsystems CA). All experiments were performed in triplicate. Cell inhibitory rate was calculated according to the formula as follows: Inhibitory rate = [(A value of control group −A value of treated group)/A value of control group] ×100. The IC_{50} of the various samples, defined as the concentration of compound that causes 50% inhibition ratio of the tumor cell tested in 48 h and 72 h, was calculated.

Morphological studies of KB cells treated with the samples

The cultures of the KB cells in 6-well plates (Costar), after exposure to the various samples at concentrations of 40 or 50 μg/ml for 48 h, were washed twice with PBS at
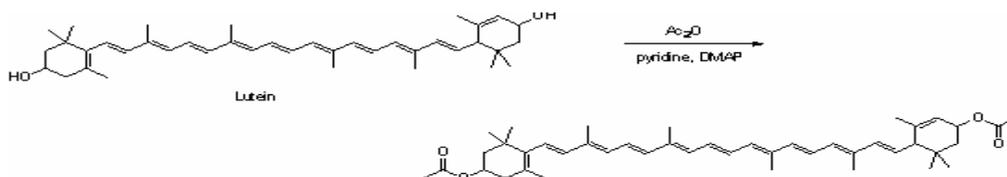
Figure 1. Reaction for Preparation of Lutein Diacetate
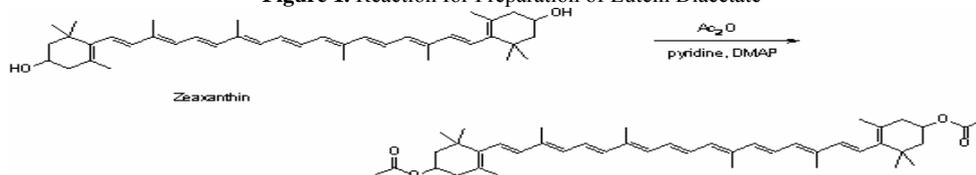
Figure 2. Reaction for Preparation of Zeaxanthin Diacetate

(1)lutein

(2) zeaxanthin

(3) lutein modification product

(4) zeaxanthin modification product
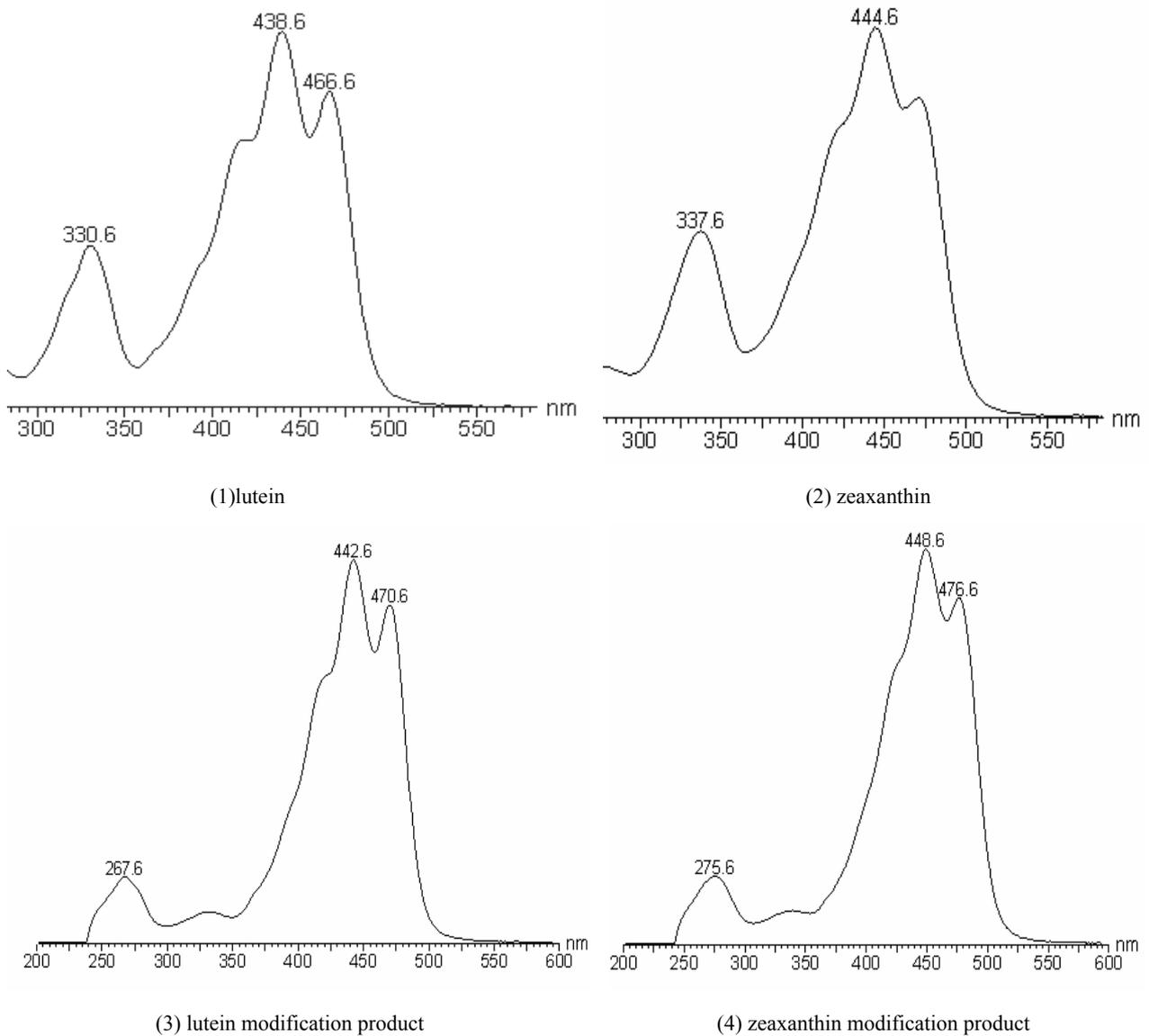
Figure 3. The UV-Visible absorption spectra of lutein-3-3`-diyl diacetate and zeaxanthin-3-3`-diyl diacetate (in alcohol）
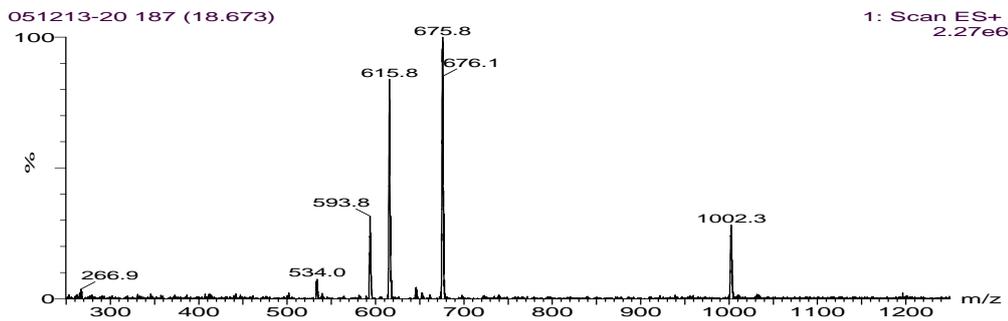
Figure 4. Positive-ion ESI mass spectra of lutein-3-3'--diyl diacetate
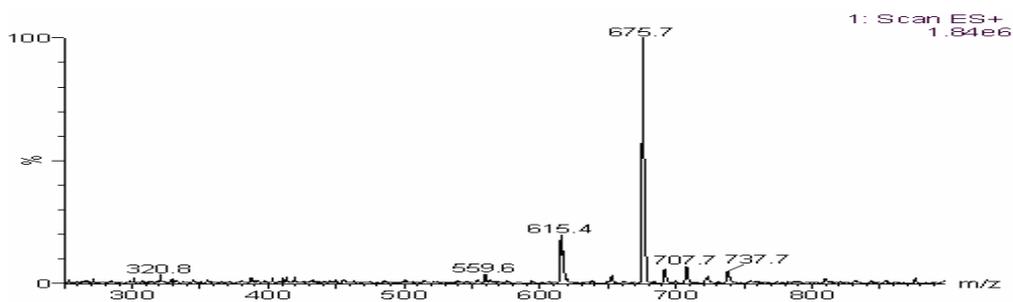
Figure 5. Positive-ion ESI mass spectra of zeaxanthin-3-3'--diyl diacetate

room temperature. After washing with PBS, the morphological changes were observed using phase contrast microscope (Olympus, Tokyo, Japan) at 200×.

Statistical analysis
Microsoft office excel 2003 statistical analysis software was used for statistical analysis. Data are expressed as means ± SD. t-test was used to identify significance of treatment effects on cell proliferation ($p<0.05$).

Results
UV/Visible spectra comparisons between lutein, zeaxanthin and their derivates
The UV/Visible absorbance of the di-acetylated products of lutein and zeaxanthin were presented in Fig 3. The shapes of wavelength of the di-acetylated lutein and di-acetylated zeaxanthin were similar to those of original controls; however, their maximum absorption wavelengths were 442.6 nm and 448.6nm, respectively, which little shift from the original samples [15], which indicated that the di-acetylating reaction of lutein and zeaxanthin have been conducted.

HPLC/Mass Spectroscopy of the di-acetylated lutein and di-acetylated zeaxanthin
Positive-ion ESI mass spectra of lutein and zeaxanthin showed a strong peak at m/z 568.6 (data not shown) that is consistent with the $(M+H)^+$ ion of the compound. Whereas the peak at m/z 568.6 disappeared and peaks at m/z 676.1 [M+23+1, M+H+Na], 675.8[M+23、M+Na] and 1002.3[M+1/2 M+ Na +H] (Fig 4), 675.7[M+23, M+Na] (Fig 5) appeared in the mass spectra of the di-

Table 1. IC_{50} of KB cells by lutein or zeaxanthin or their modification products (μ mol/L)

	lut	di-lut	zea	di-zea
48h	61.0	90.4	60.9	89.6
72h	53.5	70.2	36.5	51.3

acetylated products, which mean that the di-acetylation derivatives of lutein and zeaxanthin, namely, lutein-3-3'-diyl diacetate (molecular weight 652.7) and zeaxanthin-3-3'--diyl diacetate (molecular weight 652.8), were obtained. Purities of the two di-acetylation derivatives were revealed by HPLC at 87.37% and 79.06%, respectively.

Effects on morphology of KB cells by lutein, zeaxanthin and their derivates
The morphological examinations of the KB cells treated with or without lutein, zeaxanthin and their derivates were illustrated using phase contrast microscopy. The control group cells showed a typical polygonal and cobblestone monolayer appearance. Significant decrease of the number of KB cells treated with high concentration of samples for 48 h was observed compared to the control group. Furthermore, the cells treated with samples for 48 h appeared as round-shaped cells poorly adhered to the culture flasks (Fig 6).

Cell inhibitory rate caused by lutein, zeaxanthin and their derivates
Both lutein and zeaxanthin derivatives at low contentrations (20, 30 μmol/L at 48 h) had lower inhibitory rate against KB cells compared with their corresponding natural conterpart, but at higher concentrations (40, 50 μmol/L at 48 h), especially at 50 μmol/L at 48 h, had more significant inhibitory effects on proliferation of KB cells. The inhibitory rate was raised with increase of lutein and zeaxanthin concentration, with the highest inhibition (64.2%) observed in the KB cell line at 50 μmol/L zeaxanthin ($p< 0.05$) (Fig 7).

After 72 h of culturing of KB cells, all the test groups, except the groups with 20 μmol/L lutein and lutein-3，3'-diacetate, showed significant inhibition effects in a concentration-dependent manner. However, at same concentrations, the di-acetylated products of lutein and zeaxanthin showed lower antiproliferation acitivity in KB cells compared with natural lutein and zeaxanthin. At lower

Control   50 μmol/L lutein-3, 3'- diacetate   40 μmol/L zeaxanthin-3, 3'- diacetate

50 μmol/L lutein   40 μmol/L zeaxanthin
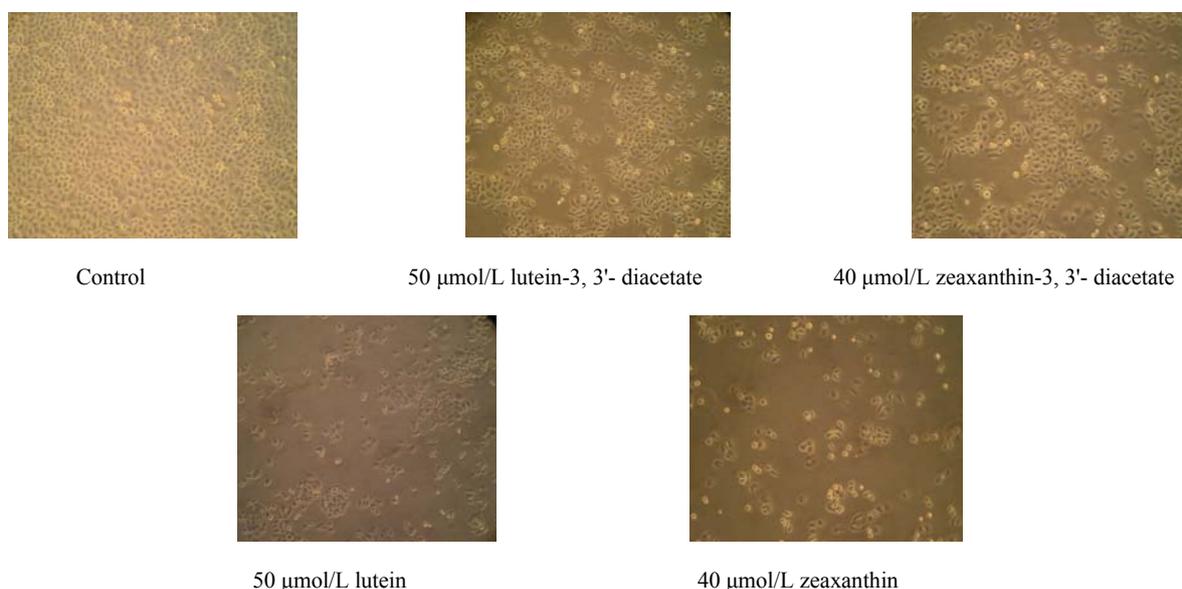
Figure 6. The influence on morphological changes of cells treated with lutein or zeaxanthin by phase contrast microscope (×200)
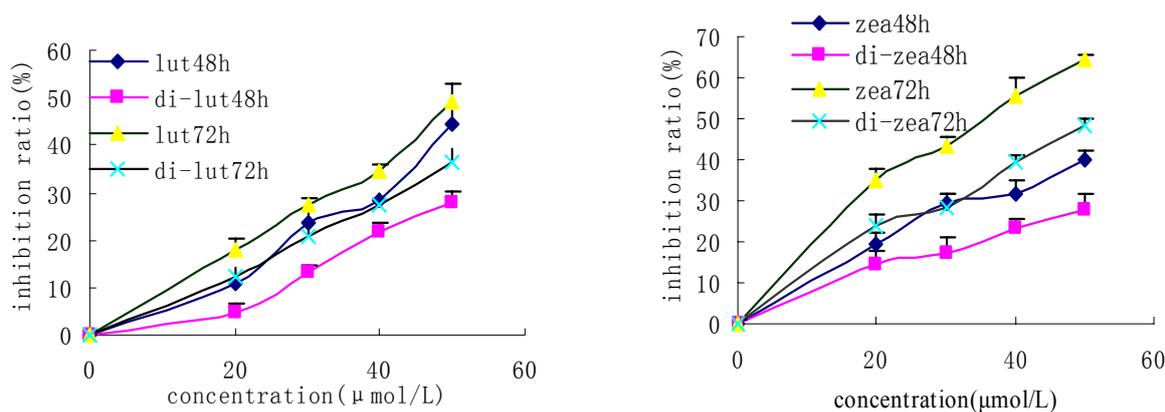
Figure 7. Effect of structure modification of lutein or zeaxanthin on their anti -proliferation activity
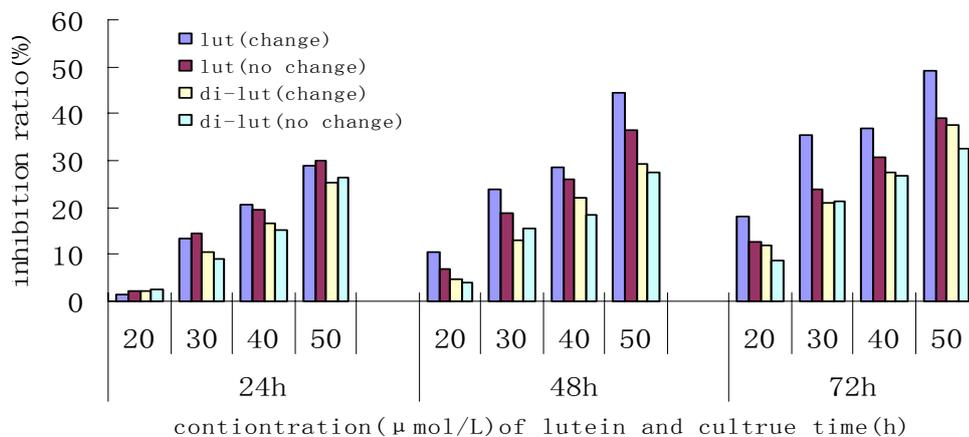
Figure 8. The influence of lutein stability on the proliferation of KB cell

concentrations, the di-acetylated products of lutein and zeaxanthin did not exhibit significant difference in inhibitory effects on KB cell proliferation. In this study, only 50 μ mol/L zeanxanthin after 48 h, 72 h of culturing of KB cells or 50 μ mol/L lutein could significantly inhibit the proliferation of KB cells ($p<0.05$).

IC_{50} change of KB cells caused by lutein, zeaxanthin and their derivates

Viability of KB cells exposed to lutein, zeaxanthin and their derivates for 48 h and 72 h decreased linearly with decrease in sample concentrations, and the IC_{50} values, calculated using the linear regression equation, were shown in Table 1. Results showed that sensitivity of samples on the KB cells was time-dependent, and the ranking order of cellular sensitivity towards the test tumor cells was: zeaxanthin > lutein > di-acetylated zeaxanthin > di-acetylated lutein. The data indicated the IC_{50} value of lutein or zeaxanthin was lower than corresponding di-acetylation products.

The influence of sample stability on the proliferation of KB cell

Lutein and zeaxanthin are heat- unstable, and also light sensitive and easily oxidized therefore it is necessary to carry out each above-mentioned experiment with a fresh sample.

In the next experiment we used lutein and its di-acetylated products, to turn an outcome, adopt to keep on development in the continuous development, add kind once to investigate the stability of the sample to the in-

fluence that the KB cell proliferation (Fig 8).

It was observed that at 37 ℃ and under 5% CO_2 conditions, the sample increased the influence of the free lutine most strongly. If a fresh sample was not used, after 72 hours, 50 μ mol/L of lutine reducing the KB cell rate will lower by 15.4 %, but the concentration of lutine di-acetate ester to repress the ratio of the KB cell will lower only by 6 %.

Discussion

Multiple conjugate double bonds exist in carotenoids, which confer specific biological characteristics on the family of carotenoids. On introduction of oxygenated groups including hydroxyl groups onto the six-element ring, however, ionone-ring-containing carotenoids gain stronger free radical scavenging capacity. Further investigations proved the significant dependence of anti-oxidative activity on the orientation of carotenoids within the cell membrane. Furthermore, the orientation of carotenoids depends on the molecular structure.[16] β-carotenoid is a kind of non-polar member of carotenoids, which occurs in the hydrophobic moiety of bi-layer lipid membrane in parallel to the membrane. When peroxidation is induced in the membrane surface, β-carotenoid has lower capturing efficiency towards chain transmitted free radicals.[17] While introduction of hydroxyl groups into lutein or zeaxanthin makes the latter project through the bi-layer lipid membrane and vertically riveted onto the membrane surface [18], so that their polar hydroxyl groups are located on both hydrophilic surfaces of the membrane to capture free radicals attacking the membrane surface with higher

efficiency.[19]

Fig 1, 2 shows the reaction route of lutein, zeaxanthin to their diacetate esters. Lutein is an α-carotene derivative, which has two different ionone rings: β- and ε-ionone rings. Zeaxanthin is a β-carotene derivative, which has two same ionone rings: β-ionone rings. In each ionone ring, two -OH groups are bonded with carbon atom number 3 and 3', respectively. However in this study, we only investigated the antitumor activities of lutein and zeaxanthin and their diacetate esters.

Di-acetylated products of lutein and zeaxanthin showed decreased anti-proliferation activities and little morphological disturbance towards KB cells under certain concentrations. Theoretically, di-acetylation of the ionone ring –OH groups reduces the polarity of lutein or zeaxanthin molecules, which in turn alters the existence of the latter in the cell membrane. Free lutein and zeaxanthin contact the cell in a trans-membrane mode to induce apoptosis of tumor cells; while diacetated lutein and diacetated zeaxanthin might act on receptors located on the membrane surface to reduce the possibility of inducing the apoptosis of tumor cells.

The further research express that lutein and zeaxanthin are very unstable to heat, under prolonged incubation times displaying the development of the tumor cell, it oxidized as a result in to propagate the ability of repress to descend to the tumor cell towards foster under condition of because single body, therefore, the general demand replaces experiment with the fresh and single body everyday.

The diacetated lutein and diacetate zeaxanthin with hydroxyl groups can increase the stability to light, oxygen and the temperature, make up because of the hydroxyl polish result in repress the KB cell proliferation ability to die down.

Carotenoids without pro-vitamin A activity still show biological and pharmaceutical activity, which is primarily attributed to their physical and chemical properties. Such properties are determined by the long, highly conjugated system of double bonds in the carotenoid molecule. For effective physical interaction of the carotenoid molecule with lipids in a lipid bilayer membrane, the presence of polar groups on both ends of the long, stick-like molecule as well as the ratio of the carotenoid molecule length to the membrane thickness is also very important.

In conclusion, we have first investigated the anti-proliferation activity of lutein and zeaxanthin and their di-acetylation derivates on the KB tumor cells and proved that the hydroxyl groups made significant contribution to the anti-tumor-proliferation activity of zeaxanthin and lutein molecules.

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