Carotenoids Suppressed Proliferating Cell Nuclear Antigen and Cyclin D_1 Expression in Oral Carcinogenic Models

Hsin-Chung Cheng[a], Hsin Chien[b], Chia-Hui Liao[b], Yi-Yuan Yang[c], Shih-Yi Huang[d]*

[a]Orthodonic Department, Taipei Medical University Hospital,

[b]School of Pharmaceutical Science,

[c]School of Medical Technology and Biotechnology,

[d]School of Nutrition and Health Sciences, Taipei Medical University, 110, Taipei, Taiwan

* To whom correspondence should be addressed.

Address: 250 Wu-Hsing Street, School of Nutrition and Health Sciences, Taipei Medical University, Taipei, 110 Taiwan

Phone: 886-2-27361661 ext. 6551~120

Fax: 886-2-27373112

E-mail: sihuang@tmu.edu.tw

Abstract

The purpose of this study was to investigate the chemopreventive effect of carotenoids on proliferating cell nuclear antigen (PCNA) and cyclin D_1 expression in the betel quid extract (BQE)-induced hamster oral cancer and human KB cell models, respectively. In animal study, forty-one hamsters were divided into six groups and were treated with 0.3 mL of 0.5% of DMBA, BQE, α-tocopherol, β-carotene, lycopene, lutein, and a mixture of carotenoids for 12 weeks. After treatment, the pouches were excised and graded using an immunohistochemical assay of PCNA. In cell experiment, KB cells were cultured and evaluated the inhibitory effect of carotenoids (β-carotene, lycopene and lutein) on cell proliferation. Cyclin D_1 and PCNA were evaluated as cell differentiation. In results, most of the animal lesions showed no overexpression of PCNA. However, in the dysplastic lesions, PCNA expressions of the β-carotene, lutein, lycopene, mixture, and vitamin E groups were less than that of the control group. In papilloma lesions, PCNA expressions of the β-carotene, mixture, and vitamin E groups were less severe than of the control group. PCNA expressions of vitamin E-treated group were less severe than that of the controlled group. No carcinoma was found in the lycopene and mixture groups. In cell study, all carotenoids exerted a significant inhibitory effect on KB cell proliferation. Although lycopene suppressed KB cell proliferation at G_0/G_1 phase with significant decrease of PCNA expression, β-carotene and lutein possessed less inhibited effect and even elevated the cell proliferation at G_2/M phase. These results indicate that differ carotenoids presented the various suppressive ability of PCNA and cyclin D_1 expression in cell proliferation. In conclusion, carotenoids suppressed the carcinogenesis of induced hamster oral cancer and cancer cell line by acting as a suppressor for inhibiting the expression of PCNA and cyclin D_1.

Key words: carotenoids, betel quid extract, oral cancer, proliferating cell nuclear antigen, cyclin D_1.

1. Introduction

Betel quid chewing is thought to contribute to leukoplakia, submucosal fibrosis, and oral cancer in Taiwan, India, and many Southeast Asian countries [1]. The traditional formula of the Taiwanese betel quid contains the tender areca nut, slaked lime, catechu (an astringent), inflorescence of the piper betel, or piper betel leaves. Some studies have reported that the extract of areca quid is associated with the onset of early malignant changes in hamster cheek pouch [2,3]. The components of betel quid accelerate the proliferation and differentiation of oral mucous cells, especially in combination with slaked lime and the nitrosative products of areca nut -- arecoline (i.e., guvacoline, guvacine, and arecaidine) [4]. One of the possible mechanisms of submucosal fibrosis might be the elevation of reactive oxygen species and then induction of DNA damage in vitro. However, the mechanism of BQ chewing induced oral cancer still remains unknown.

The immunohistochemical assay of proliferating cell nuclear antigen (PCNA) has been utilized by many investigators as a useful marker of cell proliferation and differentiation. Proliferating cell nuclear antigen (PCNA) is one of the helper proteins of DNA polymerase σ, which is a key protein in cell cycle regulation [5,6] and a trigger in cell proliferation and differentiation [7,8]. Some studies have indicated that PCNA shows different expression degrees in normal, precancerous stage, and malignant tissues: the greater the degree of metaplastic tissue, the greater the PCNA expression is. In cancer progression, e.g., from normal mucosal tissues to metaplasia, then dysplasia to squamous cell carcinoma in neck and oral tissues, PCNA expression is elevated [9], and the assay can be used to diagnose throat cancer progression [10]. Cyclin D_1 was an important nuclear protein in G_1/S phase of cell cycle. Bova and colleagues revealed that the cyclin D_1 overexpression and cyclin D_1 gene amplification were found in the patients with oral squamous cell carcinoma. It implied that the cyclin D_1 expression may be as an early indicator in oral carcinogenesis [11].

In our previous studies, betel quid was shown to have a promoter role during the carcinogenesis of hamster buccal pouch carcinoma, and carotenoids presented inhibitory effect in induced hamster oral carcinogenesis [12,13]. Carotenoids have been indicated to present a strong ability to protect against active oxygen species in cells. Carotenoids were common lipotrophic phytochemicals in nature which prevent cancers by interfering protein expression in cell cycle. Dietary carotenoids may serve in lowering the risk of cancer in humans based on their reported antioxidant capability to quench singlet oxygen and other oxidizing species. If such a free radical mechanism is involved in the initiation and promotion of carcinogenesis, carotenoids may undergo oxidation in order to protect against cellular oxidative damage. Thus, In this study, we investigated the chemopreventive effect of carotenoids on proliferating cell nuclear antigen (PCNA) and cyclin D_1 expression in the betel quid extract (BQE)-induced hamster oral cancer and KB cell (human oral epidermoid carcinoma cell line) models, respectively.

2. Materials and methods

2.1 Animal Protocol

During the experiment, forty-one 8-week-old adult male hamsters (National Science Council, Taipei, Taiwan), weighing between 110~120 g, were housed under a 12-h light-dark cycle, a temperature- (22~24°C) and humidity-controlled (60%) room. Rodent chow (*Labdiet* # 5001) and water were received *ad libitum*.

2.1.1 Experimental design

Animals were divided into six experimental groups and were treated with 0.3 mL 0.5% of the experimental agents, which included 9,10-dimethyl- 1,2-benz[a]-anthracene (DMBA), betel quid extract (BQE), α-tocopherol, β-carotene, lycopene, lutein, and a mixture of equal amounts of these carotenoids, respectively. In the 16-week experimental period, the buccal pouches of animals in the test groups were initially daubed with DMBA for 4 weeks. In the subsequent 12 weeks, groups were daubed with either BQE or a mineral oil solution containing carotenoids three times per week on alternate days. The process of manufacturing BQE was modified from the description by Lin et al. [14]. Vitamin E and carotenoids were purchased from Roche (Nutley, NJ, USA). All chemicals used in this study were obtained from Sigma (St Louis, MO, USA). The components of BQE were purchased from a local market.

2.1.2 Sample preparation

At the end of the experiment, all animals were anesthetized under a CO_2 atmosphere. Hamster buccal pouch carcinoma and esophageal tissues were removed, extended, and fixed on a 25-cm^2 paperboard under a 10% formaldehyde solution for at least 24 h. The tumor number and volume of the tissue lesions were recorded and photographed. After the paraffin fixation process, the tissue lesions were sliced into 5-μm sections under poly-L-lysine treatment for further immunohistochemical staining and were evaluated by a senior pathologist.

2.1.3 Immunohistochemical Staining of PCNA

For immunohistochemical staining analyses, tissue sections cut from the paraffin blocks were first deparaffinized and rehydrated in graded alcoholic solutions and phosphate buffered saline (PBS). The specific antibody staining for various antigens (LSAB2 System, HRP, DAKO, Carpinteria, CA, USA) were used. Primary antibody incubations of PCNA (DAKO-PCNA

PC10 monoclone antibody) were carried out at room temperature for 45 min with non-immune mouse IgG; after careful washing, sections were incubated in biotinylated secondary immunoglobulin of appropriate species specificity (Sigma; diluted with PBS at 1:200) for 30 min at room temperature. After incubation with the secondary antibody, sections were washed in PBS three times for 10 min each and then incubated with an avidin-biotin complex for 30 min at room temperature. Histochemical localization was accomplished using an avidin-biotin horseradish peroxidase complex (DAKO Liquid DAB+ Substrate-Chromogen System) with DAB (3-3' diaminobenzidine tetrahydrochloride) as the chromagen. Normal goat serum was applied to the sections for 30 min to bind nonspecific sites. After immunohistochemical staining, tissue sections were counterstained with hematoxylin. Control sections were processed in parallel with mouse nonimmune IgG at the same concentration as the primary antibodies. After sectioning and staining, multiple degrees (score, 1~5) were examined to determine the percentage of epithelial cells showing positive immunoreactivity against PCNA. For each individual degree, the percentage of expressing cells was counted in five separate, low-magnification microscopic fields.

2.2 KB Cell culture

2.2.1 Chemicals

Bovine serum albumin (BSA), tetramethylene diamine (TEMED), dimethyl sulfoxide (DMSO), β-carotene and lutein were commercially obtained from Sigma Chemical (St. Louis, MO) while lutein needed partial purification. Lycopene, propidium iodium and ribonuclease A (RNase A) were purchased from MP Biomedicals, France. Minimum essential medium (MEM), fetal bovine serum (FBS), trypsin/EDTA solution and penicilline-streptomycin were purchased from GIBCO (Grand Island, NY). Cyclin D_1 mouse monoclone IgG_1 antibody and PCNA mouse monoclone $IgG_{2\alpha}$ antibody and goat anti-mouse IgG-HRP antibody were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). β-Actin mouse monoclone IgG1k antibody was offered from Chemicon International, Inc. (Temecula, CA). Absolute ethanol was purchased from Merck (Darmstadt, Germany). All other laboratory chemicals were of the highest quality available and were purchased from Sigma, TEDIA (Fairfield, OH) and USB (Cleveland, OH).

2.2.2 Cell culture condition

Human KB cell line (human oral epidermoid carcinoma cell line, American Type Culture Collection number [ATCC]: CCL-17) originated from the Food Industry Research and Development Institute, Hsin-Chiu, Taiwan. Cells were grown as monolayers in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 100 IU/ml of penicillin-streptomycin in an atmosphere of 95% air and 5% CO_2 at 37°C. β-Carotene, lycopene and lutein were dissolved in absolute ethanol, and the concentration of absolute ethanol added to the media never exceeded 0.2% (v/v). Because β-carotene, lycopene and lutein (5, 10, 20, & 30 μM) showed no significant cytotoxicity, these concentrations were used in this study.

2.2.3 MTT assay

The inhibition of carotenoids on KB cell proliferation was determined by the below described MTT assay [15], where in cells were plated onto 96-well tissue culture dishes at density of 5×10^3 $well^{-1}$ in 100 μL medium. After plating, the cells were allowed to attach for 24 h. Cells were incubated with various concentrations of the agents for 24 h, at which time 20 μL of 2 mg/mL MTT was added, and the absorbance at 492 nm was determined by a microtiter plate reader. The extract was added using THF as the vehicles, at maximum concentration of 0.1%. Live cells convert 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2- (4-sulfophenyl)- 2H-tetrazolium (MTS) to a formazan dye that can be detected at 492 nm by a microplate reader. The absorbance at 690 nm was also measured as reference. The same aliquot of carotenoid-free emulsion alone was added to the control cells. Experiments were conducted in six times.

2.2.4 Protein Extraction and Western Blot

At the termination of cultures, the treated KB cells were incubated at 4°C for 60 min in the presence of lysis buffer consisting of 150 mM/L NaCl, 2 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, 1% sodium deoxycholate (DOS), 1 mg/L aprotinin, 0.1% sodium dodecylsulfate (SDS) and 50 mM Tris-HCL (pH 7.4). Cells were subsequently scraped off the plates. The lysates were centrifuged at 4°C, 15,000×g for 15 min and the supernatants were collected. Protein contents in the supernatants were determined using a protein assay kit (Bio-Rad Dc Protein Assay Reagent; Bio-Rad Laboratories, USA). Proteins (50 μg) were subjected to 12.5% SDS-PAGE and electrophoretically transferred to a polyvinylidene fluoride membrane (Immobilon-P, Millipore, Bedford, MA). Membranes were blocked with nonfat dried milk and immunoblotting was done using anti-cyclin D_1 mouse monoclone IgG_1 antibody (1:500) (Santa Cruz Biotechnology), anti-PCNA mouse monoclone $IgG_{2\alpha}$ antibody (1:2000) (Santa Cruz Biotechnology) and anti-β-actin monoclone IgG_{1K} antibody (1:1000) (Chemicon) in Tween 20 base saline buffer (PBS-T). Membranes were probed with a goat anti-mouse IgG-horseradish peroxidase antibody (Santa Cruz Biotechnology) as a second antibody. The antigen-antibody complexes were detected with the secondary antibodies using an ECL chemiluminescence detection system (Amersham Pharmacia Biotech). Membranes were visualized by exposure to X-Omat film (Eastman Kodak Co., Rochester, NY). The experiments were repeated triplicate analysis in different cultured specimens with the similar results, and the reported results are representative.

2.2.5 Cell Cycle Analysis

Cell sample preparations for cytometry analysis were performed according to the method reported where else [16]. Cell cycle distribution was determined using a FACScan laser flow cytometer (Becton Dichenson, San Jose CA). Cells were trypsinized, collected, and washed twice with PBS. The pellets were resuspended in 1 mL of PBS, fixed in 2 mL of ethanol-PBS solution (75/25), and stored at -20°C. Cells were washed twice with PBS, then incubated for 30 minutes in 1 ml of PBS containing and 3 μL ribonuclease A (Rnase A, 10 mg/mL) at room temperature. One mL propidium iodide (PI, 40 μg/mL) was added, and the suspension was incubated in the dark at room temperature for 30 min and was then filtered through a 60-μm mesh filter. The percentage of cells in different phases of the cell cycle was determined by flow cytometry with a ModFit (Verity Software House) computer program.

2.3 Statistical analysis

Values are presented as the mean ± SD. All data were analyzed using the SAS system. ANOVA with least significant difference test was performed to analyze differences among different ethanol durations. The acceptable level of significance was established at $p < 0.05$ except when otherwise indicated.

3. Results

3.1 Effect of Carotenoid on PCNA Expression in Hamster Buccal Pouch Lesion

The results indicated that most of the normal tissues showed low expression of PCNA. The expression of PCNA merely appeared in the basal layer of the epithelium. In basal layers, the expression of PCNA was significant, especially in peripheral cells of the basal layer. The expression of PCNA was obvious in lesions with dysplasia, carcinoma and hyperplasia and papilloma in the control group. Cells along the in border of the tumor and anterior tissue of invasive carcinomas also showed significant positive expression of PCNA. Precancerous lesions such as hyperplasia, dysplasia and keratocytes showed strong PCNA expression, especially in lesions with papilloma and squama cell carcinoma. The PCNA expression with carotenoid treatment is summarized in Table 1.

Figure 1A~D shows morphologic changes in lesions treated with mixed carotenoids. In the control, cells showed disorientation during proliferation and chromosomal aberrations, such as micronuclei and sister chromatid exchanges. Under the low microscopic field, lesions appeared dysplastic (Fig. 1A). Not surprisingly, lesions exhibiting PCNA expression showed inconsistencies (Fig. 2A~D). In hyperplastic lesions, PCNA expressions of the lutein, lycopene, mixture, and vitamin E groups were less severe compared with that of the control group. In papilloma lesions, PCNA expressions of the β-carotene, mixture, and vitamin E groups were less severe compared with that of the control group. In dysplastic lesions, PCNA expressions of the carotenoid and vitamin E groups were less severe compared with that of the control group, but in carcinoma lesions, only the PCNA expression of the β-carotene group was less severe than the of control group. There was no carcinoma production in the lycopene and mixture groups, thus, no PCNA expression in those groups. For most of the lesions, vitamin E and β-carotene showed inhibitory effects on PCNA expression in buccal mucosal lesions.

3.2 Carotenoid on PCNA and Cyclin D_1 Expression in KB Cells

3.2.1 MTT assay

The inhibitory effects of carotenoids (β-carotene, lycopene and lutein) on the growth of KB human oral tumor cells were detected in 24 h (Fig. 2). The carotenoid solvent (THF) did not affect KB cell growth relative to growth in the control media. The cell-growth inhibition induced by carotenoids presented dose-dependent manner. Surprisingly, the cell-growth suppression induced by lutein (30 μM) was significantly more potent than that by β-carotene and lycopene. β-Carotene and lycopene presented the similar concentration-dependent inhibitory effect on cell proliferation. The lowest dose (5 μM) of ß-carotene and lycopene caused 12 ± 7% and 21 ± 9% inhibition, respectively, the highest dose (30 μM) resulted in a much higher and significant inhibition (35 ± 8% and 37 ± 10%) after 24 hr. A lutein concentration of 5 μM inhibited growth by 21 ± 9% after 24 hr of incubation, whereas the highest concentration (30 μM) led to 45 ± 8% inhibition after this time (Fig. 3).

3.2.2 Carotenoids on PCNA & Cyclin D_1 expression

The inhibitory effect of carotenoids on KB cell was additionally confirmed using the immunohistochemical marker, PCNA. PCNA/cyclin regulates the initiation of cell proliferation by mediating DNA polymerase and is elevated in the G_1 and S phases of cell cycle. Under cultural conditions, lycopene remarkably suppressed the PCNA expression KB cells in higher doses (10 μM ~ 30 μM) statistically. However, β-carotene and lutein presented the less inhibitory effects on PCNA expression beyond 20 μM. THF did not affect PCNA expression. Determination of PCNA expression in control and treated cells demonstrates that lycopene did affect proliferation in KB cells in dose-dependent manner (Fig. 4). Surprisingly, β-carotene and lutein suppressed the cyclin D_1 expression in dose-dependent manner but no in lycopene group (Fig. 5). THF did not affect PCNA expression. Although THF affected the cyclin D_1 expression in lycopene group, the higher concentrations did not show any inhibitory effectiveness.

3.2.3 Carotenoids on cell cycle progression

The cell cycle progression of KB cell under various carotenoids treatment was shown in Figure 6. There were no significant differences in any concentration while treated with β-carotene and lutein, however, lycopene (20 μM and 30μM) presented the significant alteration in the phase of G_0/G_1 and G_2/M.

Discussion

In this study, PCNA expression obviously showed dramatic alterations in each cancer stage. Due to the cancer development process, the morphologic changes in lesions are in progressed; nevertheless, PCNA expression presented more activity in lately stages such as the transformation and translocation stages. The expression of PNCA was lower in the vitamin E and β-carotene groups than in the control, but PCNA presented overexpressed action in most of the developing cancerous lesions. The invasive and metastatic potential of carcinomas has been considered to be regulated by alterations in cell morphology and motility [17]. Compared to HE staining, there was no such difference between groups, in contrast to specific protein expression (such as PNCA and cytoskeletons), while the control showed much protein expression, especially in the late stage of cancerous cell proliferation and displayed various extents levels of tissue disorientation.

Meanwhile, PCNA expression was found not only in the lesions of papilloma, dysplasia, and squamous cell carcinoma, but also in normal basal cells and peripheral tissues. Thus, from the viewpoint of cell differentiation, some cells of the suprabasal layer had proceeded and even bypassed normal cell-cycle regulation. Cell proliferation by normal cells and squamous cell carcinoma showed little PCNA expression; the reason for this situation could have been that cancer cells released some transforming growth factors or epithelium growth factors, which stimulated irregular cell differentiation or activated some DNA excision-repair actions [5,6]. Betel quid extract (BQE) has been demonstrated to promote oral carcinogenesis, it shows strong genotoxicity, and DNA breakage may contribute to the major cause of oral carcinoma [18,19]. Thus, it is suggested that the expression of PCNA is highly correlated with the genotoxicity of DNA and hyperproliferation of buccal mucosal tissues [20]. PCNA was expressed to a greater extent in the control group than in the experimental groups in our study. These results could imply two aspects of clinical significance: 1) some components of betel quid extract contribute to PCNA's overexpression via genotoxicity; and 2) carotenoids attenuated the DNA-breaking process and PCNA expression, and thus may provide a chemopreventive effect against oral carcinogenesis.

The results indicated that buccal and esophageal histological symptoms (data not shown) in the mixture group were less severe compared with those of the control group. With DMBA induction, the BQE intervention indeed promoted the development of oral cancer. Carotenoids effectively inhibited the development of oral mucosal malignant tumors, especially in the lycopene and the mixture group. Compared with the esophageal and buccal mucosal pathology, there were fewer buccal mucosal tumors than esophageal mucosal tumors. However, the volume of tumors in the esophagus was smaller than that of buccal tumors. Furthermore, the buccal mucosal tumor burden was much greater than that in the esophagus. From the aspect of carotenoids preventive effect ability, the tumor number and burden in the esophagus and the buccal mucosa were the most severe in the control group. The application of carotenoids significantly inhibited the development of both esophageal and buccal tumors, especially in the mixture group. Kozuki et al. reported the anti-invasive effects of carotenoids on rat ascites hepatoma AH109A cells [21]. Cancer cells cultured with hypoxanthine and xanthine oxidase showed highly invasive activity. β-Carotene and astaxanthin, at up to 5 μM, suppressed the reactive oxygen species-potentiated invasive capacity. The results suggested that the antioxidative property of carotenoids may be involved in this anti-invasive action.

The impact of carotenoids on cell proliferation has been discussed [22-24]. Cellular micronuclei have been suggested that involved in the DNA damage and replicated as end products. β-Carotene (10 μM) has been reported that indeed decreased the prevalence of micronuclei in KB cell [22]. Livny et al., (23) indicated the lower lycopene concentration (7 μM) possessed the suppressed capability on KB cell proliferation. Another study showed alternative anti-cancer actions. β-Carotene (1 μM) and its derivatives (9-cis β-carotene isomers, 10 μM) have suppressing effects on proliferation and gene expression in murine 10T1/2 cells and human HaCaT keratinocytes [24]. The 9-cis isomer was less active than all-trans β-carotene in reducing proliferation and in upregulating expression of connexin 43 in 10T1/2 cells and suppressing expression of keratin K1. Surprisingly, the 9-cis isomer of retinoic acid was approximately 10-fold more active in suppressing neoplastic transformation and inducing connexin 43 expression in both cell types than the all-trans. In our study, carotenoids represented the significant inhibitory effect on the cell proliferation and showed with dose-dependent manner. β-Carotene showed the suppressive action on S phase and stayed on G_1 phase of cell proliferation in fibroblasts [25]. Nahum et al., [26] also reported the lycopene suppressed the cyclin D_1 expression, cyclin-dependent kinase 4 (cdk-4) activity, and retinoblastoma protein (pRb) phosphorylation of breast (MCF-7) and endometrial cancer cells.

It is noteworthy that lycopene showed greater chemopreventive effectiveness than other carotenoids in this study, especially on PCNA expression. Cell-cell interaction via gap junctions has been considered to be a key factor in tissue homeostasis, and its alteration is associated with the neoplastic phenotype. Lycopene up-regulates both the transcription and expression of connexin 43 [27]. The report implied that the pattern of cellular uptake and incorporation into cancer KB-1 cells differed between the carotenoids. β-Carotene was rapidly incorporated into KB-1 cells, whereas lycopene uptake into the cells took place after longer incubation periods and only at the highest concentrations. Regarding to the change in PCNA expression correlated with the phase of the cell cycle that the agents acted upon. In our study, although lycopene suppressed KB cell proliferation at G_0/G_1 phase with significant decrease of PCNA expression, β-carotene and lutein possessed less inhibited effect and even elevated the cell proliferation at G_2/M phase. These results indicate that differ carotenoids presented the various suppressive ability of PCNA and cyclin D_1 expression in cell proliferation. Furthermore, with the exception of its cellular regulatory property, lycopene also demonstrated strong free radical-quenching ability. In some *in vitro* studies, lycopene possessed the most-powerful quenching capacity for singlet oxygen. Lycopene also showed an ability to deactivate some free radicals such as H_2O_2, NO_2, RS and RSO_2 [28]. Such potential mechanisms of the anticancer reveal the molecular regulation aspect of carotenoids may contribute the major role in these chemopreventive actions.

In conclusion, carotenoids suppressed the carcinogenesis of induced hamster oral cancer and cancer cell line by acting as a suppressor for inhibiting the expression of PCNA and cyclin D_1. The results suggested that diverse anticancer characteristics of carotenoids may involve in the cell cycle progression.

Acknowledgements

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References

1. Stich HF, Mathew B, Sankaranarayanan R, Nair MK. Remission of precancerous lesions in the oral cavity of tobacco chewers and maintenance of the protective effect of beta-carotene or vitamin A. Am J Clin Nutr 1991;53:298S-304S.
2. Suri K, Goldman HM, Wells H. Carcinogenic effect of a dimethyl sulphoxide extract of betel nut on the mucosa of the hamster buccal pouch. Nature 1971;230:383-4.
3. Odukoya O, Shklar G. Two-phase carcinogenesis in hamster buccal pouch. Oral Surg Oral Med Oral Pathol 1982;54:547-52.
4. Pool P. In: Parkin DM, Whelan SL, Ferlay J, Raymond LA, Young J, editors. Cancer incidence in five continents. IARC Science Publication No. 143 vol. VII. IARC, 1997.
5. McCormick D, Hall PA. The complexities of proliferating cell nuclear antigen. Histopathology 1992;21:591-4.
6. Hall PA, Coates PJ, Goodlad RA, Hart IR, lane DP. Proliferating cell nuclear antigen expression in non-cycling cells may be induced by growth factors in vivo. Br J Cancer 1994;70:244-7.
7. Bravo R, Frank R, Blundell PA, Macdonald-Bravo H. Cyclin/PCNA is the auxiliary protein of DNA polymerase-delta. Nature 1987;326:515-7.
8. Jaskulski D, deRiel JK, Mercer WE, Calabretta B, Baserga R. Inhibition of cellular proliferation by antisense oligodeoxynucleotides to PCNA cyclin. Science 1988;240:1544. Science 1988;240:1544-6.
9. Shin DM, Voravud N, Ro JY, Lee JS, Hong WK, Hittelman WN. Sequential increases in proliferating cell nuclear antigen expression in head and neck tumorigenesis: a potential biomarker. J Natl Cancer Inst 1993;85:971-8.
10. Krecicki T, Jelen M, Zalesska-Krecicka M, Rak J, Szkudlarek T, Jelen-Krzeszewska J. Epidermal growth factor receptor (EGFR), proliferating cell nuclear antigen (PCNA) and Ki-67 antigen in laryngeal epithelial lesions. Oral Oncol 1999;35:180-6.
11. Bova RJ, Quinn DI, Nankervis JS, Cole IE, Sheridan RL, Jensen MJ, Morgan GJ, Hughes CJ and Sutherland RL (1999) Cyclin D_1 and $p16^{INK4a}$ expression predict reduced survival in carcinoma of the anterior tongue. Clin Cancer Res 5:2810-9.
12. Chien H, Huang SY, Lin YH, Hsieh YS, Hsieh MJ, Shieh MJ. The preventive effect of carotenoids on DMBA induced oral carcinoma in male hamsters. J Nutr Sci 2000;25:199-206. [in Chinese]
13. Bhuvaneswari V, Velmurugan B, Balasenthil S, Ramachandran CR, Nagini S. Chemopreventive efficacy of lycopene on 7,12-dimethylbenz[a]anthracene- induced hamster buccal pouch carcinogenesis. Fitoterapia 2001;72:865-74.
14. Lin LM, Chen YK, Lai DR, Huang YL, Chen HR. Cancer-promoting effect of Taiwan betel quid in hamster buccal pouch carcinogenesis. Oral Dis 1997;3:232-5.
15. Twentyman PR, Fox NE, Rees JK. Chemosensitivity testing of fresh leukaemia cells using the MTT colorimetric assay. Br J Haematol 1989;71:19-24.
16. Hotz MA, Gong J, Traganos F, Darzynkiewicz Z. Flow cytometric detection of apoptosis: comparison of the assays of in situ DNA degradation and chromatin changes. Cytometry 1994;15:237-44.
17. Volk T, Geiger B, Raz A. Motility and adhesive properties of high- and low-metastatic murine neoplastic cells. Cancer Res 1984;44:811-24.
18. Sundqvist K, Liu Y, Nair J, Bartsch H, Arvidson K,

Grafstrom RC. Cytotoxic and genotoxic effects of areca nut-related compounds in cultured human buccal epithelial cells. Cancer Res 1989;49:5294-8.
19. Dave BJ, Trivedi AH, Adhvaryu SG. Role of areca nut consumption in the cause of oral cancers. A cytogenetic assessment. Cancer 1992;70:1017-23.
20. Chiang CP, Lang MJ, Liu BY, Wang JT, Leu JS, Hahn LJ, Kuo MY. Expression of proliferating cell nuclear antigen (PCNA) in oral submucous fibrosis, oral epithelial hyperkeratosis and oral epithelial dysplasia in Taiwan. Oral Oncol 2000;36:353-9.
21. Kozuki Y, Miura Y, Yagasaki K. Inhibitory effects of carotenoids on the invasion of rat ascites hepatoma cells in culture. Cancer Lett 2000;151:111-5.
22. Toma S, Albanese E, Palumbo R, Nicolo G, Morerio C, Magiante PE, Bonatti S, Cancedda R, Santi L. In vitro effects of beta-carotene on human oral keratinocytes from precancerous lesions and squamous carcinoma. Anticancer Drugs 1991;2:581-9.
23. Livny O, Kaplan I, Reifen R, Polak-Charcon S, Madar Z, Schwartz B. Lycopene inhibits proliferation and enhances gap-junction communication of KB-1 human oral tumor cells. J Nutr 2002;132:3754-9.
24. Hieber AD, King TJ, Morioka S, Fukushima LH, Franke AA, Bertram JS. Comparative effects of all-trans beta-carotene vs. 9-cis beta-carotene on carcinogen-induced neoplastic transformation and connexin 43 expression in murine 10T1/2 cells and on the differentiation of human keratinocytes. Nutr Cancer 2000;37:234-44.
25. Stivala LA, Savio M, Cazzalini O, Pizzala R, Rehak, Bianchi L, Vannini V, Prosperi E. Effect of beta-carotene on cell cycle progression of human fibroblasts. Carcinogenesis 1996;17:2395-401.
26. Nahum A, Hirsch K, Danilenko M, Watts CK and Prall OW. Lycopene inhibition of cell cycle progression in breast and endometrial cancer cells is associated with reduction in cyclin D levels and retention of p27(Kip1) in the cyclin E-cdk2 complexes. Oncogene 2001;20:3428-36.
27. Zhang LX, Cooney RV, Bertram JS. Carotenoids up-regulate connexin43 gene expression independent of their provitamin A or antioxidant properties. Cancer Res 1992;52:5707-12.
28. Bohm F, Tinkler JH, Truscott TG. Carotenoids protect against cell membrane damage by the nitrogen dioxide radical. Nat Med 1995;1:98-9.

Table 1: PCNA expression of epithelial cells treated with different carotenoids

Treatment	Animal Number	Normal epithelium	Hyperplasia	Dysplasia	Papilloma	Carcinoma
DMBA+BQE	7	1.6±0.3 (0~2)	2.6±0.7 (2~3)	4.4±0.8 (4~5)	3.1±0.5 (3~4)	4.6±0.6 (4~5)
DMBA+BQE+ Vitamin E	6	0.3±0.0 (0~1)*	1.6±0.2 (1~2)*	1.3±0.2 (1~2)*	2.3±0.3 (2~3)*	2.8±0.5 (2~4)*
DMBA+BQE+ β-Carotene	7	1.5±0.4 (0~2)	2.1±0.2 (2~3)	2.1±0.1 (2~3)*	2.1±0.4 (2~3)*	3.2±0.4 (2~4)*
DMBA+BQE+ Lycopene	7	1.1±0.2 (0~2)	2.3±0.1 (2~3)	2.4±0.6 (2~3)*	2.5±0.6 (2~3)	0*
DMBA+BQE+ Lutein	7	0.3±0.0 (0~1)*	2.2±0.1 (2~3)	2.6±0.4 (2~3)*	3.2±0.5 (3~4)	3.9±0.7 (2~4)
DMBA+BQE+ Mixture	7	1.1±0.0 (0~2)*	1.7±0.7 (1~3)	2.2±0.2 (2~3)*	2.1±0.5 (2~3)*	0*

1. The expression and distribution of proliferating cell nuclear antigen (PCNA) were graded as five degrees (scores 1~5, a higher number indicated greater severity). Values are presented as the mean ± SD. * p<0.05 comparing to control group (DMBA+BQE).
2. The Mixture consisted of equal amounts of β-carotene, lycopene, lutein, and α-tocopherol.
3. '--' Indicates no carcinoma production in the group.

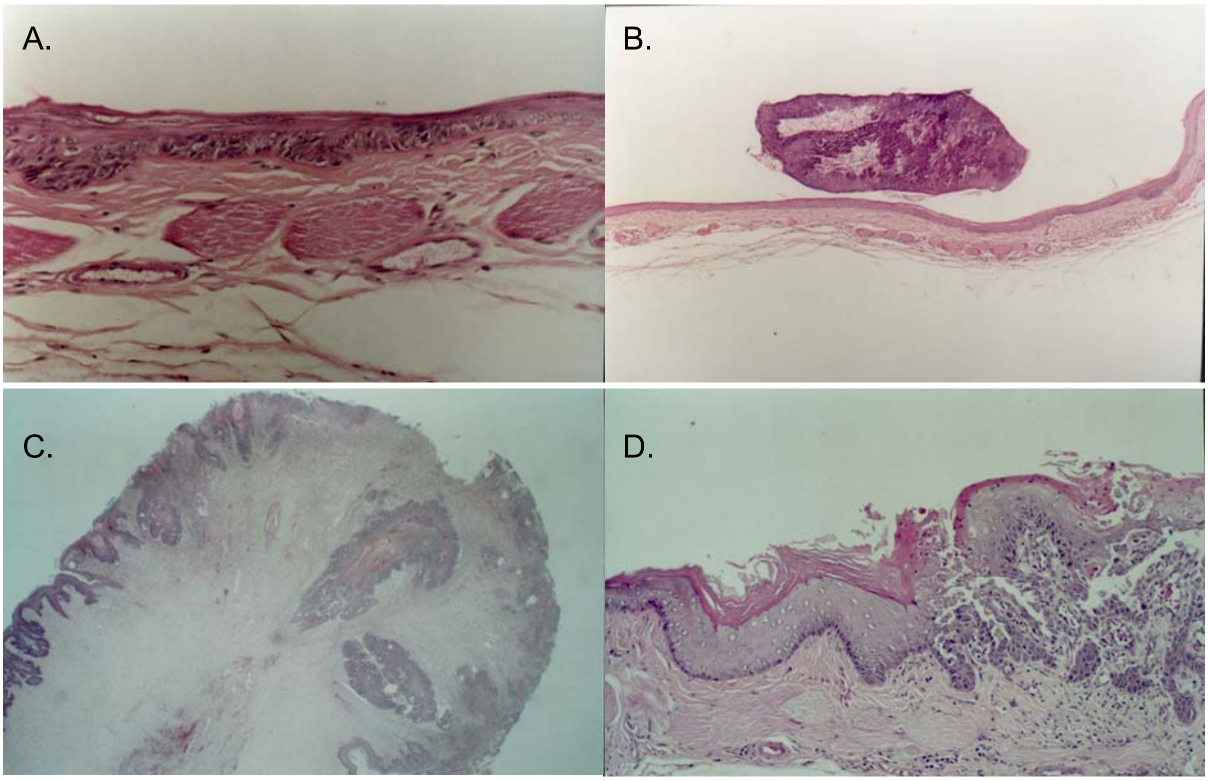

Figure 1: Lesions characteristics of the control and experimental groups with H&E stain. In the control group, some lesions presented with a disordered cell arrangement, condensed chromosomes of the basal layer, and dysplasia (A, 100X); some lesions developed papilloma with hyperplasia (B, 20X); and some lesions exhibited squamous cell carcinoma with keratin overexpression (C, 20X). In the carotenoids mixture group, lesions showed less hyperplasia and more-normal tissue morphology (D, 40X).

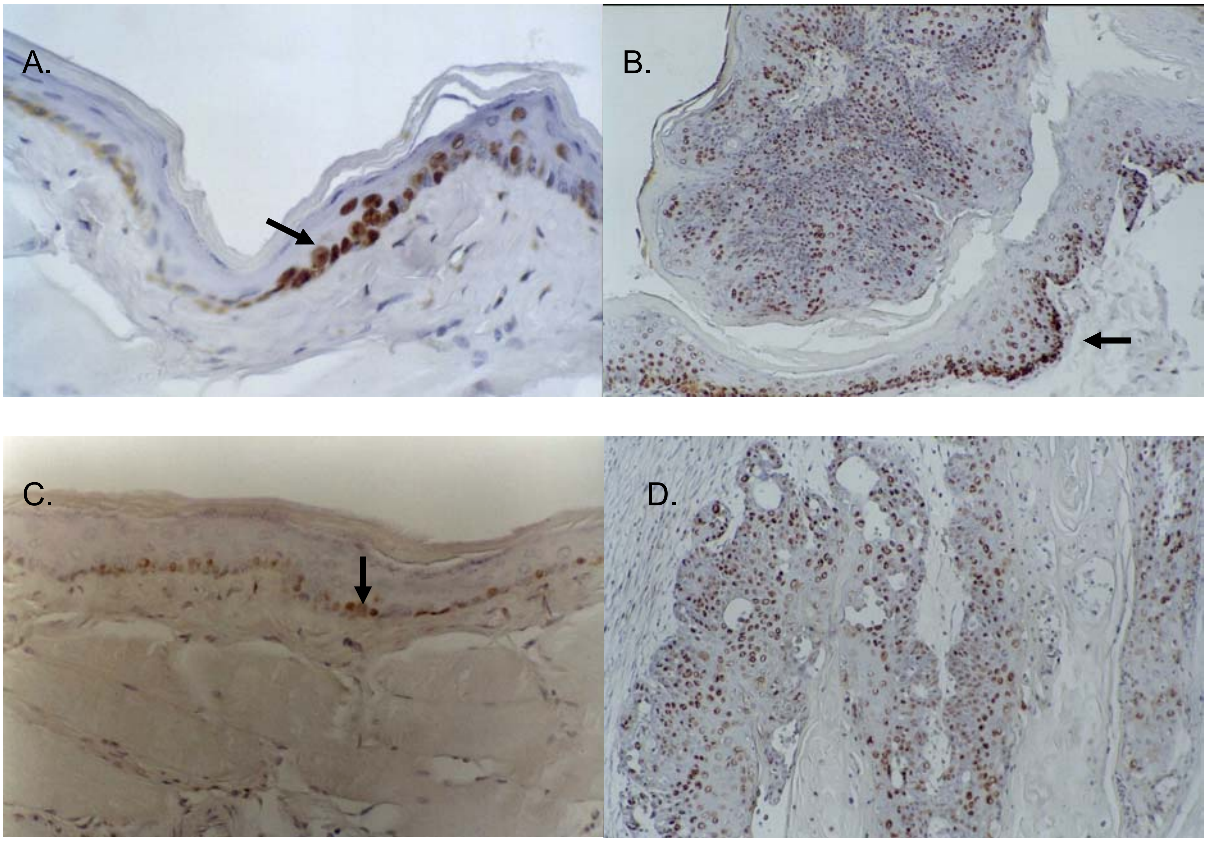

Figure 2. PCNA expression of lesions. In the control group, the basal with dysplasia presented greater PCNA expression (A, 100X); some lesions developed papilloma with hyperplasia (B, 40X); and some lesions with carcinoma also showed high PCNA expression (C, 100X). In the carotenoid mixture group, PCNA was only evident in the basal layer (D, 40X).

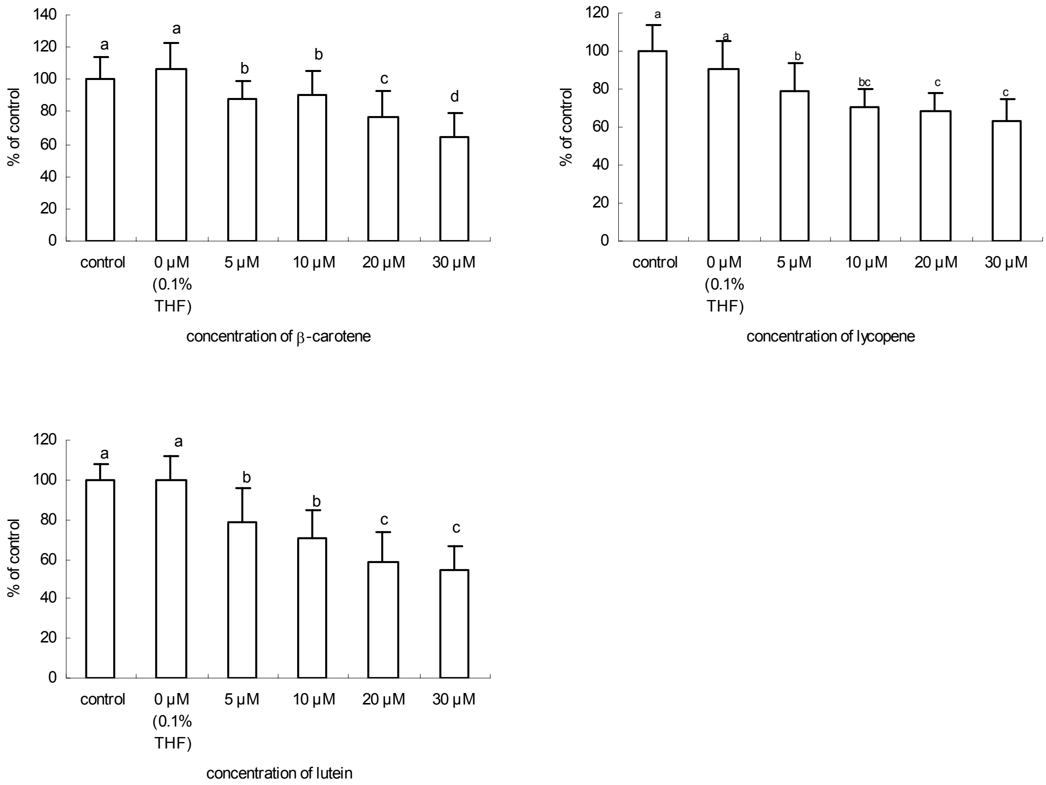

Figure 3. Effect of catotenoids (β-carotene [A]; lycopene [B] and lutein [C]) on KB cell proliferation at 24h incubation. Cells were incubated in medium (control), in the presence of the carotenoid solvent tetrahydrofuran (THF) at final concentration of 0.1% (0 μM), or in the presence of 5, 10, 20, 30μM carotenoids. Results are mean ± SD, n=6. Values were evaluated by One-Way ANOVA and the Fisher's test. Data with different superscripts indicate significantly differ between groups, $p < 0.05$.

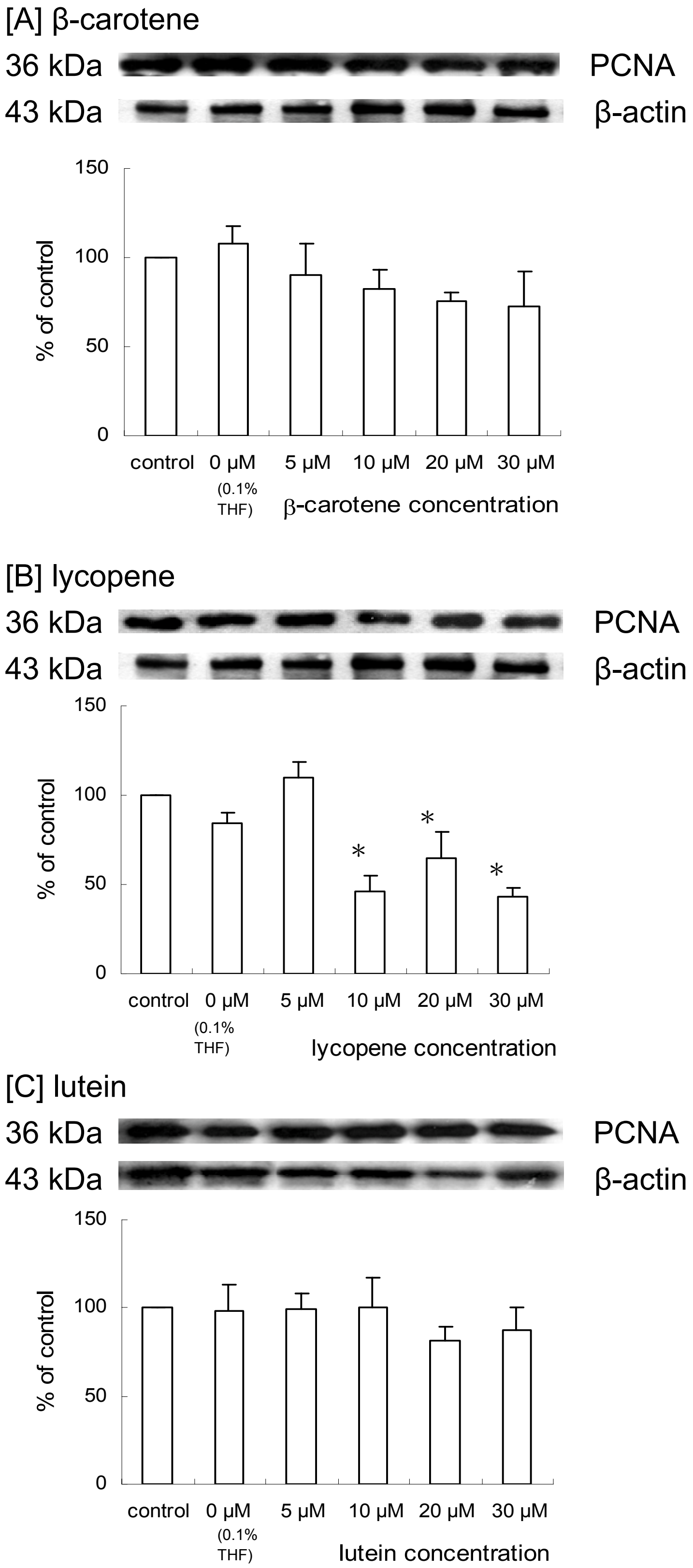

Figure 4. Effects of catotenoids (β-carotene [A]; lycopene [B] and lutein [C]) on PCNA (36 kDa) protein expression of KB cell at 24h incubation. β-Actin (43 kDa) serves as internal control. Results are mean ± SEM, n=3. Values were evaluated by Student's t-test. Data with "*"indicates significantly different from 0 μM group, $p < 0.05$.

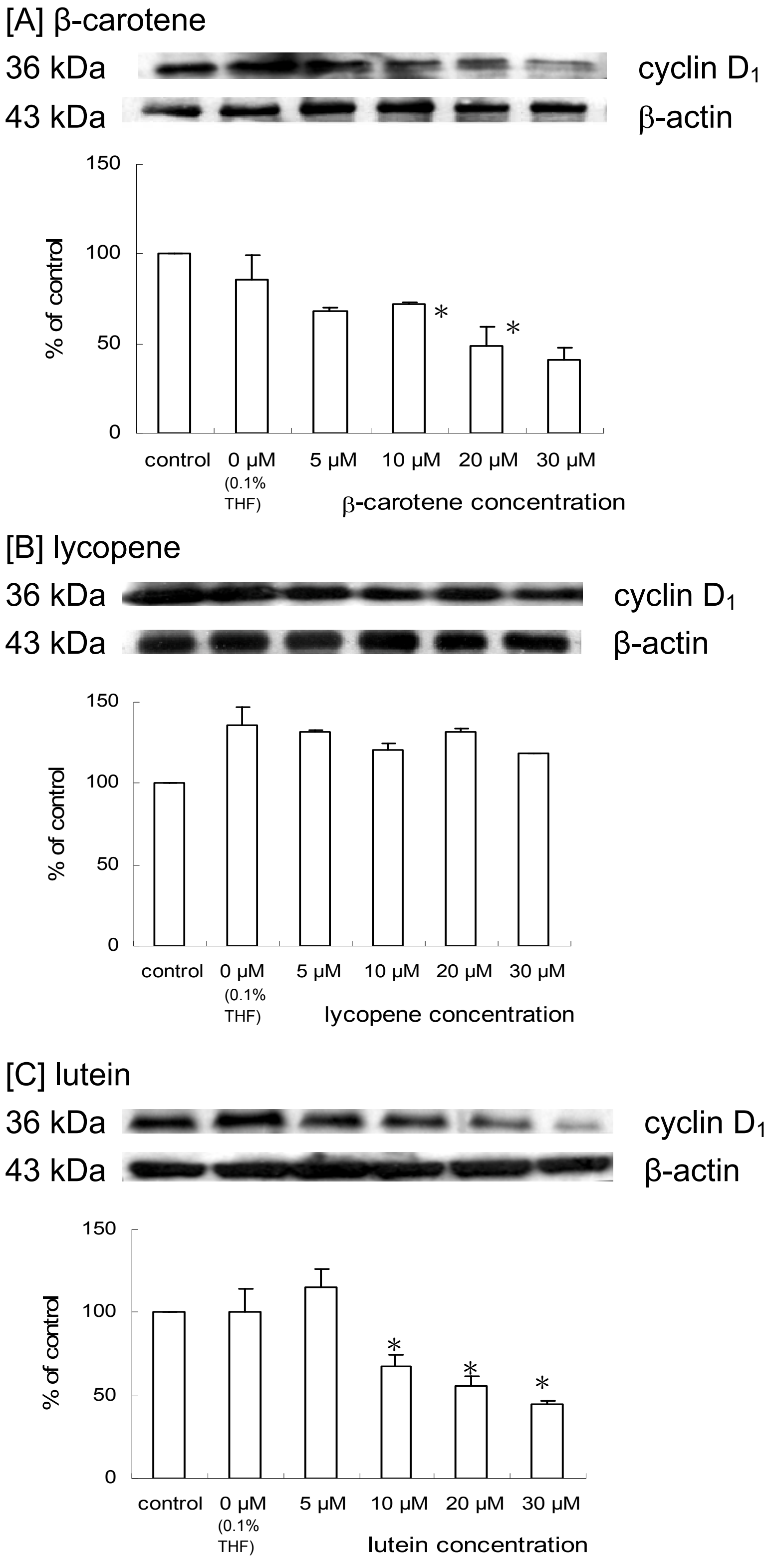

Figure 5. Effects of catotenoids (β-carotene [A]; lycopene [B] and lutein [C]) on cyclin D_1 (36 kDa) expression of KB cell at 24h incubation. β-Actin (43 kDa) serves as internal control. Data with "∗"indicates significantly different from 0 μM group, $p < 0.05$.

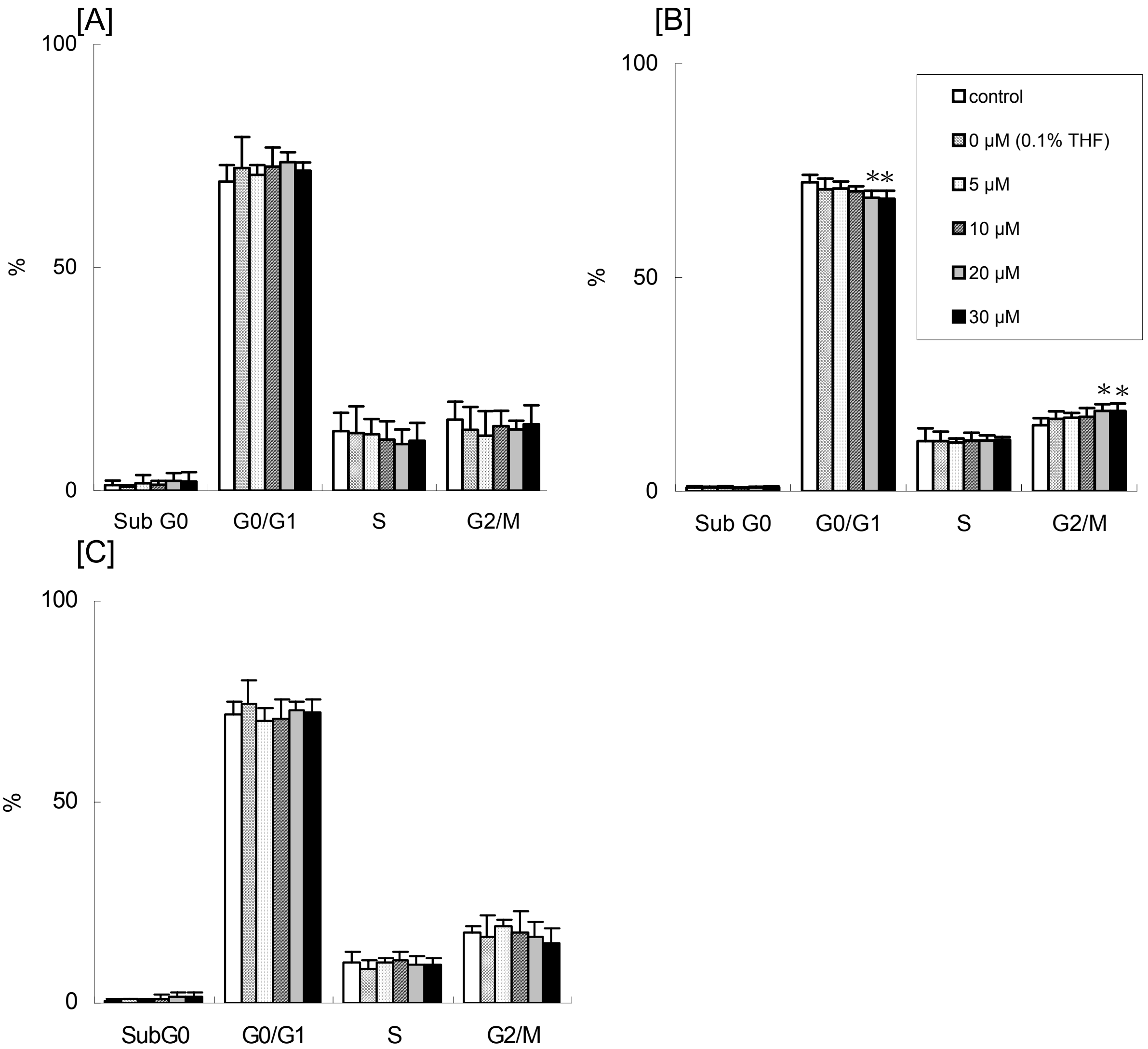

Figure 6. Effect of carotenoids ([A] β-carotene, [B] lycopene and [C] lutein, on cell cycle progression of KB cell for 24 h incubation. Data with "∗" indicates significantly different from 0 μM group, $p < 0.05$.