

# Suppressive Effects of Carotenoids on Proliferation and Differentiation of 3T3-L1 Preadipocytes

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**Abstract** Obesity results from excessive growth and expansion of adipose tissue due to preadipocyte proliferation and differentiation as well as excess lipid accumulation. The aim of the study was to assess the effects of bixin, lycopene, and  $\beta$ -carotene on proliferation and differentiation of 3T3-L1 preadipocytes. The cell viability was determined by MTT assays and their differentiation was evaluated by Oil red O staining and monitoring the expression of peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) and fatty acid binding protein 4 (FABP4). The expression levels of leptin and acetyl-CoA carboxylase (ACC) were measured by West blots. The results showed that bixin,  $\beta$ -carotene and lycopene reduced the viability of 3T3-L1 preadipocytes and inhibited intracellular lipid accumulation.  $\beta$ -Carotene had stronger suppressive effects on lipid accumulation than bixin and lycopene. Treatments of these carotenoids obviously reduced expression levels of PPAR $\gamma$  and FABP4 proteins in 3T3-L1 cells. The carotenoids down-regulated leptin expression, but up-regulated ACC expression during adipocyte differentiation. Bixin, lycopene and  $\beta$ -carotene suppressed differentiation of 3T3-L1 preadipocytes through down-regulating protein expressions of PPAR $\gamma$  and FABP4 as well as leptin.

**Keywords:** 3T3-L1 preadipocytes, carotenoids, proliferation, differentiation

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## 1. Introduction

Nowdays, obesity has become one of the most serious public health problems in countries worldwide because it may increase the risk of numerous diseases, such as heart disease, type II diabetes, obstructive sleep apnea, and so on [1,2]. Obesity results from excessive growth and expansion of adipose tissue due to generation of new adipocytes and adipocyte hypertrophy caused by excess lipid accumulation. Proliferation and differentiation of preadipocytes lead to the increases of adipocyte number. The differentiation of preadipocytes into adipocytes, also called adipogenesis, is a complex biological process, in which a variety of transcription factors such as C/EBPs and PPAR $\gamma$  are involved and genetic programming for lipid synthesis and storage changes. C/EBPs and PPAR $\gamma$  regulate the expression of genes implicated in the induction of adipocyte phenotypes and promote adipocyte differentiation [3]. As a result, the number of adipocytes increases in adipose tissues. Suppression of excessive preadipocyte proliferation and differentiation may have preventive and/or therapeutic potential against obesity and its associated pathologies.

Recently, natural compounds from dietary and edible plants have been investigated to reduce the pathogenesis of obesity and metabolic syndrome by inhibiting adipocyte differentiation. As one of the main micronutrients in

vegetables and fruit carotenoids are almost daily intaken in significant quantity. The pharmacological roles of carotenoids in the prevention and reduction of obesity have received a great deal of attention [4]. Suppressive effects of carotenoids on adipocyte differentiation have been studied in 3T3-L1 preadipocytes. It has been reported that  $\beta$ -carotene,  $\beta$ -apo-8'-carotenal, and retinal markedly suppressed the differentiation of 3T3-L1 preadipocytes to adipocytes through the retinoic acid receptor (RAR) up-regulation and the suppression of PPAR $\gamma$ 2 [5]. PPAR $\gamma$  is known to be a master regulator of 3T3-L1 adipocyte differentiation [6]. Similarly, other carotenoids such as astaxanthin,  $\beta$ -cryptoxanthin, fucoxanthin and its metabolites, fucoxanthinol and amarouciaxanthin A, neoxanthin, and siphonaxanthin were reported to suppress adipocyte differentiation and lipid accumulation via the down-regulation of PPAR $\gamma$  in 3T3-L1 cells [7-13]. Xanthigen (brown marine algae fucoxanthin + pomegranate seed oil) was demonstrated to suppress adipocyte differentiation and lipid accumulation through down-regulating the protein levels of PPAR $\gamma$  as well as a key enzyme involved in adipogenesis [14]. In addition,  $\beta$ -apo-14'-carotenal, an apo-carotenoid, repressed PPAR $\gamma$  and PPAR $\alpha$  responses. During 3T3-L1 adipocyte differentiation, it inhibited PPAR $\gamma$  target gene expression and adipogenesis [15].

However, the inconsistent results have been observed on some carotenoids.  $\beta$ -Carotene treatment at a concentration of 20  $\mu$ M, but not 10  $\mu$ M, induced the expression of genes

for several important transcriptional factors involved in adipocyte differentiation (PPAR $\gamma$ 1, PPAR $\gamma$ 2, C/EBP $\alpha$ , and C/EBP $\beta$ ) in 3T3-L1 adipocytes [16]. Paprika pigments (containing capsanthin and capsorubin in main) were demonstrated to promote 3T3-L1 adipocyte differentiation and upregulate PPAR $\gamma$  expression [17]. Bixin and norbixin activated PPAR $\gamma$  and induced mRNA expression of PPAR $\gamma$  target genes in differentiated 3T3-L1 adipocytes [18]. Apo-10'-lycopenoic acid, a lycopene metabolite, did not affect the PPAR $\gamma$  mRNA expression levels in 3T3-L1 cells [19]. Treatment of 3T3-L1 adipocytes with either  $\beta$ -apo-13-carotenone or  $\beta$ -apo-10'-carotenoid acid stimulated adipocyte marker gene expression. The former upregulated PPAR $\gamma$  expression [20]. It is quite evident that this discrepancy about effects of carotenoids on 3T3-L1 adipocyte differentiation would deserve further investigation and the mechanism underlying the carotenoid effects would need to be explored, although it is reported that fucoxanthin exerted differing effects on 3T3-L1 cells of different differentiation stages [21].

In the current study, we evaluated the proliferation-inhibitory and anti-adipogenic effects of bixin, lycopene and  $\beta$ -carotene by using a 3T3-L1 preadipocytes culture system, as well as effects on the protein levels leptin and acetyl-CoA carboxylase and explored the possible molecular mechanism.

## 2. Materials and Methods

### 2.1. Materials

Mouse 3T3-L1 preadipocytes, the well-established cultured cell model of preadipocytes, were purchased from Cell Resource Center (Beijing, China). Dulbecco's modified Eagle's medium (DMEM) was from Gibco BRL (Grand Island, NY, USA).  $\beta$ -Carotene, bixin, 3-isobutyl-1-methylxanthine (IBMX) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich Chemical Co. (Shanghai, China). Lycopene (purity 90%) was from Pisite Biological Manufacture (Chengdu, China). Dexamethasone (DEX), insulin, Oil red O staining, trypsin-EDTA solution, RIPA lysis solution and fetal bovine serum (FBS) were obtained from Solarbio Biological Manufacture (Beijing, China). Primary antibodies against PPAR $\gamma$ , FABP4, leptin, acetyl-CoA carboxylase (ACC), and  $\beta$ -actin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemicals were purchased from Sangon Biotech (Shanghai, China).

### 2.2. Cell Culture

3T3-L1 Preadipocytes were cultured in DMEM supplemented with 10% decomplexed fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100  $\mu$ g/mL) until confluent. The cells were maintained at 37°C in a 5% CO<sub>2</sub> humidified atmosphere. For the differentiation of preadipocytes, 2 days after confluence, the cells were stimulated for 48 h with DMEM containing 10% FBS, 10  $\mu$ g/mL insulin, 1  $\mu$ M dexamethasone, and 0.5 mM isobutylmethylxanthine (IBMX) (differentiation medium I). The cells were then incubated in in DMEM

containing 10% FBS and 10  $\mu$ g/mL insulin (differentiation medium II) for another 48 h, thereafter, were maintained in postdifferentiation medium (DMEM containing 10% FBS), which was refreshed every 2 days. Adipocyte differentiation was monitored by Oil Red O staining and by examining the levels of the adipocyte marker FABP4.

To examine the effects of test compounds on the differentiation of preadipocytes to adipocytes, the cells were cultured in the differentiation medium II, respectively, in the presence of various concentrations of carotenoids for 48 h and then incubated in postdifferentiation medium for 6 days. Carotenoids (bixin, lycopene and  $\beta$ -carotene) were delivered to the cell using dimethyl sulfoxide (DMSO) as a solvent. The concentration of DMSO was adjusted to be the same in all experiments and final concentration was no more than 0.5% (V/V). Control groups received the same amount of DMSO without carotenoids.

### 2.3. Oil Red O Staining of 3T3-L1 Adipocytes

0.5% Oil Red O stock solution was prepared by stirring Oil Red O in isopropanol overnight, and filtered through a 0.22 mm filter and stored at 4°C. Fresh Oil Red O working solutions were obtained by mixing 0.5% Oil Red O stock solution with distilled water (6:4, v/v), and incubating for 20 min and further filtration.

After adipocyte differentiation, the cells were stained using Oil Red O, an indicator of the cell lipid content. In brief, the cells were twice washed with PBS, then fixed using 4% formaldehyde in PBS for 1 h at room temperature. Subsequently, the cells were washed with PBS and 60% isopropanol, respectively, and dried and stained with fresh Oil Red O working solution (1.5 mL/dish) for 2 h. Dishes were washed extensively with distilled water, and dried and photographed. For lipid content measurement, stained lipid droplets in 3T3-L1 adipocytes were extracted with isopropanol, then read at 490 nm for their absorbance. Results were represented as a relative percentage of differentiated 3T3-L1 cells without carotenoid treatments (controls).

### 2.4. Cell Survival

3T3-L1 Preadipocytes were cultured at a density of 10<sup>4</sup> cells/well in 96-well microplates. After 24 h, the cells were treated with fresh medium containing carotenoids at indicated concentrations for 3 days. MTT (500  $\mu$ g/mL) was added to each well and the plate was incubated for 4 h at 37°C. The liquid in the plate was then removed, and DMSO added to dissolve the MTT-formazan complex formed. The absorbance at 570 nm (630 nm) was measured by a BioRad model 680 microplate reader (Richmond, CA, USA). Samples were measured in triplicate and each experiment was performed independently at least three times. The effects of carotenoids on cell viability was evaluated as absorbance relative to that of control cultures.

### 2.5. Western Blot

To detect PPAR $\gamma$ , FABP4, leptin and ACC, 3T3-L1 cells were washed twice with ice-cold PBS, collected, and centrifuged. The cell pellets were resuspended in lysis

buffer (50 mM Tris-Cl, 150 mM NaCl, 0.02% (w/v)  $\text{NaN}_3$ , 100  $\mu\text{g}/\text{mL}$  PMSF, 1  $\mu\text{g}/\text{mL}$  aprotinin 1  $\mu\text{g}/\text{mL}$  pepstatin A, 2  $\mu\text{g}/\text{mL}$  leupeptin and 1% (v/v) Triton-X 100) and incubated on ice for 30 min. 3T3-L1 Cell debris were then removed by centrifugation, and the protein concentrations in the lysates were measured using BCA kit (Pierce Inc). The lysates were then subjected to electrophoresis on polyacrylamide gels containing sodium dodecyl sulfate (15% for FABP4 and leptin, 10% for  $\beta$ -actin and PPAR $\gamma$ , 6% for ACC ) and were transferred to NC membranes (PALL, USA). The membranes were blocked in 0.1 M Tris-buffered saline containing 0.1% Tween-20 and 5% no-fat milk at room temperature for 1 h. After incubation overnight at 4°C with primary antibodies, the membranes were washed four time, and then incubated with the appropriate secondary antibodies at room temperature for 2 h. The antigen was detected using the Super Signal West Pico Chemiluminiscent system (Pierce, USA), and visualized by autoradiography on Kodak-XAR film.

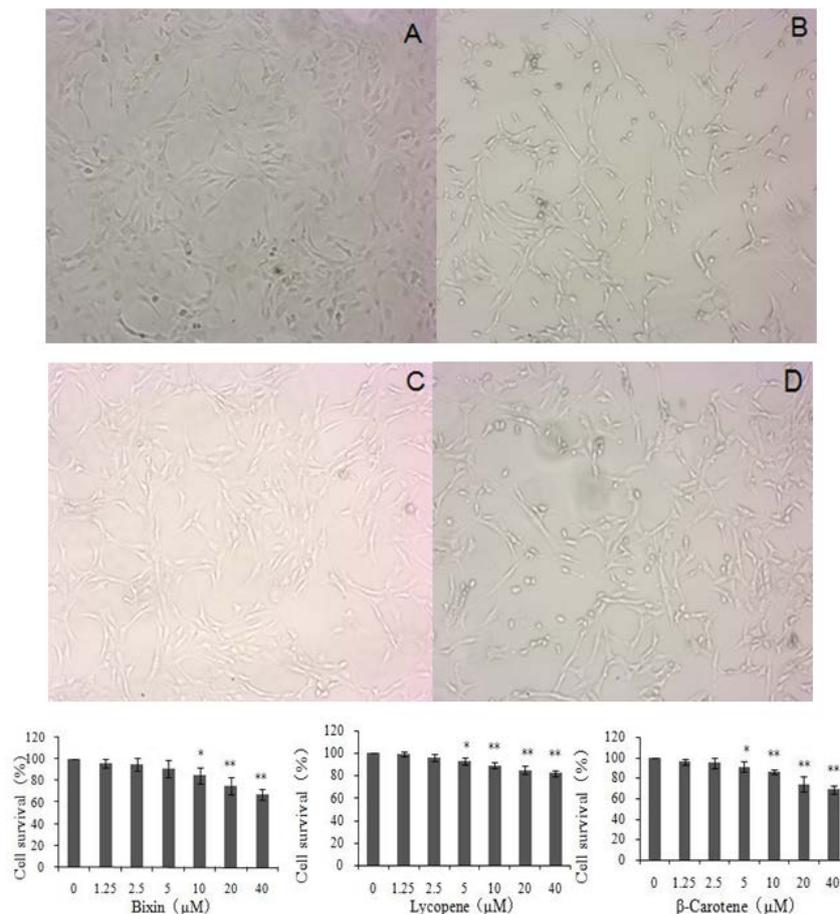
## 2.6. Statistical Analysis

Data are presented as the means  $\pm$ S.D. Significant differences between control and treated groups were determined by Student's t-test or one-way ANOVA, followed by a Bonferroni-type multiple t-test. Values of  $P < 0.05$  were considered statistically significant.

## 3. Results

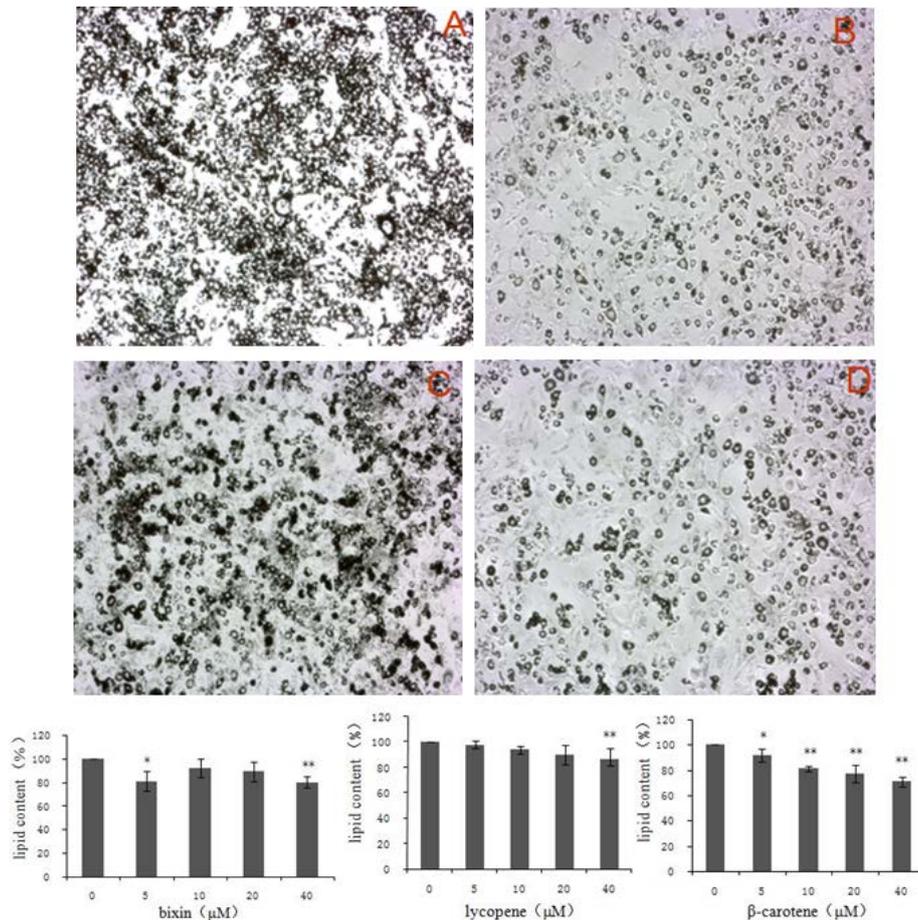
### 3.1. Inhibitory Effects of Carotenoids on the Proliferation of 3T3-L1 Preadipocytes

3T3-L1 Preadipocytes were treated with increasing concentrations (1.25–40  $\mu\text{M}$ ) of carotenoids (bixin, lycopene and  $\beta$ -carotene), respectively, for 3 days. Many dead cells and cellular debris which were in suspension were observed under a microscope after 48 h treatment. As can be seen in Figure 1, the cell numbers decreased significantly under 40  $\mu\text{M}$  carotenoid treatments compared with controls. The bar chart in Figure 1 shows the effects of changing carotenoid concentrations on the viability of 3T3-L1 preadipocytes as determined by MTT proliferation assays. Carotenoid treatments suppressed the cell proliferation and the cell survival loss induced by carotenoids was dose-dependent. When the cells were treated with low concentrations of carotenoids (1.25  $\mu\text{M}$  and 2.5  $\mu\text{M}$ ), the reduction of the cell survival was not very obvious. However, notably inhibitory effects on the 3T3-L1 preadipocyte proliferation were observed at higher concentrations (5  $\mu\text{M}$  and over for  $\beta$ -carotene and lycopene, 10  $\mu\text{M}$  and over for bixin) of carotenoids. At 40  $\mu\text{M}$ ,  $\beta$ -carotene exhibited the most effectively inhibitory effects on the cell proliferation, followed by bixin and lycopene in order.



3T3-L1 preadipocytes were treated with carotenoids for 72 h. Photomicrographs (40 $\times$  magnification) of 3T3-L1 cells treated with 40  $\mu\text{M}$  carotenoids (A control; B bixin; C lycopene; D  $\beta$ -carotene). The cell viability (bar chart) measured by MTT assay after treatment of carotenoids. Data are presented as the means  $\pm$  SD, n = 3, \* $P < 0.05$  and \*\* $P < 0.01$  versus control group.

**Figure 1.** Effects of carotenoids on the cell viability of 3T3-L1 preadipocytes



3T3-L1 cells were differentiated for 48 h, and subsequently treated with carotenoids for 6 d, after which lipid accumulation in the adipocytes was measured using Oil-red O staining. Photomicrographs (40 $\times$  magnification) of 3T3-L1 cells treated with 40  $\mu$ M carotenoids (A control; B bixin; C lycopene; D  $\beta$ -carotene). Data (bar chart) are presented as the means  $\pm$  SD. \* $P < 0.05$  and \*\* $P < 0.01$ , compared with control.

**Figure 2.** Effects of carotenoids on lipid accumulation in 3T3-L1 adipocytes

### 3.2. Effects of Carotenoids on the Intracellular Lipid Content and the Differentiation of 3T3-L1 Preadipocytes

To investigate the anti-adipogenic effects of carotenoids, differentiation of 3T3-L1 preadipocytes was assessed by Oil Red O staining, measurements of the intracellular lipid content, and by monitoring the expression of the adipocyte markers PPAR $\gamma$  and FABP4. Mature adipocytes were identified and characterized based on oil droplets in the cells which are not seen in undifferentiated cells. Each carotenoid was added, respectively, 2 day after 3T3-L1 preadipocytes were induced to differentiate and through exposure for 6 days in differentiation medium II and postdifferentiation medium. Oil Red O staining test showed that many lipid droplets were formed in 3T3-L1 cells in induction medium on day 8 of cell differentiation (Figure 2 A). However, 3T3-L1 preadipocytes treated with 40  $\mu$ M carotenoids for 6 days contained less lipid droplets strikingly than the control cells (Figure 2 B, C, D). Apparently, carotenoids at 40  $\mu$ M largely reduced the amounts of lipid droplets formed.

Next we further tested carotenoid 's lipid-lowering effects on 3T3-L1 adipocytes. The cells were exposed to increasing concentrations of carotenoids for 6 days starting 2 day after differentiation induction. Consistently,

as the bar chart shown in Figure 2, the treatments of carotenoids dose-dependently reduced intracellular lipid contents, compared with controls. The observations thus show that the bixin, lycopene and  $\beta$ -carotene inhibited formation of adipocytes and adipogenesis.

Then we assessed protein expression levels of the adipocyte markers FABP4 and PPAR $\gamma$  in 3T3-L1 adipocytes by immunoblots using the ECL detection reagent. As shown in Figure 3, expression levels of PPAR $\gamma$  and FABP4 proteins in 3T3-L1 cells were obviously down-regulated after 6 day treatments of bixin, lycopene and  $\beta$ -carotene. Reducing the expression was dose-dependent except effect of bixin on protein expression levels of FABP4. The reduced expression of these adipocyte markers further demonstrated that bixin, lycopene and  $\beta$ -carotene suppressed adipocyte differentiation and adipogenesis.

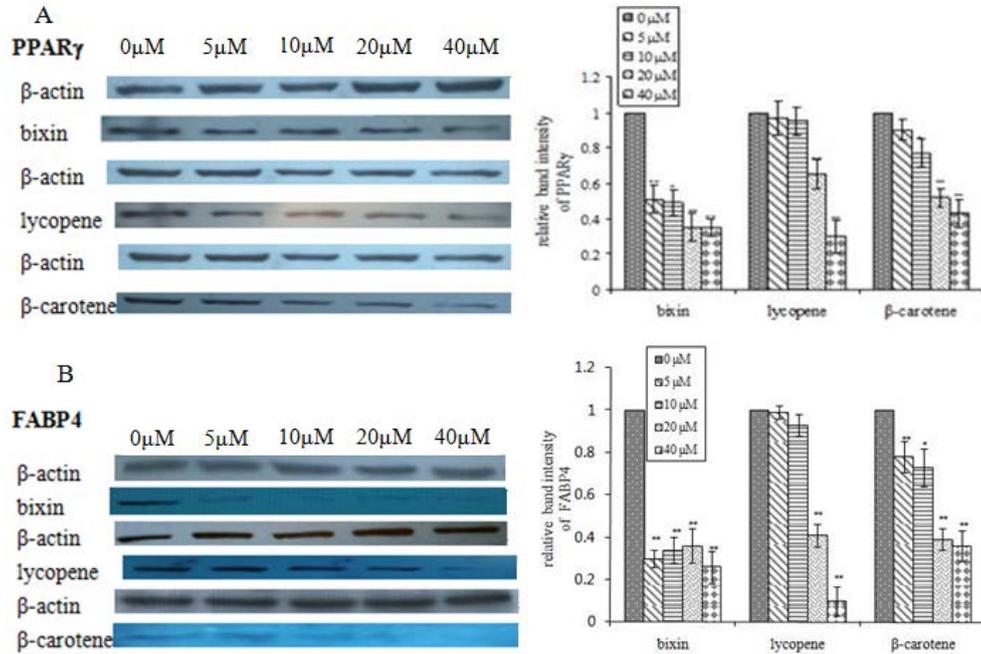
### 3.3. Effects of Carotenoids On Expression of Leptin and ACC during Differentiation of 3T3-L1 Preadipocytes

Expression and secretion of adipocyte-specific leptin occur during 3T3-L1 preadipocyte differentiation. Therefore, whether carotenoids regulate the expression of leptin, during preadipocyte differentiation was examined.

As shown in Figure 4 A, bixin, lycopene and  $\beta$ -carotene strongly suppressed the protein levels of leptin as compared with controls. At high concentrations (20  $\mu$ M and 40  $\mu$ M) the suppressive effects of carotenoids were very striking on leptin protein levels.

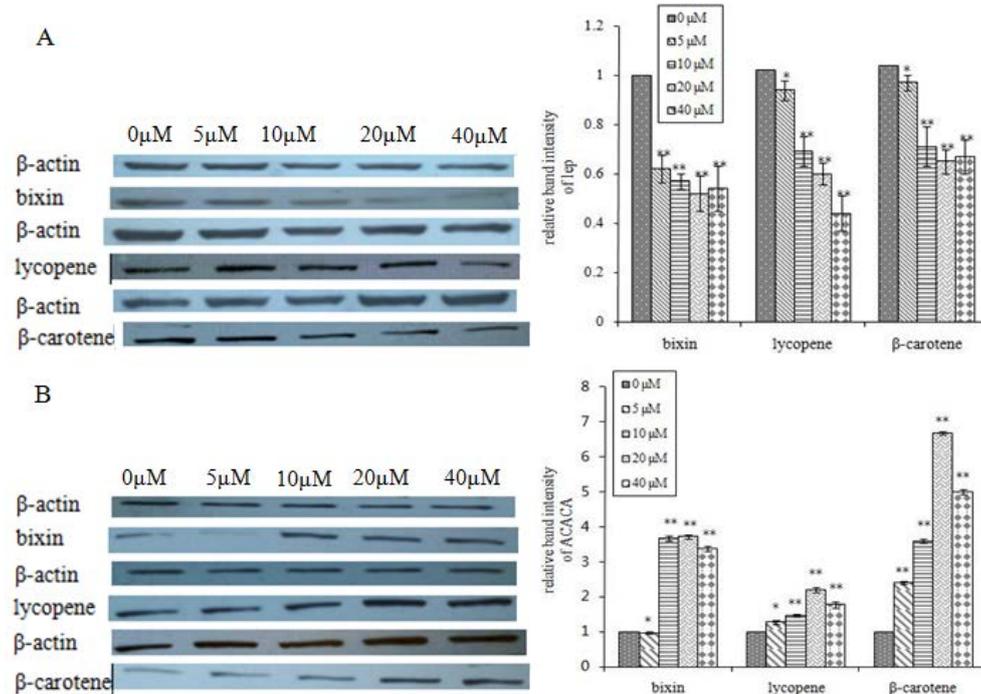
In order to decipher how carotenoids inhibited lipid accumulation, we tested the regulatory effects of carotenoids on protein expressions of acetyl-CoA

carboxylase (ACC) involved in adipogenesis and lipid metabolism. As shown in Figure 4 B, carotenoids increased ACC protein expression in 3T3-L1 cells when compared to controls. At 5  $\mu$ M, 10  $\mu$ M and 20  $\mu$ M the up-regulatory effects of carotenoids were dose-dependent on expression levels of ACC proteins, but at 40  $\mu$ M of carotenoids ACC protein levels enhanced were slightly attenuated when compared to 20  $\mu$ M carotenoid treatment groups.



3T3-L1 cells were exposed to differentiation inducer for 48 h, and subsequently treated with carotenoids for 6 d. The expression levels of PPAR $\gamma$  proteins were normalized to the corresponding levels of  $\beta$ -actin. Data are presented as percent of control and the bars are the means  $\pm$  SD for three independent experiments. \*P < 0.05 and \*\*P < 0.01 versus control groups.

**Figure 3.** Carotenoids down-regulate the expression of PPAR $\gamma$  (A) and FABP4 (B) in 3T3-L1 cells



3T3-L1 cells were exposed to differentiation inducer for 48 h, and subsequently treated with carotenoids for 6 d. The expression levels of leptin and ACC proteins were normalized to the corresponding levels of  $\beta$ -actin, respectively. Data are presented as percent of control and the bars are the means  $\pm$  SD for three independent experiments. \*P < 0.05 and \*\*P < 0.01 versus control groups.

**Figure 4.** Effects of carotenoids on protein expression levels of leptin (A) and ACC (B) in 3T3-L1 cells

## 4. Discussion

Epidemiologic studies have shown that increased consumption of diets with a relatively large amount of fruits and vegetables is associated with a decreased risk of various diseases including certain cancers, eye diseases and atherosclerosis [22]. Carotenoids, one of major micronutrients in fruits and vegetables, have attracted substantial attention. In addition to some carotenoids being provitamin A dietary carotenoids have been shown to play an essential role in the prevention and reduction of numerous chronic diseases such as cardiovascular disease, age-related macular degeneration and cancer [23,24,25]. Recent accumulating evidence has suggested beneficial applications for carotenoids to obesity.  $\beta$ -Carotene and lycopene have been most studied and also occur typically in human diet and plasma [24,25]. Bixin, as a natural pigment extracted from annatto, is extensively used in many processed foods. Its activities have been well studied [26]. Thus, these carotenoids were chosen to focus on their effects on adipocyte proliferation and differentiation in 3T3 L1 cells and possible molecular mechanisms although there have been some results about bixin and  $\beta$ -carotene.

In the present study, as shown in Figure 1, lycopene and  $\beta$ -carotene treatments at the concentrations of 5  $\mu$ M and over as well as bixin treatments at 10  $\mu$ M and over significantly reduced 3T3-L1 preadipocyte viability. It was reported that the concentrations (10  $\mu$ M, 20  $\mu$ M and over) of  $\beta$ -carotene used in 3T3-L1 adipocytes were considered to be within a physiologic range [16]. Fucoxanthin treatments at the concentrations of 10  $\mu$ M and 15  $\mu$ M did not affect viability or cause cytotoxicity in 3T3-L1 cells [10,21]. It is evident that bixin, lycopene and  $\beta$ -carotene are more effective in attenuating adipocyte number increase via cell proliferation than fucoxanthin. In any case the inhibitory effects on preadipocyte proliferation are helpful for controlling obesity development.

Similarly to the results obtained for astaxanthin,  $\beta$ -cryptoxanthin, fucoxanthin and its metabolites, fucoxanthinol and amarouciaxanthin, neoxanthin, siphonaxanthin, violaxanthin, zeaxanthin, Xanthigen, and  $\beta$ -apo-14'-carotenal [7,8,10,12,13,14,15], bixin, lycopene and  $\beta$ -carotene treatments largely inhibited intracellular lipid accumulation 48 h after stimulation of 3T3-L1 preadipocyte differentiation (Figure 2). Takahashi et al reported that the treatments with bixin (30  $\mu$ M and 70  $\mu$ M) for 10 days increased intracellular lipid contents in 3T3-L1 adipocytes [18]. But very little is known about the specific time of bixin addition during the differentiation of 3T3-L1 preadipocytes in their report. Moreover, 70  $\mu$ M of bixin is much larger than the concentrations of bixin used in the present study. It was shown that suppressive effects of siphonaxanthin on lipid accumulation were largely limited to the early stages of 3T3-L1 preadipocyte differentiation, siphonaxanthin treatments at the intermediate and late stages did not significantly reduce lipid accumulation [13]. Perhaps, in our experiment and the report by Takahashi et al the different results about effects of bixin on intracellular lipid accumulation in 3T3-L1 cells may be related to differing effects on 3T3-L1 cells of different differentiation stages. In addition, fucoxanthin

was reported to have different behaviour in the effects on intercellular lipid accumulation during the preadipocyte differentiation. When being administered during the early stage of differentiation (days 0–2) of 3T3-L1 cells, fucoxanthin increased triglyceride accumulation [21]. It inhibited intercellular lipid accumulation during the intermediate stage of differentiation (hours 48–120) of the cells [10].

Growing evidence shows that PPAR $\gamma$  is a central determinant of the transcriptional cascade inducing adipocytes, its expression level is considerably up-regulated when preadipocyte converts to adipocyte, and it has an important role in the early stages of 3T3-L1 adipocyte differentiation [3,27]. FABP4 (aP2) is involved in adipocyte differentiation and a predominant fatty acid binding protein found in adipocytes and has been considered as an adipocyte marker [28]. In addition, FABP4 enhances the transcriptional activity of PPAR $\gamma$  through delivering specific ligands from the cytosol to the nuclear receptor PPAR $\gamma$  in the nucleus [29]. Effects of bixin, lycopene and  $\beta$ -carotene on the differentiation of 3T3-L1 preadipocytes were assessed by immunoblotting PPAR $\gamma$  and FABP4 protein expression levels. The down-regulation of PPAR $\gamma$  and FABP4 expression in 3T3-L1 cells by bixin, lycopene and  $\beta$ -carotene in Figure 3 showed that the carotenoids-mediated suppressive effects on 3T3-L1 adipocyte differentiation is closely linked to the reduced expression of PPAR $\gamma$  and FABP4. The similar results were obtained previously for neoxanthin and siphonaxanthin. Neoxanthin showed significantly suppressive effects on expression of PPAR $\gamma$  and aP2 mRNAs in the 3T3-L1 differentiation [12]. After 8 days of adipocyte differentiation, siphonaxanthin significantly lowered gene expression of PPAR $\gamma$  and FABP4 [13]. The GPDH activity indicates the rate phase of adipocyte differentiation and is an indicator of induced adipocyte differentiation [30]. After treatments with neoxanthin relative GPDH activity in 3T3-L1 cells became significantly lower than that of control [12]. In addition, fucoxanthin and metabolites, fucoxanthinol and amarouciaxanthin A decreased GPDH activity and down-regulated mRNA and protein expression of PPAR $\gamma$  as well as transcription of adipocyte fatty acid-binding protein aP2 [10,11].  $\beta$ -Carotene and  $\beta$ -apo-8'-carotenal treatments at 50  $\mu$ M both suppressed GPDH activity and PPAR $\gamma$  mRNA expression [5]. Xanthigen down-regulated the protein levels of PPAR $\gamma$  in differentiated 3T3-L1 adipocytes [14].  $\beta$ -apo-14'-carotenal repressed PPAR $\gamma$  target gene aP2 mRNA levels and suppressed PPAR $\alpha$  and PPAR $\gamma$ -ligand binding domain activation during 3T3-L1 preadipocyte differentiation [15]. These results described above show that blockage of PPAR $\gamma$  and FABP4 expressions and/or GPDH activities by bixin, lycopene,  $\beta$ -carotene, neoxanthin, siphonaxanthin, fucoxanthin and metabolites, fucoxanthinol and amarouciaxanthin A, xanthigen,  $\beta$ -apo-8'-carotenal and  $\beta$ -apo-14'-carotenal leads to inhibition of 3T3-L1 preadipocyte differentiation, namely inhibition of adipogenesis.

On the other hand, Kameji et al reported that  $\beta$ -carotene treatment at a concentration of 20  $\mu$ M, but not 10  $\mu$ M, significantly induced the expression of PPAR $\gamma$ 1 and PPAR $\gamma$ 2 genes in 3T3-L1 adipocytes from day 4 to 8

after the onset of differentiation [16]. It is very significant that such results are not enough to show the effects of  $\beta$ -carotene on 3T3-L1 preadipocyte differentiation because at such stages the cellular events, such as the expression of PPAR $\gamma$  has been in progress. Although it was reported that 3T3-L1 adipocytes treated for 144 h with paprika pigments 48 h after the onset of differentiation increased the activity of GPDH and enhanced mRNA expression and protein levels of PPAR $\gamma$  [17]. Fucoxanthin upregulated the expression of PPAR $\gamma$  and aP2 at the initial stage of 3T3-L1 preadipocyte differentiation, however, and inhibited their expression at intermediate and late stages [21]. With respect to bixin, lycopene and  $\beta$ -carotene, the repressing effects on PPAR $\gamma$  and FABP4 expression were limited to the early stages of 3T3-L1 preadipocyte differentiation, which were similar to the results obtained from many or most of other carotenoids discussed above.

As an adipokine synthesized and secreted by differentiated adipocytes, leptin influences adipocyte lipid and glucose/insulin metabolism and is involved in the endocrine control of energy homeostasis [31]. Increasing evidence suggests that excessive expression/secretion of leptin is linked to obesity and obesity related diseases [32]. We observed that bixin, lycopene and  $\beta$ -carotene treatments largely reduced protein expression of leptin during 3T3-L1 adipocyte differentiation (Figure 4 A), demonstrating the capacity of the carotenoids to suppress adipogenesis. Moreover, this was in parallel to their suppression of PPAR $\gamma$  and FABP4 expression. It should be investigated in future works whether or not there is a possible link between the two pathways in the effects of carotenoids on 3T3-L1 adipocyte differentiation. Moreover, to the best of our knowledge, effects of bixin, lycopene and  $\beta$ -carotene on expression of leptin during 3T3-L1 preadipocyte differentiation have not been still understood well yet.

There had been little information about regulation of expression and activity of acetyl-CoA carboxylase (ACC) by carotenoids during adipocyte differentiation, although fucoxanthin was reported to increase the phosphorylation of ACC and not affect its protein levels in mature 3T3-L1 adipocytes [33]. In this study, we found that treatments with carotenoids significantly increased ACC protein levels (Figure 4 B), accompanied with suppression of 3T3-L1 adipocyte differentiation. As a lipogenic enzyme controlling fatty acid synthesis, ACC protein expression increased by carotenoids should facilitate lipid accumulation in differentiated adipocytes. However, carotenoid treatments largely inhibited intracellular lipid accumulation (Figure 2). The cause of the discord between ACC protein expression and intracellular lipid accumulation is still unknown. Further studies are needed to determine whether or not there are some events or mechanisms not still known yet from fatty acid synthesis to intracellular lipid accumulation during adipocyte differentiation.

In conclusion, the present study demonstrated that bixin, lycopene and  $\beta$ -carotene significantly reduced the viability of 3T3-L1 preadipocytes and suppressed the preadipocyte differentiation through the reduced intercellular lipid accumulation and expression of PPAR $\gamma$ , FABP4 and leptin. Although there are still important issues including the up-regulating effects of carotenoids on ACC

expression that remain to be resolved, our findings presented here suggest that bixin, lycopene and  $\beta$ -carotene may be nutritional regulators for the prevention and treatment of obesity and related metabolic disorders.

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