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ARTICLE

Cell-Based Assay To Quantify the Antioxidant Effect of Food-Derived Carotenoids Enriched in Postprandial Human Chylomicrons

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We developed a new method to evaluate the antioxidant effect of food products in a biological system. The antioxidant status of HepG2 cells was quantified after incubation with postprandial human chylomicrons after the intake of vegetable products. Three subjects consumed in a meal a vegetable soup containing 8.4 mg of β-carotene and 9 mg of lycopene. After 5 h, the subjects consumed a second meal without carotenoids. Blood samples were collected at basal time and every hour for 9 h. Chylomicrons were isolated from serum samples and used for both carotenoid quantification and HepG2 stimulation. Carotenoid in chylomicrons followed an inter-individual and bimodal carotenoid response. We demonstrated the antioxidant effect of postprandial chylomicrons in HepG2 at the time of maximum carotenoid concentration of chylomicrons with respect to basal time. This cell-based assay seems to be a useful method to evaluate the antioxidant effect of fruit and vegetable products in a biological system.

KEYWORDS: Reactive oxygen species; HepG2 cells; chylomicron; lycopene; β-carotene; vegetable and fruit food; postprandial

INTRODUCTION

Reactive oxygen species (ROS) play a critical role in cardiovascular and inflammatory diseases, cancer, and aging. Diets rich in fruits and vegetables, such as a Mediterranean diet, contain antioxidant compounds, such as carotenoids, that could help to prevent chronic diseases (*1*, *2*). Carotenoids are lipid-soluble plant pigments, and a significant amount can be found in the human diet. The most abundant carotenoids in human plasma are lycopene and β-carotene, occurring mainly in tomato and carrots, respectively (*3*).

Four challenges are related to the interpretation of carotenoid bioavailability: bioaccessibility, absorption, interpreting plasma response, and inter-individual variation (*4*). Bioaccessibility or the release of carotenoids is governed by characteristics of the food matrix, which affects the efficiency of physical, enzymatic, and chemical digestion. In the enterocyte, lipid compounds are packaged into chylomicrons, enter into the blood via lymph (*5*), and are delivered to the liver to be processed; thus, chylomicrons are the responsive pool of exogenous carotenoids.

Carotenoids have an antioxidant effect (*6*) and present trapping activity of ROS (*7*,*8*). However, contradictory results on the antioxidant effect of carotenoids in human plasma have been reported. Paterson et al. (*9*) investigated the effect of a daily increase of 400 g of fruits and vegetables upon markers of cardiovascular disease in healthy subjects. In comparison to the control treatment (with carotenoid-poor food), plasma concentrations of carotenoids increased significantly, whereas the plasma oxidant status and markers of oxidative stress were not affected by the treatment. Whereas after consumption of higher amounts of, e.g., lycopene, the oxidative stability seems to be enhanced because it was reported that the intake of 75 mg/day of lycopene extract for 1 week resulted in more than a 2-fold increase in serum lycopene concentration and a 20−25% decrease in serum lipid biomarkers of oxidative stress (*10*, *11*). However, Deniss et al. did not find any effect on the oxidative stress inflammatory biomarkers after the consumption of a similar dose of lycopene (*12*). The authors proposed that the common measurements of markers for vascular oxidative stress and inflammation used in the cohort was possibly not sensitive enough to detect such changes and that better biomarkers of oxidative status are needed.

The liver plays an important role in the lipid metabolism. The human liver cell line HepG2 was previously used as a biological model to study the effect of phytochemicals and remnant-like chylomicrons enriched in bioactive compounds (*13*−*16*);

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nevertheless, they have not been incubated directly with the postprandial human chylomicron serum fraction. In HepG2 cells, it is easy to detect changes on the antioxidant status in response to the bioactive compounds of different vegetables (*17*), but this method does not reflect the physiological situation because liver cells are not in direct contact with vegetable products but with chylomicrons. The objective was to develop a new method to study the antioxidant effect of vegetable food products, stimulating HepG2 cells with human postprandial chylomicrons produced after the intake of the selected food products.

MATERIALS AND METHODS

Cells and Reagents. HepG2 cells [American Type Culture Collection (ATCC) catalogue number HB-8065] were grown and maintained in minimum essential medium (MEM) with Earle's salts (Paisley, Scotland, U.K.), supplemented with 10% heat-inactivate fetal bovine serum from Sigma-Aldrich (St. Louis, MO), 2 mM L-glutamine from Gibco (Paisley, Scotland, UK), 50 units/mL penicillin, and 50 μg/mL streptomycin (Paisley, Scotland, U.K.). The cells were detached with trypsin (0.1%) plus ethylenediaminetetraacetic acid (EDTA, 0.2%) from Sigma-Aldrich (St. Louis, MO). For ROS evaluation, 2′,7′-dichlorofluorescein diacetate (DCFH-DA) and catalase were used from Sigma-Aldrich (St. Louis, MO). 3-(4,5-Dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and butylated hydroxytoluene (BHT) were purchased from Sigma-Aldrich (St. Louis, MO). Dimethylsulfoxide (DMSO) was purchased from Merck (Darmstadt, Germany). *iso*-Hexane (< 5% *n*-hexane) was purchased from Kemetyl. Acetone (HPLC) and methanol (MeOH, HPLC) were purchased from Fischer. Methyl *tert*-butyl ether (MTBE, HPLC) was purchased from Lab-scan. Milli-Q H_2O was purchased from Millipore. To optimize the cell culture conditions, we used a chylomicron sample from a subject with hypertriglyceridemia containing 680 mg/dL triglycerides (TGs).

Chylomicron Sterility. The sterility of the chylomicrons was evaluated by incubation of 25 μL of human chylomicrons in 5 mL of cell culture medium (complete MEM) for 1 week. No detectable growth of microorganisms was observed by microscopy (Nikon TMS) after 1 week of incubation, and also the cell culture medium characteristic, such as turbidity or pH, remained stable during this period.

MTT Assay. The MTT assay was used to evaluate the chylomicron antiproliferative activity. The assay was based on the cleavage of the yellow tetrazolium salt MTT into purple formazan by metabolically active cells, which can be photometrically quantified. An increase in the number of living cells leads to a purple color formation. For the assay, 9×10^3 cells/well in 100 μL of complete MEM were placed in a 96-well flat-bottom plate. Cells were allowed to be adhered for 24 h, and then 100 μL of complete cell growth medium without phenol red with an increasing concentration of chylomicron was added to the cells. The concentration of total chylomicrons in the assay ranged from 0 to 100 μg/mL. Blank wells contained the above concentration of chylomicrons but without cells. After 8, 24, and 48 h of cell incubation, we added 100 μL of MTT solution (2 mg/mL) and post-incubated for 2 h. The medium was replaced for 100 μL of DMSO to solubilize the formazan, and the plate was agitated for 5 min in darkness. The plate was read at a wavelength of 690 nm using a Labsystem Multiscan MCC/340 microplate reader (Thermo Electron Corporation, Barcelona, Spain).

Osmolality Test. The osmolality of complete cell medium plus chylomicrons was measured at different concentrations (0, 2, 5, 10, 25, 50, and 100 μg/mL) using a Vapro vapor pressure osmometer model 5520 (Wescor, Claremont, Ontario, Canada).

Postprandial Assay. Three healthy women aged 23 ± 2 years old with a body mass index (BMI) of 20.2 ± 1.7 kg/m^2 and normolipemic were recruited. The subjects followed a diet free of carotenoids 24 h before the postprandial assay. On the day of the experiment, subjects consumed a breakfast with 600 mL of soup containing tomatoes, carrots, and broccoli (Unilever, The Netherlands) and with a carotenoid content of 8.4 mg of β-carotene and 9 mg of lycopene. The breakfast also included 125 g of toasted white bread, 20 g of olive oil, and water *ad libitum*. The broccoli was used as a potential source of folate to design a healthy soup. At 5 h after the breakfast, the same subjects consumed a second meal without carotenoids, to release the carotenoids that could be retained by the enterocyte. The meal without carotenoids contained fish and chips, white bread, and water *ad libitum*. Blood samples were collected from the subjects at basal time (fasting) and every hour for 9 h (total 10 blood samples/subject) using an indwelling venous line (*18*). Serum was obtained by blood centrifugation at 1500*g* for 10 min. Chylomicrons were isolated according to the method described by Borel et al. (*19*), with some modifications; 2 mL of serum samples was added to sodium chloride (3 mL) and ultracentrifuged for 90 min at 15000*g* and 10 °C using a vertical rotor 50.3Ti (Beckman, Palo Alto, CA). Chylomicrons, appearing on the top of the tube, were separated into three aliquots. The first aliquot was used to quantify triglycerides in the chylomicrons. The second aliquot was used for the analyses of lycopene and β-carotene contents in the chylomicron serum fraction. The third aliquot was used to stimulate hepatocytes (HepG2 cells). The study protocol was performed in accordance with "The Helsinki Declaration of Human Studies" and approved by the Ethical Committee of the University of Murcia, Murcia, Spain. Each volunteer gave written informed consent before participating.

TG Quantification. TGs were quantified in both human serum samples and human serum chylomicrons using a commercial kit (Roche), using a Modular Hitachi and Cobas 711 (Roche Diagnostic).

Extraction and Quantification of Lycopene and β-Carotene in Chylomicron Samples. Thawed chylomicron samples (200 μL) were vortexed and denaturized by the addition of 200 μL of ethanol containing 0.1% butylated hydroxytoluene (BHT, added as an antioxidant). After homogenization with a vortex blender, 1 mL of hexane solution containing 0.1% BHT was added. The sample was homogenized again with a vortex blender, followed by sonication for 1 min, and then vortexed again. Samples were centrifuged by allowing the centrifuge to reach 3000*g* and then turned off. The upper hexane phase was transferred to a glass tube. The extraction procedure was repeated, except for the sonication step. Lycopene and β-carotene were quantified by high-performance liquid chromatography (HPLC) according to Sadler and Dezman (*20*). Extracted chylomicron samples were evaporated under a flow of nitrogen (35 °C). Residues were dissolved in 100 μL of methanol (MeOH)/MTBE (50:50, v/v), transferred to HPLC vials, loaded onto an autosampler (Waters 700_{plus}), and analyzed within 12 h. The injection volume was 40 μL, and flow rate was 1 mL/min. Samples were separated by reverse-phase elution on a C_{30} column (4.6 mm × 25 cm, particle size of 5 μm, YMC Europe GmbH, Schermbeck, Germany) and detected with a Waters 996 photodiode array detector. A gradient elution using MeOH and MTBE was set up. The gradient [initial conditions of 70% (v/v) MeOH and 30% (v/v) MTBE] was built up over 17 min (end concentrations of 30% MeOH and 70% MTBE). This was followed by isocratic elution for 18 min. The gradient was selected and applied to allow for separation of all-*trans*-β-carotene and its isomers as well as all-*trans*-lycopene and most of its geometrical isomers. Identification and quantification of all-*trans*-lycopene and -β-carotene was made at 452 nm and based on linear calibration curves with 8 points.

Stimulation of HepG2 with Chylomicrons and Evaluation of Cellular Antioxidant Activity. HepG2 cells (9×10^3 cells/well) were seeded on 96-well plates with completed MEM and incubated at 37 °C in a 5% CO_2 atmosphere for 24 h to be adhered. The cells were incubated for 24 h with the chylomicron samples obtained during the postprandial study, after dilution of 1:100 with complete MEM.

To evaluate oxidative status, the cells were treated for 20 min with 12.5 μM DCFH-DA diluted in DMSO at 20 mM. The nonpolar and non-ionic DCFH-DA crosses cell membranes and is hydrolyzed to non-fluorescent 2′,7′-dichlorofluorescein (DCFH) by intracellular esterases (*14*, *21*). Antioxidants in the chylomicrons, such as carotenoids, may prevent oxidation of DCFH and decrease the fluorescence intensity. Thus, a lowering of the fluorescence intensity indicates a higher antioxidant activity. Catalase (1000 units/mL) was used as a positive control to check that the method worked properly; fluorescence decreased in the cells after the addition of the catalase enzyme (*22*). Finally, the stimulated cells were trypsinized and resuspended in cold phosphate-buffered saline (PBS) in darkness. The fluorescence intensity of the cells was measured using flow cytometry (Coulter, Epics XL, Miami, FL). A total of 5000 hepatocytes/3 wells were acquired and analyzed. The signal intensity was analyzed using the Cell Quest Software from Applied Cytometry System (Miami, FL). Triplicate experiments were performed for these *in vitro* studies.

Statistical Analysis. Data were analyzed by Statistical Package SPSS 11.0 version for Windows (SPSS, Inc., Chicago, IL). Differences

Table 1. Cell Proliferation (%, with Respect to the Control) in HepG2 Stimulated with Different Concentrations of Chylomicrons for 8, 24, and 48 h[a]

	concentration of TGs in chylomicron (μg/mL)							
time (h)	0	2	5	10	25	50	100	*p*
8	100 ab	106 b	98 ab	104 ab	97 a	101 ab	135 b	0.027
24	100 a	101 a	107 a	115 a	117 a	105 a	165 a	0.150
48	100 a	140 abc	138 abc	139 bd	152 cb	172 bd	210 d	0.000

[a] Chylomicrons were obtained from a diluted series of a 500 μg/mL chylomicron sample. Results were calculated from five replicates.

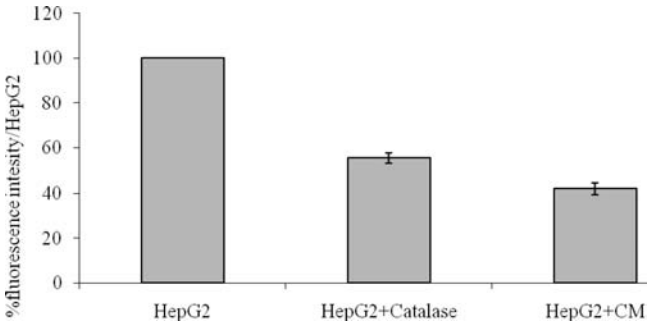

Figure 1. Antioxidant effect of catalase and chylomicrons (CM) in HepG2 as a percentage of fluorescence intensity with respect to non-stimulate HepG2. The results were expressed as the mean ± standard error of the mean (SEM). Each treatment was tested in four different replicates.

by concentrations on the cell proliferation study were evaluated by one-way analysis of variation (ANOVA). $p < 0.05$ values were considered significant.

RESULTS

Optimization of the Cell Culture Conditions. Cell culture medium osmolality ranged between 285 and 300 mmol/kg, and it did not change with respect to the control medium range after the addition of human serum chylomicrons in a concentration from 2 to 100 μg/mL. Thus, under these experimental conditions, HepG2 cell growth variation was not osmotically mediated.

To determine the cytotoxic concentration of human postprandial chylomicrons, we incubated HepG2 cells with different concentrations of chylomicrons (from 0 to 100 μg/mL; **Table 1**). We can observe that, after 8, 24, and 48 h of incubation, HepG2 cell proliferation increased with the time and according to a dose-dependent manner. Thus, human postprandial chylomicrons do not have a cytotoxic effect on HepG2 at the studied concentration, and the best conditions point toward incubation with chylomicrons for 24 h and ≤50 μg/mL.

Stimulation of HepG2 cells with chylomicrons diluted 1:100 decreased fluorescence intensity 42 ± 2% compared to the control (100%). This decrease was even higher than those using a positive control with catalase (**Figure 1**); thus, chylomicron serum fractions per se have an antioxidant effect on HepG2.

Concentration of Both TGs and Carotenoids in Postprandial Chylomicrons. The TG concentration in human chylomicrons is described in **Figure 2A**. We found a bimodal response for TG in chylomicrons with two peaks: the first one at 2−3 h with respect to breakfast intake and the second one at around 7 h after lunch intake.

Concerning both lycopene and β-carotene concentrations in chylomicrons, we observed a bi- or trimodal carotenoid response with a main peak at 3 h and a two other pulses at 6 and 8 h in the postprandial assay (panels **B** and **C** of **Figure 2**). The results also indicated a wide inter-individual variability in the postprandial carotenoid response (panels **B** and **C** of **Figure 2**).

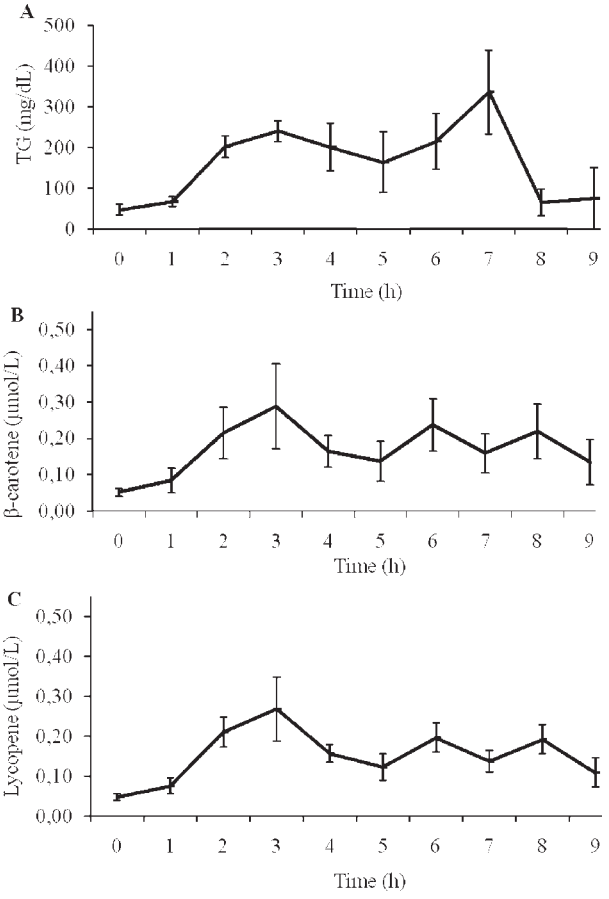

Figure 2. Concentration of (A) TGs, (B) β-carotene, and (C) lycopene in postprandial human serum chylomicrons. The results were expressed as the mean ± SEM. Each treatment was tested in four different replicates.

Antioxidant Effect of Human Postprandial Chylomicrons in HepG2 Cells. The highest antioxidant activity in HepG2 cells was found after exposure with the chylomicrons collected at 3 h after the intake of the meal (diluted 1:100) compared to chylomicron samples obtained at basal time (0 h; **Figure 3**). Because the maximum TG concentration in postprandial chylomicrons was found at 7 h, while the carotenoid concentration peaked at 3 h, carotenoids rather than TGs are likely to be responsible for the antioxidant activity of the chylomicron fractions in the HepG2 cells.

DISCUSSION

We have developed a method to study the cellular antioxidant activity of food products based on the antioxidant effect of postprandial human chylomicrons in HepG2 cells. The assay is intended as an improvement of the chemical methods used to evaluate and predict *in vivo* antioxidant activity.

Our data showed that the human chylomicrons isolated did not contain microorganisms that could contaminate the cell culture medium and also that their effect on cell culture was not osmotically mediated. Moreover, no cytotoxic effect of human chylomicrons was detected in HepG2 cells; on the contrary, we observed a proliferative effect on HepG2 that was both time- and dose-dependent. This proliferative effect might be explained by the fact that internalized chylomicrons may provide lipid substrates to HepG2 (*23*) that could enhance cellular proliferation or that might reduce cellular apoptosis. The human chylomicron serum fraction significantly reduced the fluorescence intensity of DCFH in the HepG2 compared to the higher fluorescence values

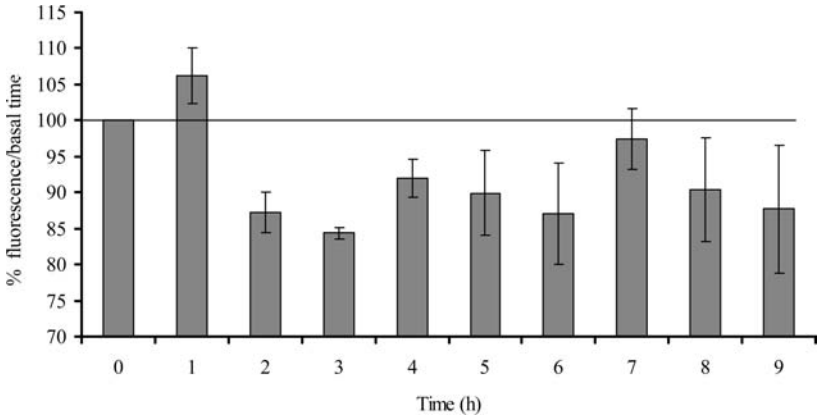

Figure 3. Antioxidant effect of human chylomicrons from the postprandial study on HepG2 cells represented as a percentage of the fluorescence intensity with respect to basal time (0 h). The results were expressed as the mean ± SEM. Each treatment was tested in three different replicates.

obtained in cells that were not stimulated, indicating that their oxidative status was decreased. It has been reported that chylomicron remnants enriched in *n*-3 or *n*-6 polyunsaturated fatty acids modulate cellular oxidative status (*24*). We propose that the human chylomicron serum fraction contains some molecules that can be metabolized by the HepG2 cells and their content promotes the reduction of intracellular ROS.

Because it is possible that carotenoids can be not only incorporated into the chylomicrons secreted in the first postprandial period after a meal with lipids but also secreted after subsequent meals, we gave a second meal to the subjects to avoid underestimating the carotenoid response (*25*, *26*). We detected an increase in postprandial chylomicron β-carotene and lycopene concentrations with a trimodal response in the postprandial period (panels **B** and **C** of **Figure 2**). The first peak, at around 3 h, could be due to the process of gastric emptying of the breakfast containing the carotenoids. The second peak, with two increments (at 6 and 8 h in the postprandial assay), within about 2 h after the second meal, appears because some lipids and, hence, lycopene and β-carotene can be released from the enterocyte after the ingestion of a second carotenoid-free meal.

In agreement with several authors, we found a high inter-individual variability in the postprandial carotenoid response in serum after a single oral meal. The inter-individual variability in serum response could be due to differences in digestion and absorption that could even depend upon age, sex, health status, and the rate of clearance of the chylomicrons and probably similar to the variability observed for triglyceride responses (*12*, *25*−*27*). Borel et al. (*28*) also suggested that polymorphism in genes related to lipid metabolism, absorption, intracellular trafficking, and plasma transport of carotenoid, such as apoB, SR-BI, and apoA-IV, could be involved.

Oxidative stress produced by some dietary substrates is one of the major causes of liver cell injury (*29*). Several studies have demonstrated the protective effect of carotenoids in hepatocytes; lycopene reduced ethanol-induced oxidative stress and apoptosis in HepG2 cells (*30*) and also attenuated arachidonic acid toxicity in HepG2 cells overexpressing CYP2E1 (*31*). In addition, lycopene has been reported to reduce genotoxicity and mutagenicity of H_2O_2 in HepG2 cells (*32*). Moreover, other studies have described the antiproliferative and cytoprotective activities of fruit and vegetable products rich in polyphenols and carotenoids in HepG2 (*17*, *33*), although liver cells are not in contact directly with food products. Because newly absorbed carotenoids are transported by chylomicrons, we stimulated the HepG2 cells with human postprandial chylomicrons enriched in carotenoid from the consumption of food and vegetable products.

The present study showed that HepG2 cells treated with postprandial chylomicrons at 3 h showed the lowest fluorescence intensity and, thus, the lowest ROS concentration compared to cells treated with chylomicrons collected at 0 h (basal sample). It is noteworthy that postprandial chylomicrons were diluted 1:100 for the stimulation of the HepG2, and thus, the effect could be measured even at very low concentrations.

The maximum carotenoid concentration in chylomicrons was produced at 3 h, the same time point as the maximum cellular antioxidant status of postprandial chylomicrons on HepG2 cells. These results indicated that the highest antioxidant effect in the cells seems to be related to the carotenoid content instead of the TG content. The protective effect of carotenoids is ascribed to their ability to act as antioxidants, thereby inhibiting the negative effects of ROS (*11*) in the form of oxidative damage (*9*). This result supports the concept that dietary carotenoids scavenge ROS directly (*34*). In contrast, at 7 h, when the concentration of TG was highest, we found an increase in fluorescence intensity and, hence, ROS in the cells. A possible explanation is that the increase in oxidative stress is produced by the postprandial hyperlipidemia (*34*) with chylomicrons poor in carotenoids.

Other liposoluble compounds present in the vegetable soup, such as tocopherols, might also affect the antioxidant status in the HepG2 cells; unfortunately, all of these compounds were not quantified in this study. In addition, the few human subjects could be a limitation of this study.

In summary, we have developed a biological system to evaluate the antioxidant effect of carotenoids in food products. We can conclude that the proposed cell-based assay, stimulating HepG2 cells with human postprandial chylomicrons, seems to be a useful method to evaluate the antioxidant potential of fruit and vegetable products.

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