

## Lycopene and Beta-carotene Induce Cell-Cycle Arrest and Apoptosis in Human Breast Cancer Cell Lines

NATHALIE FONSECA GLORIA<sup>1</sup>, NATHALIA SOARES<sup>2</sup>, CAMILA BRAND<sup>2</sup>,  
FELIPE LEITE OLIVEIRA<sup>2</sup>, RADOVAN BOROJEVIC<sup>3</sup> and ANDERSON JUNGER TEODORO<sup>1</sup>

<sup>1</sup>Laboratory of Nutritional Biochemistry, Program of Food and Nutrition, UNIRIO, Rio de Janeiro, Brazil;

<sup>2</sup>Laboratory of Cell Proliferation and Differentiation, Institute of Biomedical Sciences,  
Federal University of Rio de Janeiro, Rio de Janeiro, Brazil;

<sup>3</sup>Excellion Biomedical Services, Petrópolis, Rio de Janeiro, Brazil

**Abstract.** Lycopene and beta-carotene are carotenoids widely distributed in fruits and vegetables, with potential anticancer activity. Epidemiological trials rarely provide evidence for the mechanisms of action of these compounds, and their biological effects at different times of treatment are still unclear. The aim of the present study was to determine the effect of carotenoids on the cell cycle and cell viability in human breast cancer cell lines. Human breast cell lines were treated with carotenoids (0.5-10  $\mu$ M) for 48 and 96 h. Cell viability was monitored using the MTT method (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue). The cell cycle was analyzed by flow cytometry, and apoptotic cells were identified by annexin/propidium iodide (PI) biomarkers. Our data showed a significant decrease in the number of viable breast cancer cells on treatment with carotenoids. Carotenoids also promoted cell-cycle arrest followed by decreased cell viability in the majority of cell lines after 96 h, compared to controls. Furthermore, an increase in apoptosis was observed in cell lines when cells were treated with carotenoids. Our findings show the capacity of lycopene and beta-carotene to inhibit cell proliferation, arrest the cell cycle in different phases, and increase apoptosis. These findings indicate that the effect was cell type-dependent and that carotenoids are potential agents for biological interference with cancer.

*Correspondence to:* Anderson Junger Teodoro, Universidade Federal do Estado do Rio de Janeiro, Escola de Nutrição, Departamento de Tecnologia de Alimentos CEP 22290-240, Rio de Janeiro, RJ, Brazil. Tel: +55 2125427276, Fax: +55 2125427752, e-mail: atteodoro@gmail.com

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Breast cancer is the most frequently diagnosed type of cancer globally (1), and is a complex disease caused by progressive genetic mutations, associated with other factors. The increased incidence of breast cancer follows the behavioral changes of urbanization, such as a sedentary lifestyle, excess of processed food in the diet, overweight and obesity (2, 3).

Diets enriched in fruits and vegetables are associated with reduced rates of cancer and coronary heart disease. Bioactive compounds present in the diet have been reported as substances responsible for these effects. In this context, carotenoids, such as lycopene and beta-carotene, are being studied to correlate diet and decreased risk of breast and other types of cancer (4, 5).

Lycopene, the major carotenoid component of tomato, exhibited potential anticancer activity in distinct types of cancer (6, 7). Epidemiological studies have reported a statistically significant inverse association between tomato consumption and risk of several types of cancer, including lung, prostate and colon cancer (8-10).

On the other hand, beta-carotene, a carotenoid precursor of vitamin A, an essential nutrient with antioxidant activity, is suggested to be an important factor in reduction of cancer risk. It has been demonstrated that human cancer risks are inversely correlated with high blood levels of retinol or beta-carotene and intake of food containing retinol (11-13). Even when administered at high doses during long periods of time, beta-carotene does not cause toxicity, whereas high doses of vitamin A and retinoids, as possible prophylactic agents for cancer prevention, can lead to acute hepatotoxicity and cause other adverse effects (14).

Beta-carotene inhibited the growth of many human colon adenocarcinoma cell lines, inducing cell-cycle arrest and apoptosis. It has been reported that beta-carotene was responsible for reducing cyclin A, an important regulator of cell-cycle progression (15). *In vitro* studies support the hypothesis that this carotenoid is chemopreventive or

chemotherapeutic against some kinds of cancer, including of the prostate (16) and melanoma (17).

Lycopene has an anti-proliferative effect on prostate and breast cancer cell-lines. In breast cancer models, reduced expression of cell cycle regulatory proteins, such as cyclins (D1 and E) and cyclin-dependent kinases (-2 and -4), as well as suppression of insulin-like growth factor (IGF-I) action, have been correlated with the effects of lycopene on cell proliferation (18, 19). Other studies reported that lycopene had limited effect on cell proliferation of cancerous and non-cancerous cell lines in an *in vitro* system with doses within the physiological range, but only rarely have studies reported no effect of lycopene on cell proliferation (20, 21).

Lycopene and beta-carotene have been proposed to negatively affect cancer cells or the development of cancer by modulating cell-cycle progression and cell proliferation. The de-regulated cell cycle is one of the hallmarks of cancer cells. These cells have disturbed cell cycle and inability to control their rate of proliferation. A rate-limiting step in the cell cycle that is often disturbed in cancer is the progression of cells through the first gap ( $G_1$ ) phase (22). Recently, several reports have shown that lycopene can induce cell-cycle arrest at the  $G_1$  phase in human hepatocarcinoma cells, and  $G_0/G_1$  arrest and S phase block in human prostate cancer cell lines LNCAP and PC3.

Although the role of carotenoids in the prevention of breast cancer has been studied more extensively, human studies with lycopene and beta-carotene on cancer are now being undertaken. The majority of studies on the effects on the cell cycle were carried out after 48 h of carotenoid treatment, which can lead to underestimation of the effect of these substances. The aim of the present study was to determine the effect of lycopene and beta-carotene on cell cycle and cell viability in three different human breast cancer cell lines at different time points.

## Materials and Methods

**Cell culture reagents.** All-trans lycopene and all-trans beta-carotene was purchased from Sigma Chemical Company (St. Louis, MO, USA). Water-soluble (WS) lycopene (10%) and CWS beta-carotene (1%) was provided by Roche (Rio de Janeiro, RJ, Brazil). Dulbecco's cell culture medium (DMEM) and bovine serum albumin were obtained from Sigma, and fetal bovine serum (FBS) from Laborclin (Campinas, SP, Brazil). Cell culture flasks and cell scrapers were obtained from Nunc (Roskilde, Denmark). All chemicals were of analytical grade.

**Cell culture experiments.** Cell lines were obtained from the Rio de Janeiro Cell Bank that certified their identity and quality (Inmetro, Rio de Janeiro, RJ, Brazil). Human breast adenocarcinoma cell lines (MCF-7, MDA-MB-231 and MDA-MB-235) were plated in 25 cm<sup>2</sup> cell culture flasks at a density of  $5.0 \times 10^6$  cells/flask, and maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and

2 g/l HEPES buffer, pH 7.4, under a 5% CO<sub>2</sub> atmosphere. Cells were passaged by trypsinization when reaching 70-80% of confluence, about twice a week. For each experiment, all cells were seeded at a density of  $10^4$  cells/cm<sup>2</sup> in 6- and 96-multiwell plates for cell cycle and cell proliferation analyses, respectively. Lycopene (WS) was dissolved in water at 50°C within a range from 0.5 to 10 µM and beta-carotene was dissolved in a solution with water and ethyl alcohol. Carotenoids were then added to the plates. Cells untreated (controls) were included on each plate. After 24 h, the culture medium was changed. Cells were then incubated for 48 and 96 hours with daily medium replacement.

**Cell viability assay.** The status of cancer cell line viability was determined by of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue) assay (Sigma, New York, USA). Exponentially growing cell lines were adjusted to  $1.0 \times 10^4$  cells/cm<sup>2</sup> with DMEM, plated in 96-well plates at 200 µl/well and then incubated for 24 h according to routine procedure. After treatment with lycopene and beta-carotene (0.5-10 µM) and incubation for 48 h and 96 h (six wells for each sample), 20 µl of MTT (5 g/l) were added to each well. After 4 h of incubation, the medium was then removed and 100 µl/well sodium dodecyl sulfate (SDS) was added to dissolve the reduced formazan product. Finally, the plate was read in an enzyme-linked immunosorbent microplate reader (Bio-Rad 2550 at 490 nm). The cellular proliferation inhibition rate (CPIR) was calculated using the following formula:  $CPIR = (1 - \text{average absorbance value of experimental group} / \text{average absorbance value of control group}) \times 100\%$ .

**Cell cycle analysis.** Cells were rinsed briefly with calcium- and magnesium-free phosphate-buffered saline (PBS) and detached from cell culture flasks with trypsin at 37°C. The cells were washed twice with PBS  $1 \times 10^6$  cells were resuspended in 1.0 ml of ice-cold VindeLov solution containing 0.1% Triton X-100, 0.1% citrate buffer 0.1 mg/ml RNase and 50 µg/ml propidium iodide (Sigma Chemical Co.). After 15 min incubation, DNA content was measured by flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA). The relative proportions of cells with diploid DNA content  $G_0-G_1$  (2n), S phase (>2n but <4n), and  $G_2/M$  phase (4n) were acquired and analyzed using CellQuest and WinMDI 2.9, software, respectively. The percentage of the cell population at a particular phase was estimated with EXPO32 V1.2 Analysis software (Beckman Coulter, Inc., Brea, CA, USA). The cell dissociation procedure did not affect fluorescence under the experimental conditions that were used in this study.

**Apoptosis assay.** Human breast cancer cell lines ( $1.0 \times 10^4$  cells/cm<sup>2</sup>) were treated with lycopene and beta-carotene at a concentration of 5 and 10 µM in 6-well plates. After 48 and 96 h of incubation, the non-adherent cells were collected, and adherent cells were quickly washed with PBS and were detached with trypsin/EDTA 0.125% (Sigma Chemical Co.) at room temperature. Subsequently, apoptotic and necrotic cells were co-stained with annexin V- fluorescein isothiocyanate (FITC) and propidium iodide (PI) (BD Pharmingen) according to the manufacturer's instructions, quantified by flow cytometer (FACSCalibur; BD Bioscience, NJ, USA) and analyzed using WinMDI 2.9 software. Apoptotic data were reported as the percentage apoptosis, obtained by determining the numbers of apoptotic cells *versus* the total numbers of cells.

Apoptotic data are presented as the mean $\pm$ SD for three independently performed experiments.

*Statistical analysis.* The presented data are mean values $\pm$ standard error of three independent experiments performed in duplicate (n=6). Statistical comparisons were carried out by ANOVA and *post hoc* Tukey's test using Graph Pad Prism 4.0 and Statistical 6.0 program. (GraphPad, La Jolla, CA, USA) The differences were considered significant when  $p < 0.05$ .

## Results

*Effect of lycopene and beta-carotene on the number of viable cells in culture.* All cell lines showed normal growth characteristics expected under standard *in vitro* conditions. Previous studies reported that lycopene formulated as 10% WS granules was not toxic (23). Hereafter, all the results refer to lycopene with this vehicle, already used in our previous studies *in vitro* (24-26). Lycopene showed distinct effects when added to different types of cells, but reduction in cell viability was observed in all cancer cells.

We used the MTT assay to monitor cell viability. The MCF-7 cell line presented a decrease in the cell number only at the highest concentrations of lycopene (2.5-10  $\mu$ M). When the cells were treated with 10  $\mu$ M, the reduction reached 20% (Figure 1A). A clear effect was observed on the MDA-MB-235 cell line only with higher doses of lycopene, but the decrease was statistically insignificant (Figure 1B). After 48 h of treatment, a substantial decrease in the number of viable cells was observed in the MDA-MB-231 cell line, reaching a 30% reduction compared to untreated cells. (Figure 1C).

After 96 h, lycopene significantly changed the cell viability in all cell lines. Lycopene treatment inhibited cell viability of MCF-7 and MDA-MB-235 cells by 30% and 20%, respectively (Figure 1A, 1B). However, MDA-MB-231 cells were inhibited by 75% with this compound (Figure 1C). Taken together, these data indicate that the effect of lycopene is cell-specific and time-dependent, and this effect requires a relatively long incubation time for the majority of breast cell lines studied to achieve better action.

All cell lines treated with beta-carotene presented different behaviors when compared to lycopene-treated cells. After 48 h of treatment, beta-carotene caused significant changes in cell viability of the three cell lines. The reduction was in the order of 40% in the MCF-7 cell line (Figure 2A), 30% in MDA-MB-235 cells (Figure 2B) and 70% in MDA-MB-231 cells (Figure 2C) when treated with 10  $\mu$ M.

After 96 h of treatment, the effect of beta-carotene on the different cell lines was similar, except for the MCF-7 cell line, in which a stronger short-term effect was found (Figure 2A, B and C).

*Effect of lycopene and beta-carotene on cell-cycle progression.* In order to elucidate the mechanism by which lycopene and

beta-carotene regulated cell growth, we treated cells with these compounds for 48 and 96 h and quantified the percentage of cells in different cell-cycle phases.

In the presence of lycopene, we found a time-dependent influence on the MDA-MB-235 cell line, with an important decrease of the G<sub>2</sub>/M phase that reached 18%, which was explained by accumulation of cells in the G<sub>0</sub>/G<sub>1</sub> phase. MCF-7 cells exhibited the same changes but with no differences between time of exposure (48 and 96 h) (Table I).

The MDA-MB-231 cell line underwent the major changes in cell viability when treated with lycopene and showed an important change in the percentages of cells in different cell-cycle phases. After 96 h, a significant increase in the order of 10% of cells was observed in G<sub>0</sub>/G<sub>1</sub> phase with a decrease in the percentage of cells on S phase. No effect was observed after 48 h of treatment in MDA-MB-231 and MDA-MB-235 cell lines (Table I).

After treatment with 5 and 10  $\mu$ M beta-carotene, MCF-7 cells presented a reduction in the order of 30% of the percentage of cells in G<sub>2</sub>/M phase after 48 and 96 h. MDA-MB-235 cell line treated with beta-carotene demonstrated a cell-cycle arrest, with an increased number of cells in the G<sub>0</sub>/G<sub>1</sub> phase after 96 h of treatment. No changes in cell-cycle distribution was observed after 48 h of treatment in the MDA-MB-235 cell line (Table II).

Alterations in the MDA-MB-231 cell cycle were also observed. Beta-carotene (5 and 10  $\mu$ M) promoted an increase in cells retained in the G<sub>2</sub>/M phase, followed by a decrease of cells in G<sub>0</sub>/G<sub>1</sub> phase after 48 and 96 h.

Similar to the data on cell viability, these data indicate that the effect of lycopene and beta-carotene were cell-specific and time-dependent, and that this effect required a relatively long incubation time in the majority of cell lines.

*Apoptosis.* Apoptosis induction was studied by annexin V and PI biomarkers. Alteration of the balance between proliferation and apoptosis is associated with cancer and quantification of apoptosis can be a useful measure of cancer cell kinetics. In general, lycopene and beta-carotene induced apoptosis in all cell lines.

After 48 h, two cell lines (MCF-7 and MDA-MB-235) presented an increase of apoptotic cells with dose-dependent characteristics, with an average increase of 1.90- and 1.30-fold, respectively (Figure 3). In MDA-MB-231 cells, no significant difference was observed when compared to the untreated group after 48 h, results similar to those obtained in the analysis of the cell cycle (48 h).

Lycopene promoted apoptosis in all cancer cell lines after 96 h, results similar to those obtained in the analysis of cell viability and cell cycle. Lycopene induced apoptosis of MDA-MB-231 cells, with an average increase of 4.10-fold after 96 h treatment and maximum increase of 4.9-fold after 96 h, at the highest lycopene concentration (10  $\mu$ M).

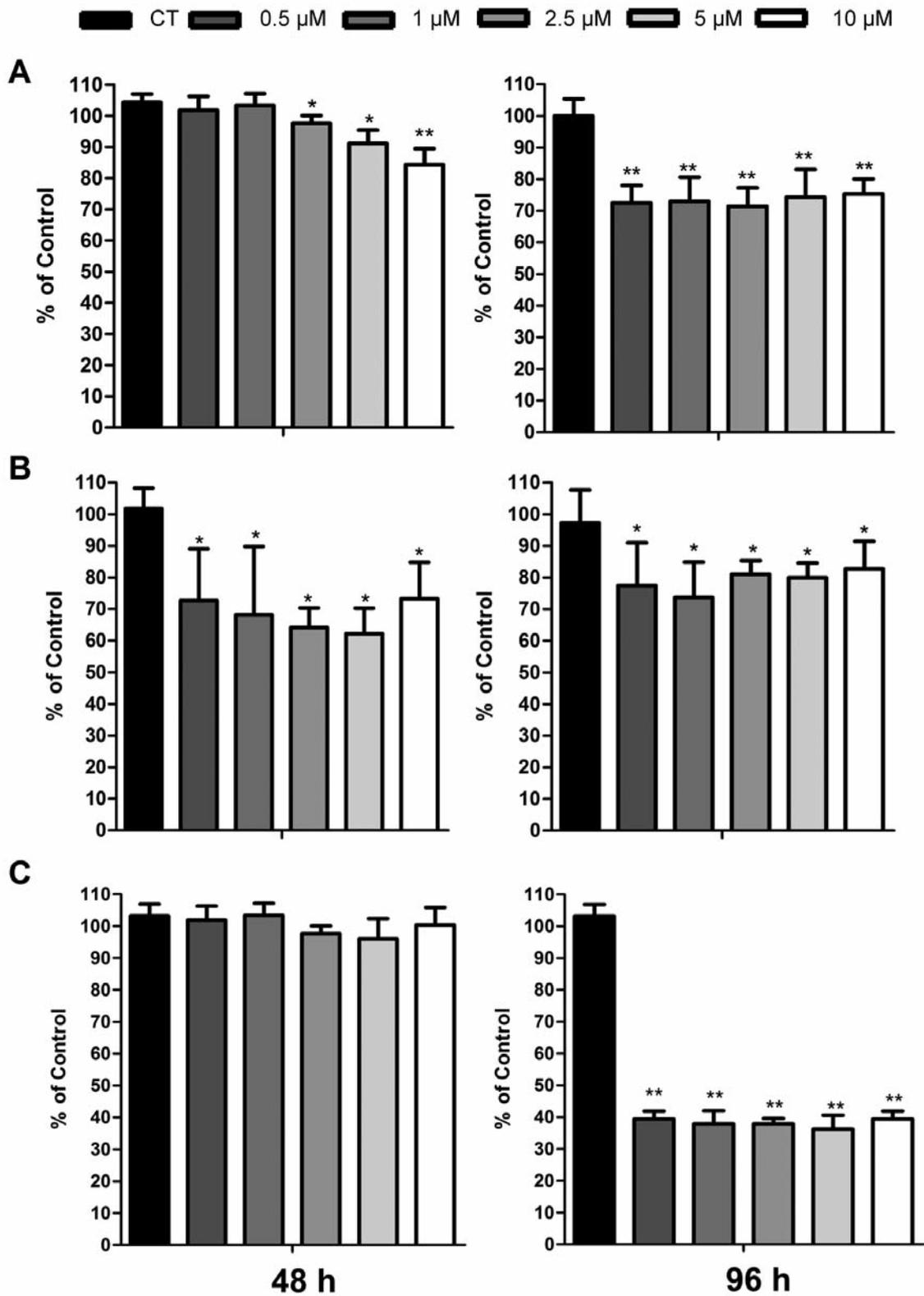


Figure 1. Cell lines were exposed to different concentrations of lycopene (0.5-10 μM) for different periods (48-96 h): (A) MCF-7; (B) MDA-MB-235; (C) MDA-MB-231. Data are presented as the means±SD, n=3, \*p<0.05, \*\*p<0.01 versus control (CT) group.

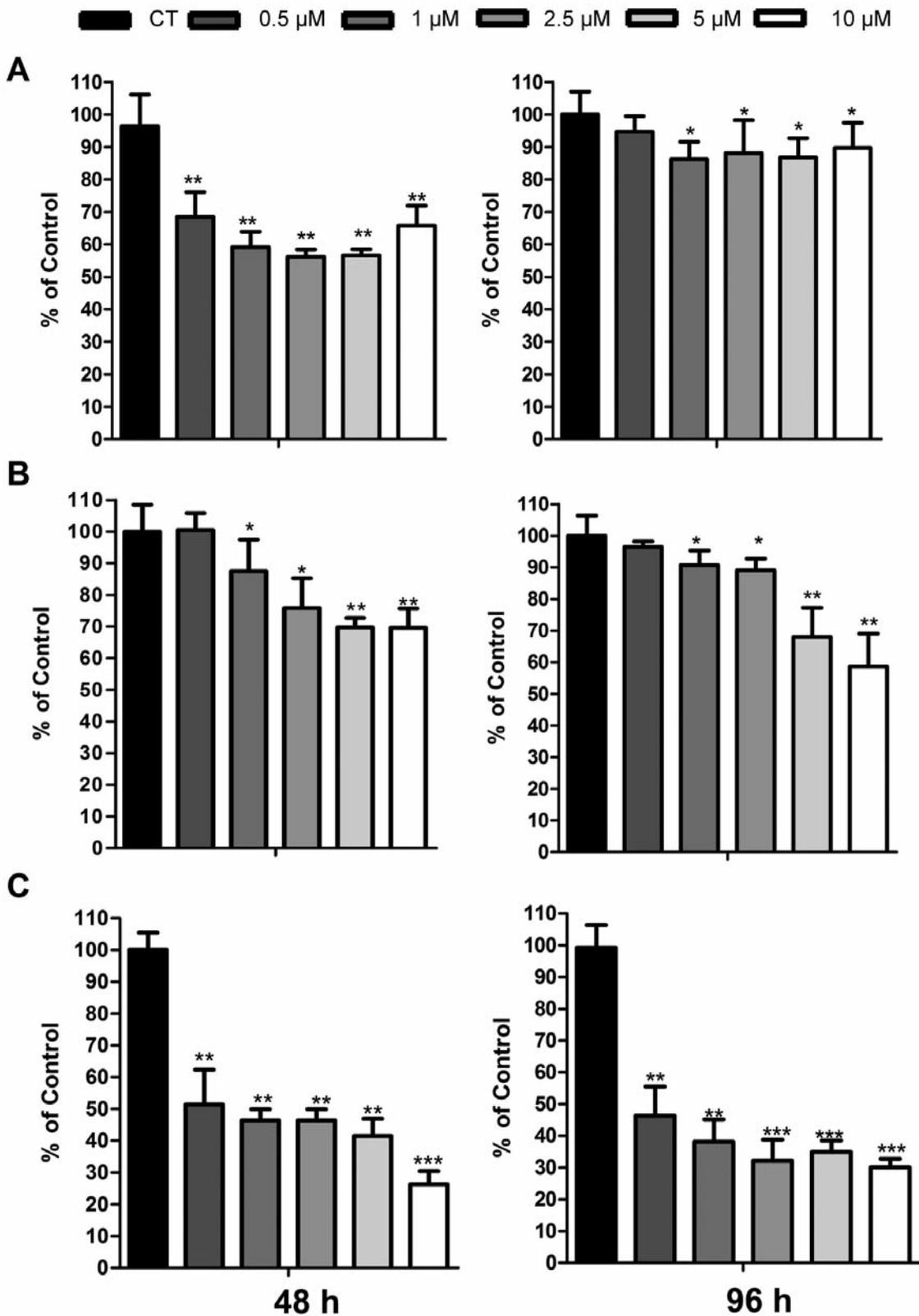


Figure 2. Cell lines were exposed to different concentrations of beta-carotene (0.5-10 μM) for different periods (48-96 h): (A) MCF-7; (B) MDA-MB-235; (C) MDA-MB-231. Data are presented as the means±SD, n=3, \*p<0.05, \*\*p<0.01, \*\*\*p<0.001 versus control (CT) group.

Table I. Effect of lycopene (5-10  $\mu\text{M}$ ) on cell-cycle progression in different human breast cancer cell lines after 48 and 96 h. Results are expressed as the percentage of total cells. Data represent the mean $\pm$ SD values of triplicate experiments.

Cell line	Time	Cell cycle phase	Control	5 $\mu\text{M}$	10 $\mu\text{M}$
MCF-7	48h	G <sub>0</sub> /G <sub>1</sub>	29.45 $\pm$ 2.05	32.65 $\pm$ 0.49*	33.20 $\pm$ 1.13*
		S	17.00 $\pm$ 4.24	16.75 $\pm$ 3.18	17.35 $\pm$ 0.92
		G <sub>2</sub> /M	36.50 $\pm$ 0.71	16.90 $\pm$ 1.27**	22.40 $\pm$ 1.98**
	96h	G <sub>0</sub> /G <sub>1</sub>	17.10 $\pm$ 1.56	19.30 $\pm$ 0.99*	20.20 $\pm$ 1.13*
		S	26.25 $\pm$ 0.35	24.15 $\pm$ 1.20	22.95 $\pm$ 1.48
		G <sub>2</sub> /M	40.50 $\pm$ 0.71	30.20 $\pm$ 0.28*	24.95 $\pm$ 0.07**
MDA-235	48h	G <sub>0</sub> /G <sub>1</sub>	63.66 $\pm$ 0.37	62.11 $\pm$ 3.35	63.62 $\pm$ 0.91
		S	18.59 $\pm$ 0.30	19.78 $\pm$ 2.11	20.01 $\pm$ 0.24
		G <sub>2</sub> /M	17.16 $\pm$ 0.28	17.66 $\pm$ 1.71	16.07 $\pm$ 0.35
	96h	G <sub>0</sub> /G <sub>1</sub>	44.31 $\pm$ 1.00	49.10 $\pm$ 1.13*	49.05 $\pm$ 1.20*
		S	29.15 $\pm$ 0.35	25.75 $\pm$ 2.76*	26.30 $\pm$ 0.85*
		G <sub>2</sub> /M	24.15 $\pm$ 0.64	20.85 $\pm$ 0.35*	19.95 $\pm$ 1.63*
MDA-231	48h	G <sub>0</sub> /G <sub>1</sub>	67.66 $\pm$ 3.72	67.11 $\pm$ 3.25	66.62 $\pm$ 0.71
		S	19.59 $\pm$ 1.30	18.78 $\pm$ 1.12	20.15 $\pm$ 2.42
		G <sub>2</sub> /M	15.16 $\pm$ 2.28	16.66 $\pm$ 1.92	16.09 $\pm$ 1.95
	96h	G <sub>0</sub> /G <sub>1</sub>	68.98 $\pm$ 1.39	75.04 $\pm$ 0.25*	75.81 $\pm$ 0.60*
		S	14.17 $\pm$ 0.05	12.06 $\pm$ 0.17*	11.39 $\pm$ 0.37*
		G <sub>2</sub> /M	14.71 $\pm$ 3.51	13.88 $\pm$ 0.23	13.70 $\pm$ 1.08

Tukey test: \* $p$ <0.05, \*\* $p$ <0.01 compared to the control.

Table II. Effect of beta-carotene (5-10  $\mu\text{M}$ ) on cell cycle progression in different human breast cancer cell lines after 48 and 96 hours. Results are expressed as the percentage of total cells. Data represent mean $\pm$ SD values of triplicate experiments.

Cell line	Time	Cell cycle phase	Control	5 $\mu\text{M}$	10 $\mu\text{M}$
MCF-7	48 h	G <sub>0</sub> /G <sub>1</sub>	62.51 $\pm$ 3.19	78.28 $\pm$ 4.79*	78.51 $\pm$ 3.19*
		S	5.03 $\pm$ 0.21	4.41 $\pm$ 0.04	5.03 $\pm$ 0.21
		G <sub>2</sub> /M	32.20 $\pm$ 3.83	27.33 $\pm$ 4.7*	23.24 $\pm$ 3.15*
	96 h	G <sub>0</sub> /G <sub>1</sub>	64.33 $\pm$ 0.16	75.04 $\pm$ 0.74*	74.33 $\pm$ 0.16*
		S	6.76 $\pm$ 1.26	8.79 $\pm$ 3.37	6.76 $\pm$ 1.26
		G <sub>2</sub> /M	28.29 $\pm$ 1.70	24.65 $\pm$ 4.48*	25.29 $\pm$ 1.70*
MDA-235	48 h	G <sub>0</sub> /G <sub>1</sub>	59.70 $\pm$ 0.48	58.18 $\pm$ 0.30	59.48 $\pm$ 0.62
		S	20.96 $\pm$ 0.96	21.27 $\pm$ 0.49	20.67 $\pm$ 0.13
		G <sub>2</sub> /M	18.84 $\pm$ 1.09	20.16 $\pm$ 0.67	19.7 $\pm$ 0.54
	96 h	G <sub>0</sub> /G <sub>1</sub>	44.31 $\pm$ 1.00	49.15 $\pm$ 1.12*	49.13 $\pm$ 1.22*
		S	29.17 $\pm$ 0.35	25.78 $\pm$ 2.73*	26.37 $\pm$ 0.88*
		G <sub>2</sub> /M	24.21 $\pm$ 0.64	24.42 $\pm$ 5.29	19.97 $\pm$ 1.64*
MDA-231	48 h	G <sub>0</sub> /G <sub>1</sub>	76.35 $\pm$ 0.16	72.89 $\pm$ 0.10*	71.47 $\pm$ 3.04*
		S	11.10 $\pm$ 0.78	10.53 $\pm$ 0.41	12.44 $\pm$ 1.43*
		G <sub>2</sub> /M	13.45 $\pm$ 0.74	16.58 $\pm$ 0.25*	17.13 $\pm$ 1.85*
	96 h	G <sub>0</sub> /G <sub>1</sub>	70.35 $\pm$ 1.66	68.89 $\pm$ 2.15*	65.47 $\pm$ 2.40*
		S	14.10 $\pm$ 0.98	12.53 $\pm$ 1.45*	13.44 $\pm$ 1.68*
		G <sub>2</sub> /M	15.45 $\pm$ 0.64	19.58 $\pm$ 2.50*	21.13 $\pm$ 1.59*

Tukey test: \* $p$ <0.05, \*\* $p$ <0.01 compared to the control.

Similar to 48 h, the effect of lycopene on apoptosis in the MDA-MB-235 cell line was lower compared to the MCF-7 cell line, with a maximal increase of 2.2- and 1.8-fold after 96 h at 10  $\mu\text{M}$  concentration, respectively.

Beta-carotene induced apoptotic effects in all breast cancer cell lines in a dose-dependent manner. Unlike lycopene, beta-carotene led to a rapid increase of

apoptosis after 48 h. Beta-carotene promoted a significant decrease in the number of live cells and significant increase in both apoptotic and dead cells in a dose-dependent manner in MCF-7, MDA-MB-235 and MDA-MB-231 cells following 48 h of treatment (5 and 10  $\mu\text{M}$ ), with a maximum increase of 2.85-, 2.10- and 4.20-fold, respectively (Figure 4).

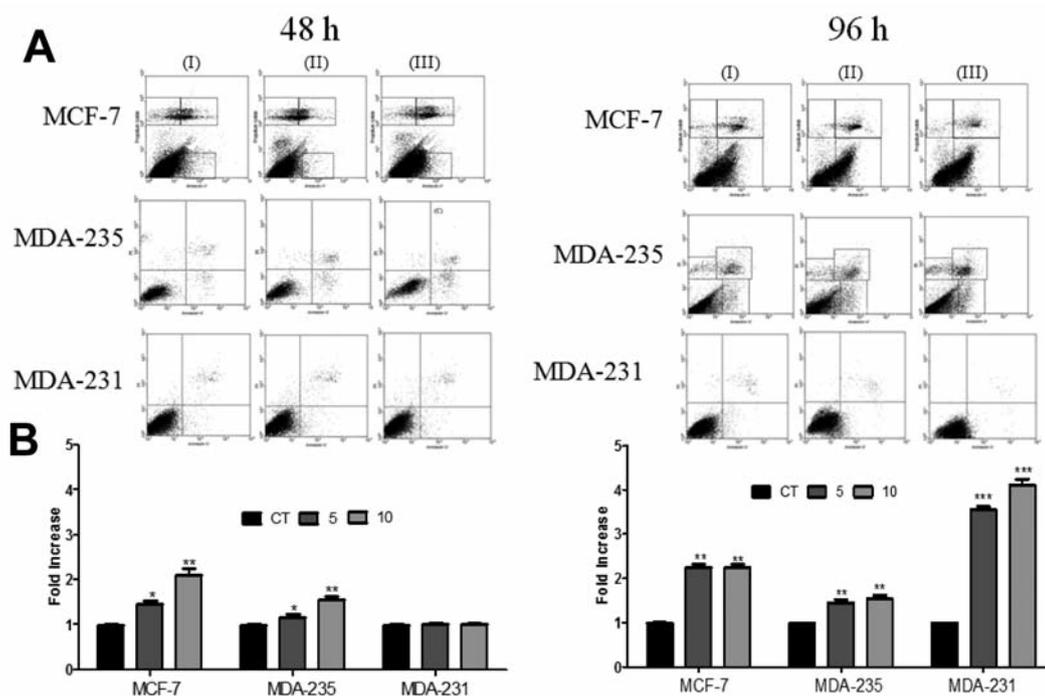


Figure 3. Cell apoptosis and necrosis induced by lycopene. A: Flow cytometric analysis: control group (I); lycopene at 5  $\mu$ M (II); 10  $\mu$ M (III). B: Fold increase relative to control. Data are presented as the means $\pm$ SEM, n=3, \* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001 versus control group.

After 96 h, two cell lines (MDA-MB-231 and MDA-MB-235) showed the same effect as that observed after 48 h of treatment; MCF-7 cells showed a smaller increase in the rate of apoptosis compared to an incubation time of 48 h (Figure 4).

## Discussion

The present study provides evidence that lycopene and beta-carotene may inhibit the growth of human breast adenocarcinoma cells. Other studies with lycopene have already established the effects of this carotenoid in several tumor types, including prostate (18), colon, liver and lung cancer (22). This has also been observed with beta-carotene, which is preventive in colon (15) and prostate (16) cancer. Although the present study showed carotenoids acting as anti-proliferative and anti-tumor substances, differences between the effects of these compounds were observed. Lycopene required a long treatment to reach its most potent effects, whereas beta-carotene had a potent effect for both periods studied. The cell line was decisive in the type of effect shown. These data re-inforce the fact that cell line sensitivity to an anti-proliferative compound must be considered.

The mechanism of action of lycopene on breast cancer cells depends upon the cell type and the treatment timing. The importance of the type of cell was reported for the expression of oncosuppressor genes *BRCA1* and *BRCA2*, which decreased

in MDA-MB-231 cells and increased in MCF-7 cells after treatment with the same dose of lycopene for 48 h (27). Beta-carotene has already been reported as a chemopreventive against colon adenocarcinoma cell lines, and distinct effects were also reported on differentiated cell lines (15).

All cell lines used in the present study have peculiarities. MCF-7 is a breast cancer cell line positive for estrogen receptor (ER), while MDA-MB-231 is negative. MDA-MB-235 cells originated external to breast tissue, although they are considered breast cancer (28-30). These data confirm that characteristics of cell properties can be determinant for the type of response of cells to carotenoids.

Our study presents many differences in the response of cell lines to the treatment. The viability of the cells treated with lycopene was time-dependent in MCF-7 and MDA-MB-231 cells, an effect not observed in MDA-MB-235 cells. MCF-7 cells showed reduction of viability, more intensive after 96 h, reaching 25% without differences in the doses. After 48 h of exposure, the minimal inhibitory concentration was 2.5  $\mu$ M, and the reduction reached 20% at the higher dose (10  $\mu$ M). In parallel, a similar study showed that MCF-7 cell line treated with lycopene did not present a dose-dependent effect after 72 h of the treatment. This study also showed that despite its effect on MCF-7 viability, lycopene does not act on the MCF-10 cell line, reinforcing the importance of cell type in carotenoid action (31).

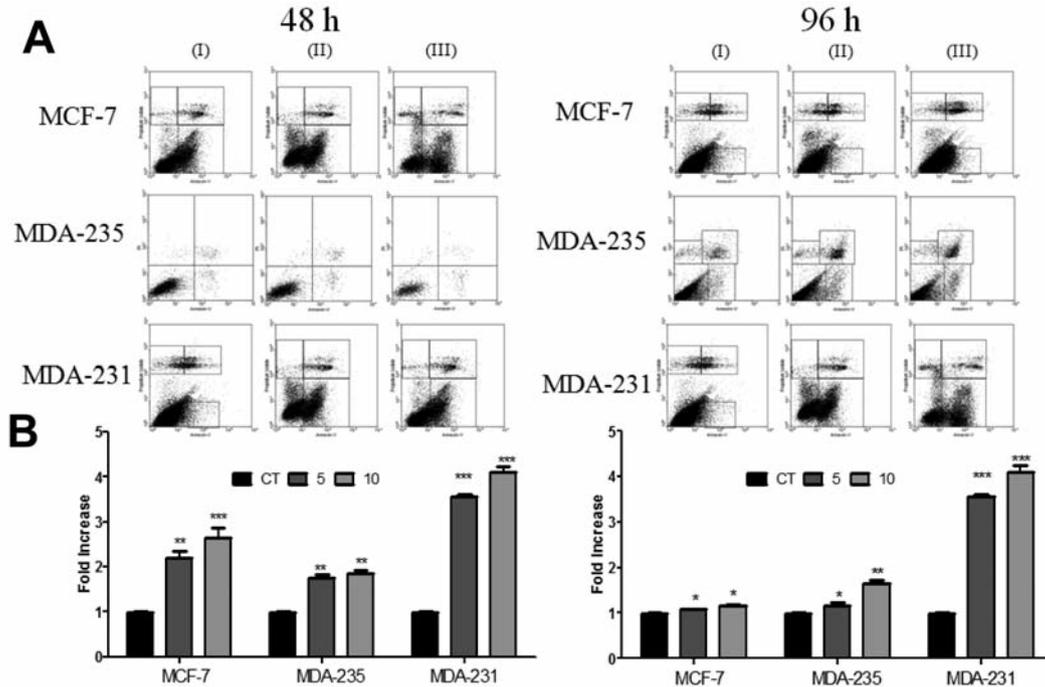


Figure 4. Cell apoptosis and necrosis induced by beta-carotene. A: Flow cytometric analysis: control group (I); beta-carotene at 5  $\mu$ M (II); 10  $\mu$ M (III). B: Fold increase relative to control. Data are presented as the means $\pm$ SEM, n=3, \* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001 versus control group.

MDA-MB-231 cells treated with lycopene exhibited potent time-dependent effects. After 48 h, no viability difference was found, but with progressive treatment, viability was reduced by an average of 75% at all concentrations after 96 h. MDA-MB-235 was the cell line that showed the best response to lycopene for 48 h for all doses (about 30%) and its inhibitory action on cell viability was indifferent to treatment progression.

The viability of cell lines changed after treatment with beta-carotene; however, time-dependent treatment did not disturb MDA-MB-231 and MDA-MB-235 cell behavior. The compound caused significant changes in cell proliferation rate, reaching a promising response in MDA-MB-231 cells, with 75% of reduction. MCF-7 cells showed different behavior from the other cell lines, since the action of beta-carotene was more intense after 48 h of treatment. Several studies have shown that carotenoids can induce cell-cycle arrest and apoptosis. Accumulation of cells in the  $G_0/G_1$  phase was already reported with carotenoid treatment, and particularly characterized as cell-cycle arrest (15, 18, 32). Our study showed that lycopene increased the percentage of cells in the  $G_0/G_1$  phase in the MCF-7 and MDA-MB-235 lines and also decreased the percentage of cells in the  $G_2/M$  phase. Cell-cycle analysis revealed similarities between other studies that consider that lycopene effect on breast cancer to be associated with the inhibition of cell-cycle progress through

$G_0/G_1$  phases. It was reported that this carotenoid is able to act as an anti-tumor agent by arresting cell proliferation and inducing apoptosis (19, 28). It has also been reported that lycopene treatment increased the percentage of cells in the  $G_0/G_1$  phases in cancer other than breast cancer, such as colon and prostate cancer (22, 33).

Our results reveal important differences between lycopene and beta-carotene regulating cell-cycle arrest in MCF-7 cells. Beta-carotene caused substantial variation in the cell cycle after 48 h. Studies with adrenocorticotrophic hormone (ACTH) secreting pituitary adenoma cells, showed the same difference in the effects of these carotenoids (34). This result may be explained by the difference in molecular polarity of the carotenoids. In hepatic stellate cells, cellular uptake of lycopene is delayed when compared to beta-carotene (35, 36).

The fact that apoptosis might influence the malignant phenotype has already been elucidated, and studies have revealed that a high frequency of apoptosis was observed in spontaneously-regressing tumors and in tumors treated with cytotoxic anticancer agents (37). A high index of apoptosis is a hallmark of important anti-tumorigenic activity. Therefore, many studies were performed in order to show the effect of carotenoids inducing programmed cell death (22, 27, 33, 38, 39, 40). In the present study, we showed that lycopene and beta-carotene induced programmed cell death. We observed similar characteristics to cell-cycle analysis, where lycopene

induced a better response with longer treatment and beta-carotene showed a more significant effect earlier (48 h). MCF-7 is the cell line that efficiently responded to the treatment in this work. Heber and Liu reported that inhibitory effects of lycopene on MCF-7 cell growth were independent of apoptotic or necrotic cell death, but dependent on IGF-I receptor signaling and cell-cycle progression (41). In our study, we did not observe the interaction of the compound with its receptor, but increase of apoptotic cells seems to be a significant factor in cell growth reduction.

Lycopene had apoptotic effects on all cell lines studied here, and after 96 h, the results showed that dose (5 or 10  $\mu\text{M}$ ) did not affect its action. MDA-MB231 cells showed an increase of 300% of the apoptosis rate (96 h) but at a shorter time (48 h) no effect was detected.

The cancer-preventive ability of lycopene by inducing apoptosis was already reported in breast, colon and prostate cell lines (22, 27, 42). Malignant T-lymphoblast cells were treated with distinct types of carotenoids to compare their effects on cell behavior and it was concluded that both lycopene and beta-carotene positively regulated apoptosis (43). It is important to note that apoptotic effects of lycopene are limited to some types of cancer. For instance, lymphocytic leukemia cell line (EHEB) was indifferent to treatment with lycopene *in vitro* (40). Moreover, gastric cancer cells presented increased apoptosis after treatment with beta-carotene at supra-physiological concentration (100  $\mu\text{M}$ ), modulating the expression of p53 (proapoptotic protein) and B-cell lymphoma-2 (Bcl-2 antiapoptotic protein) (44). Based on these observations, a study with breast cancer cells (MCF-7) confirmed that beta-carotene was able to change the expression of 21 genes related to cell apoptosis (45). As well as lycopene, we observed that beta-carotene acts on all cell lines studied here by increasing apoptosis, but under longer incubation (96 h).

These data suggest that lycopene and beta-carotene represent potential anticancer compounds depending on the type of tumor, time and frequency of treatment or consumption, concentration and bioavailability during conventional cancer therapies.

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