

## Protective effects of $\beta$ -carotene and silymarin on human lymphocytes

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**Abstract:** Beta-carotene and silymarin have antioxidant properties against oxidative damage and are used as dietary supplements. The aim of this study was to assess the protective effects of  $\beta$ -carotene and silymarin on healthy human lymphocytes exposed to L-arginine-induced oxidative damage. Study samples were lymphocyte cultures set up from venous blood obtained from 6 healthy individuals (3 males and 3 females). Oxidative DNA damage was induced by L-arginine.  $\beta$ -Carotene and silymarin were added to the cultures as antioxidants to observe their protective effects, if any. Alkaline single-cell gel electrophoresis (SCGE) was used to evaluate DNA damage. While L-arginine generated genotoxic damage in the lymphocyte cultures, both  $\beta$ -carotene and silymarin had positive effects on the reversal of the induced genotoxicity. When  $\beta$ -carotene was applied in combination with silymarin, there was no significant change in mean tail moments in comparison to the use of  $\beta$ -carotene or silymarin alone. The data presented here provide a comparative, preliminary insight into the protective role of both  $\beta$ -carotene and silymarin on L-arginine-induced genotoxicity in in vitro lymphocyte cultures.

**Key words:** L-arginine,  $\beta$ -carotene, silymarin, lymphocytes, comet assay, oxidative damage

### Beta-karoten ve silimarinin insan lenfositleri üzerindeki koruyucu etkisi

**Özet:** Diyetle ek olarak kullanılan beta-karoten ve silimarinin oksidatif hasara karşı koruyucu antioksidan etkileri bilinmektedir. Bu çalışmada insan lenfositlerinde L-arjinin ile oluşturulan oksidatif hasara karşı  $\beta$ -karoten ve silimarinin koruyucu etkilerinin varlığının değerlendirilmesi amaçlanmıştır. Çalışma kapsamına 6 sağlıklı bireyden (3 kadın, 3 erkek) alınan periferik kan örneklerinden hazırlanan lenfosit kültürleri dahil edildi. Oksidatif DNA hasarı L-arjinin ile indüklendi.  $\beta$ -karoten ve silimarin koruyucu etkilerinin varlığının araştırılması amacıyla kültürler eklendi. DNA hasarının değerlendirilmesi için alkali tek hücre jel elektroforezi uygulandı. L-arjinin kültürlerde oksidatif hasarı indüklerken  $\beta$ -karoten ve silimarin oluşan genotoksitenin geriye dönüşünde olumlu etki sağladı. Beta-karoten ve silimarin birlikte uygulandığında ise oluşan genotoksik hasarın, ajanların tek tek uygulanması ile elde edilenden farklı olmadığı görüldü. Verilerimiz, beta karoten ve silimarinin in vitro lenfosit kültürlerinde koruyucu etkilerini gösteren karşılaştırmalı ön çalışma sonuçlarını içermektedir.

**Anahtar sözcükler:** L-arjinin,  $\beta$ -karoten, silimarin, lenfosit, tek hücre jel elektroforez yöntemi, oksidatif hasar

## Introduction

Oxygen-derived species or free radical species originate from physiologic cellular reactions (e.g., mitochondrial electron transport), inflammatory reactions, and cell injury, or as a consequence of extracellular events such as exposure to toxins. To protect cells from active oxygen species, organisms have developed enzyme-dependent (superoxide dismutase, catalase, and glutathione peroxidase) and enzyme-independent (vitamins, uric acid, and glutathione) antioxidant defenses (1). Antioxidant functions are associated with lowered DNA damage, diminished lipid peroxidation, or inhibited malignant transformation (2). An imbalance between pro- and antioxidants in the intracellular microenvironment can produce oxidative stress (3). Oxidative stress is considered to play a critical role in aging and the development of various diseases, including cancer and other degenerative diseases (1).

Phytochemicals, which are found in fruits, vegetables, and plant-derived beverages, may have important roles as dietary components (4) as a result of their cytoprotective actions in many organs. Naturally occurring carotenoids and flavonoids possess free radical scavenging properties and offer cytoprotection from oxidative injury (5,6). Beta-carotene (BC) and other carotenoids exert antioxidant functions such as the quenching of singlet oxygen and other electronically excited molecules that are produced by photoexcitation or chemiexcitation reactions. They further react with peroxy or alkoxy radicals (2). Silymarin, one of the active components of milk thistle extract, has long been extensively used in patients suffering from liver diseases of different etiologies; it is reported to be one of the herbal preparations most frequently used by cancer patients on a voluntary basis (7). Silymarin comprises 4 isomers: silibinin, isosilibinin, silichristin, and silidianin (8). Silibinin is an effective free-radical quencher against OH and HOCl but has not been shown to exhibit affinity toward  $\text{H}_2\text{O}_2$  or  $\text{O}_2^{\cdot -}$  radicals (9). It was found that silibinin stabilizes cell membranes, thus preventing toxic chemicals from entering the cell and exporting toxins out of the cell before damage ensues. Silibinin has also been shown to regulate phase I and II detoxification pathways (8).

Nitric oxide, which has a wide range of important in vivo roles, is an inorganic free radical gas produced from L-arginine by nitric oxide synthases. Together with reactive oxygen species, nitric oxide is known to induce cytotoxicity and cytostasis. Nitric oxide cytotoxicity, such as the initiation of lipid peroxidation, inhibition of mitochondrial respiration, inhibition of membrane pumps, depletion of glutathione, and damage to DNA, is caused by the production of  $\text{ONOO}^-$  (10). Lymphocytes function in immunological responses by using NO. In a previous study, we demonstrated that the incubation of lymphocyte cultures with  $10^{-3}$  M L-arginine caused chromosome breaks and apoptosis together with elevated levels of NO (11). In another study, BC was shown to decrease mitomycin C-induced sister chromatid exchange frequencies in lymphocyte cultures obtained from Down's syndrome patients (12). Furthermore, we previously reported that BC and folic acid revealed positive effects on mitomycin C-induced DNA breaks and apoptosis of endometrial stromal cells, as determined by ultrastructural examinations; accordingly, BC reduced caspase-3 activity (13). In the present study, we addressed the question of whether BC and silymarin also play protective roles in healthy human lymphocytes exposed to L-arginine-induced oxidative damage.

## Materials and methods

### Human subjects, cell culture, and treatments

Six apparently healthy, age-compatible, nonsmoking volunteers (3 female and 3 male) were recruited, excluding anyone consuming a diet with supplements or taking prescribed medication. The study was approved by the local ethics committee. Venous blood samples were collected and lymphocyte cultures were set up after lymphocyte isolation with Ficoll centrifugation. The culture medium used was composed of RPMI 1640 (Biochrom AG, Germany) containing 20% fetal bovine serum (Biochrom AG), 1.5% phytohemagglutinin (Biochrom AG), 1% 200 mM L-glutamine (Biochrom AG), and 100 U/mL penicillin and 100  $\mu\text{g/mL}$  streptomycin (Biological Industries, Israel). Isolated lymphocytes were cultured at 37 °C in a humidified atmosphere of 5%  $\text{CO}_2$  (Heraeus, Germany).

Based on our previous findings (11),  $10^{-3}$  M L-arginine (Sigma-Aldrich, USA) was used to generate NO-mediated oxidative damage. Two antioxidants, BC (Sigma-Aldrich) and silymarin (Sigma-Aldrich), were added to the cultures for 72 h in previously reported relevant achievable peak plasma level concentrations of 8  $\mu$ M and 300 ng/mL, respectively, to investigate their protective effects on L-arginine-induced oxidative damage (14,15). Ethanol was added to the cultures as the solvent control.

#### **Alkaline comet assay (alkaline single-cell gel electrophoresis)**

Alkaline single-cell gel electrophoresis (SCGE) was performed in order to detect the level of genotoxicity in treated and untreated lymphocyte cultures, as previously described (16). In brief, lymphocytes were resuspended in 0.5 mL of phosphate buffered saline (PBS), and 5  $\mu$ L of cell suspension was mixed with 35  $\mu$ L of 1% (w/v) low-melting-point agarose (LMPA; Sigma-Aldrich) and added to slides coated with 0.5% (w/v) normal-melting-point agarose (NMPA; Sigma-Aldrich). Coverslips were added and slides were incubated on ice packs until solidification of the agarose. Coverslips were then removed and 40  $\mu$ L of 1% (w/v) LMPA was added to the slides. Slides were incubated in a lysis solution (2.5 M NaCl, 100 mM EDTA disodium salt, 10 mM Tris; pH 10) at 4 °C in the dark for 2 h. Slides were incubated in electrophoresis buffer (300 mM NaOH, 1 mM EDTA disodium salt; pH > 13) in the dark for 20 min and electrophoresis was performed at 24 V (300 mA) for 30 min. After neutralization (0.4 M Tris; pH 7.5), slides were stained with 2  $\mu$ g/mL of EtBr and observed under a fluorescence microscope (Eclipse 600, Nikon, Japan). A computerized image analysis system (Comet Assay IV, Perceptive Instruments, UK) was employed. Olive tail moment (TM) was used as the measure of DNA damage. A minimum of 3 SCGE slides were prepared for each treatment and, in total, 100 nuclei were analyzed per treatment (16).

#### **Statistical analysis**

All data are represented as mean  $\pm$  standard deviation (SD), and median. All statistical analyses were performed using SPSS 11.5 (SPSS Inc., USA). Differences between treatments were statistically evaluated with the Friedman test at the 0.05 level. A Mann-Whitney U-test was used at the 0.05 level for the comparison of differences between genders.

#### **Results and discussion**

The products of normal oxidative metabolism, potentially dangerous oxidants (free radicals) can damage cells and tissues in a number of ways: by damaging biomolecules and cell components, by triggering the activation of specific signaling pathways, by creating toxic products, by altering gene expression and enzyme activity, and by disrupting normal repair mechanisms. Antioxidants prevent free-radical-induced tissue damage by preventing the formation of radicals, scavenging them, or promoting their decomposition. Normal diets including antioxidants and micronutrients help cells to decrease the deleterious effects of oxidative stress. Due to their high antioxidant content, fruit- and vegetable-rich diets are inversely related to the risk of diseases related to oxidative damage (16,17).

The evaluation of lymphocyte nuclei with the comet assay demonstrated that L-arginine treatment caused significantly higher DNA damage in comparison to untreated controls (Table). Ethanol, used as a solvent control, did not increase DNA damage. After 72 h of treatment with L-arginine and BC, L-arginine-induced DNA damage significantly decreased. Previous studies demonstrated that BC protected peripheral blood lymphocytes against  $H_2O_2$ -induced oxidative DNA damage *ex vivo* (18,19). In other studies, it was also shown that using BC to pretreat various cells, including isolated human lymphocytes, protected the cells against oxidative stress-inducing agents such as  $\gamma$ -radiation that use pathways similar to that of  $H_2O_2$  (20-22). Moreover, when BC was applied together with other vitamins, such as vitamin C and vitamin E, its cytoprotective effects did not change. Authors have suggested different mechanisms of action for vitamins, and the protective effects of vitamins depend on their plasma concentrations and exposure times (22). In these studies, however, the diets of subjects were supplemented with vitamin cocktails, and then  $H_2O_2$ - or  $\gamma$ -radiation-induced DNA damage of peripheral lymphocytes was assessed by comet assay. In the present study, on the other hand, we induced oxidative damage in *in vitro* lymphocyte cultures of healthy individuals through elevated NO levels (11), and we demonstrated the protective effects of BC against DNA damage using the comet assay (Table).

Table. Effects of treatments on tail moments.

		Tail moment ( $\mu\text{m}$ )					
(n)		Untreated	EtOH	L-arginine	L-arginine + $\beta$ -carotene	L-arginine + silymarin	L-arginine + $\beta$ -carotene + silymarin
<b>Females (3)</b>	<i>Mean</i>	10.53 $\pm$ 0.61	10.66 $\pm$ 1.11	<b>27.58 <math>\pm</math> 1.23*</b>	11.72 $\pm$ 1.01	16.23 $\pm$ 1.11	10.62 $\pm$ 0.37
	<i>Median</i>	10.75	10.69	26.91	11.69	15.64	10.64
<b>Males (3)</b>	<i>Mean</i>	10.33 $\pm$ 0.30	11.84 $\pm$ 1.08	<b>23.78 <math>\pm</math> 0.36*</b>	10.63 $\pm$ 0.86	12.85 $\pm$ 2.20	11.01 $\pm$ 1.53
	<i>Median</i>	10.33	12.31	23.62	10.69	13.87	10.25
<b>Total (6)</b>	<i>Mean</i>	10.43 $\pm$ 0.41	11.25 $\pm$ 1.17	<b>25.68 <math>\pm</math> 2.23*</b>	11.17 $\pm$ 1.02	14.37 $\pm$ 2.42	10.81 $\pm$ 1.02
	<i>Median</i>	10.35	11.22	25.51	11.09	14.95	10.44

Asterisks (\*) represent a significant difference between groups.

In addition to results obtained with BC, silymarin also protected peripheral blood lymphocytes against L-arginine-induced oxidative damage. A number of studies demonstrated that silymarin regulated glutathione, glutathione peroxidase, and superoxide dismutase levels in lymphocytes (23). These enzyme systems have several important functions, including protection against oxidative stress, regulation of gene expression, induction of apoptosis, and activation and proliferation of lymphocytes (24). Concordantly, the results of the comet assay represent the in vitro protective effects of silymarin against L-arginine-induced DNA damage in the present study (Table).

In the current study, a carotenoid (BC) and a flavonoid (silymarin) were investigated for their effects on L-arginine-induced DNA damage to in vitro lymphocyte cultures by comet assay. Results of the comet assay showed that the protective effects of both BC and silymarin were similar. Furthermore, when BC was applied in combination with silymarin to L-arginine-treated cells, there was no significant change in the mean tail moments in comparison to L-arginine + BC or L-arginine + silymarin treatments (Table). Carotenoids interact with singlet  $\text{O}_2$  either via a physical quenching mechanism, in which the excited energy from singlet  $\text{O}_2$  is transferred to the carotenoid and then dissipated to the surroundings as heat, or via chemical quenching, in which the carotenoid is destroyed in the process by the addition of  $\text{O}_2$  to its

double-bond system (25). BC also quenched  $\text{O}_2^{\bullet-}$  (26). Flavonoids inhibit the enzymes responsible for  $\text{O}_2^{\bullet-}$  production; the low redox potentials of flavonoids thermodynamically allow them to reduce highly oxidizing free radicals such as  $\text{O}_2^{\bullet-}$ ,  $\text{RO}^\bullet$ , and  $\text{HO}^\bullet$ . Among the most important polyphenolic components of plants, flavonoids may stabilize free radicals by complexing with them (27,28). Based on these previous findings together with the results of this study, it may be concluded that BC and silymarin probably act on the same or related radicals. The molecules that generate oxidative damage in cells move through different mediators. Antioxidants affect these mediators or other molecules, thereby preventing damage. Different antioxidants may induce related molecules and enzyme defense systems and, as a consequence, cellular response pathways to oxidative damage-generating agents may overlap.

In conclusion, this study demonstrated the protective effects of in vitro applications of BC and silymarin on DNA damage induced by L-arginine in lymphocyte cultures of healthy individuals. These agents have direct effects on L-arginine-induced apoptosis (11) or any other molecules on apoptotic pathways, as we previously demonstrated for endometrial stromal cells (12). The data presented here provide a comparative, preliminary insight into the protective roles of both BC and silymarin on L-arginine-induced genotoxicity in in vitro

lymphocyte cultures, while many other studies on the issue have focused on the in vivo use of these agents as dietary supplements.

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