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β-Carotene and α-tocopherol concentration and antioxidant status in buccal mucosal cells and plasma after oral supplementation

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The uptake of α-tocopherol and β-carotene and their antioxidative effect in plasma and buccal mucosal cells after oral application in twelve subjects is demonstrated in our study. The effect on the antioxidative status was evaluated using a modified thiobarbituric acid-reactive substance (TBARS) method. As expected, the supplement of 134·2 mg α-tocopherol/d and 25 mg β-carotene/d for 7 d resulted in a significant increase of α-tocopherol and β-carotene concentration in plasma ($P<0·05$). In buccal mucosal cells, the concentration of β-carotene increased after supplementation ($P<0·05$), whereas the concentration of α-tocopherol remained constant. A decrease in TBARS ($P<0·05$) was found in buccal mucosal cells but not in plasma. In conclusion, an uptake of the supplemented antioxidants was detected in plasma and in buccal mucosal cells. There was significant change in β-carotene concentration and oxidative stress as measured using a modified TBARS test in buccal mucosal cells, but not in the plasma.

Buccal mucosal cells: Supplementation: α-Tocopherol: β-Carotene: Thiobarbituric acid-reactive substances

Oxidative damage to mucosal cells can lead to squamous metaplastic alterations such as oral leucoplakia, which is considered to be a precancerous lesion. Antioxidants such as α-tocopherol and partly β-carotene are able to interrupt intracellular pathogenic mechanisms and can produce regressions in oral leucoplakia (Stich *et al.* 1988; Garewal *et al.* 1990; Toma *et al.* 1992) or systemic sclerosis (Gabriele *et al.* 2000). The formation of intracellular reactive oxygen species (ROS) is one of these mechanisms which causes damage to proteins, DNA and membrane lipids. Endogenous antioxidants of the organism such as superoxide dismutase, catalase or glutathione peroxidase are able to inactivate ROS. To support the endogenous antioxidative system dietary-derived exogenous antioxidants such as α-tocopherol and to some extent β-carotene, play a major role (Barth *et al.* 1997). Direct measurement of antioxidants in plasma only gives indirect evidence for the supply to target tissues shown by Peng *et al.* (1995). It is therefore more appropriate to evaluate the level of α-tocopherol and β-carotene and in consequence their antioxidative capacity directly in the buccal mucosal cells. These cells are easily available and can therefore serve as a model system for the uptake and distribution of dietary components in target tissue.

Up to now, no systematic measurement including a variable for oxidative stress has been carried out to determine the antioxidative efficacy of supplementary vitamins on the cellular antioxidative defence in buccal mucosal cells.

This present study demonstrates the influence of a short-term supplement of β-carotene and α-tocopherol and their effect on oxidative status in plasma and buccal mucosal cells. We applied an improved thiobarbituric acid-reactive substance (TBARS) assay (Jentzsch *et al.* 1996) to determine the oxidative stress in plasma and buccal mucosal cells of twelve healthy volunteers.

Subjects and methods

Subjects and study design

Twelve healthy female volunteers (age 23–30 years, mean BMI 21·5 (SD 1·8)) were selected for the study. Exclusion criteria were chronic or acute illness, pregnancy, smoking and vitamin supplementation. During the study, foods containing high levels of α-tocopherol and β-carotene were excluded from the daily diet. The volunteers received daily doses of D-α-tocopherol (134·2 mg; Hermes, Munich, Germany) and β-carotene (25 mg; Twardy, Flörsheim, Germany) for 1 week. Prior to supplementation

Abbreviations: BHT, 2,6-di-*tert*-butyl-*p*-hydroxytoluene; ROS, reactive oxygen species; TBARS, thiobarbituric acid-reactive substances.

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(day 0), after supplementation (day 7) and after the 'wash-out phase' (day 14) blood and buccal mucosal cell samples were taken. The concentration of α-tocopherol, β-carotene, cholesterol and TBARS were determined in the blood samples. The concentration of α-tocopherol, β-carotene, TBARS and DNA were determined in the buccal mucosal cells.

One week prior to supplementation and during the study period (14 d) all foods were recorded and analysed with the aid of a nutrition program EBISpro (FEP, Esslingen, Germany) for their content of α-tocopherol and β-carotene.

Sampling of blood and buccal mucosal cells

Sampling of blood. Blood sampling by skin puncture of the finger was carried out as described recently (Erhardt *et al.* 1999) using Microtainer, Brand Safety Flow Lancets from Becton Dickinson (Franklin Lakes, NJ, USA). For the collection of blood, Microtainer Brand Plasma Separator Tubes containing lithium heparin from Becton Dickinson were used. After discarding the first drop, more than 400 μl blood was collected, the tubes were then centrifuged at 12 000 ***g*** for 1 min. The resulting plasma was transferred in 20 and 50 μl portions to several Eppendorf Safe Lock 0·5 ml Micro test tubes (Eppendorf, Hamburg, Germany) and frozen at −80°C until analysis, which was completed in less than 2 months.

Sampling of buccal mucosal cells. Buccal mucosal cells were collected by using a non-invasive method established by Gilbert *et al.* (1990). Initially the subjects rinsed their mouths with water thoroughly and then brushed the inside of their cheeks with a soft toothbrush twenty times on each side. With this procedure, contamination of the buccal mucosal cells with blood could be avoided. After the brushing the subjects were asked to rinse both cheeks with 20 ml NaCl solution (9 g/l) twice and wash the toothbrush with a further 10 ml NaCl solution (9 g/l). The solutions were collected in a 2,6-di-*tert*-butyl-*p*-hydroxytoluene (BHT)-coated 50 ml tube. Subsequently, the samples were centrifuged at 1400 ***g*** for 10 min at 4°C. The supernatant fraction was discarded and 15 ml cold PBS solution was added. The samples were vortexed and centrifuged again at 1400 ***g*** for 5 min. After removal of the supernatant fraction, 300 μl cold PBS was added, vortexed and homogenized with a spatula. The cell suspension was then divided into three aliquots for the determination of β-carotene, α-tocopherol, TBARS and DNA and treated as follows. Each sample was pipetted into a BHT-coated microcentrifuge tube and centrifuged at 12 000 ***g*** for 1 min. The supernatant fraction was discarded and the cell pellet was flushed with N_2 for 20 s; the tube was then sealed and stored at −80°C.

Analytical methods

Measurement of α-tocopherol and β-carotene. The analysis was performed according to Erhardt *et al.* (1999) using slight modifications.

Sample preparation and determination in plasma. The proteins were denatured by mixing 20 μl plasma in a 0·5 ml vessel with 100 μl ethanol–butanol (1:1, v/v), containing 5 mg BHT/ml and 4 μmol tocol/l as internal standard). Lipophilic vitamins were extracted by vigorous mixing for 10 s. The mixture was then centrifuged for 5 min at 12000 ***g*** and 30 μl supernatant fraction was analysed by using reversed-phase HPLC.

Sample preparation and determination in buccal mucosal cells. First, the cell pellet was redissolved in 400 μl sodium dodecyl sulfate (10 g/l, containing 10 ml/l BHT/l). Saturated NaCl solution (20 μl) was added for improved separation. α-tocopherol and β-carotene were extracted with 2 × 300 μl hexane (containing 5 mg BHT/ml). The combined organic supernatant fractions were evaporated using N_2 flow and the residue was redissolved in 100 μl ethanol–butanol (1:1, v/v, containing 5 mg BHT/ml and 4 μmol/l Tocol (Matreya Inc., Pleasant Gap, PA, USA) as internal standard). Of each sample, 80 μl were analysed by reversed-phase HPLC.

HPLC determination. The HPLC system consisted of the following components: intelligent-pump L6200; UV-VIS Detector L4250 (Merck, Darmstadt, Germany), injection valve (Rheodyne, Rohnert Park, CA, USA); HPLC software (Berthold, Pforzheim, Germany). The UV-VIS spectrophotometer was programmed as follows: 292 nm from 0 to 4 min and 450 nm from 4 to 8 min. A Nucleosil 125 × 3 mm, 100 RP-18 (3 μm) column from Macherey Nagel (Dueren, Germany) was used. An isocratic mobile phase consisting of acetonitrile–tetrahydrofurane–methanol–ammonium acetate (10 g/l) (684:220:68:28, by vol.) was used in recirculation mode for approximately 250 samples. The flow rate was maintained at 1 ml/min. For quantification we used the international certified NIST standards (National Institute of Standards and Technology, Gaithersburg, MD, USA).

Measurement of thiobarbituric acid-reactive substances. The analysis was previously described by Jentzsch *et al.* (1996). For plasma, 50 μl was mixed with 3 μl 0·1 M-BHT in ethanol, 50 μl 0·2 M-phosphoric acid and 6 μl 0·1 M-thiobarbituric acid. The mixture was then incubated at 90°C for 45 min. The samples were cooled on ice, 125 μl butanol and 10 μl saturated NaCl solution were added and the mixture was vortexed for 60 s. After centrifugation (1 min, 12 000 ***g***) 62·5 μl supernatant fraction was measured in a fluorometer (λ 530 nm excitation, λ 590 nm emission).

Buccal mucosal cells. The cell pellet was mixed with 10 μl BHT in 0·1 M-ethanol and 200 μl 0·2 M-phosphoric acid and 25 μl 0·1 M-thiobarbituric acid. The mixture was then incubated at 90 °C for 45 min. The samples were cooled on ice, and 500 μl Butanol and 50 μl saturated NaCl were added and the mixture was vortexed for 60 s. After centrifugation (1 min, 12 000 ***g***) 250 μl supernatant fraction was analysed in a fluorometer (λ 530 nm excitation, λ 590 nm emission). For quantification malonaldehyde-*bis*(dimethyl acetate) in phosphoric acid was used.

Measurement of DNA in buccal mucosal cells. A method according to Natarajan *et al.* (1994) was used. The cell pellet was mixed and incubated for 24 h at 37°C with 200 μl acetaldehyde in perchloric acid (160 g/l) and 320 μl diphenylamine in acetic acid (40 ml/l). After centrifugation, 200 μl supernatant fraction was analysed at λ

750 nm in a microtitre plate reader. For quantification a standard DNA from herring sperm was used.

Measurement of cholesterol. The analysis was performed as previously described (Erhardt *et al.* 1999) by a standard clinical chemistry method.

Statistical evaluation

The data were analysed using the software SPSS for Windows (version 8.0; SPSS Inc., Chicago, IL, USA). For the comparison of days 0, 7 and 14 the Wilcoxon matched pairs test was used, a P value of $<0{\cdot}05$ was assumed to be significant.

Ethical considerations

The individuals provided informed consent and the study was performed in accord with the 1983 revision of the Helsinki Declaration. The study was approved by the Ethical Committee of the Landesärztekammer/Stuttgart, Germany.

Results

Fig. 1 demonstrates a significant increase of β-carotene and α-tocopherol concentration in plasma in the first 7 d. After the following 7 d of the wash-out phase (days 7–14), the vitamin levels decreased significantly but they still showed a significant higher level compared with day 0. In buccal mucosal cells a significant increase could be detected only for β-carotene concentration as shown in Fig. 2. A significant peak level ($P<0{\cdot}05$) was determined at day 14. This is in contrast to the situation in plasma where β-carotene level had decreased between days 7 and 14. There was only a marginal increase in the concentration of α-tocopherol ($P=0{\cdot}07$) following 7 d supplementation. On day 14 α-tocopherol concentration in buccal mucosal cells had reached nearly the basal level of day 0.

The TBARS method was applied to assess the oxidative stress in plasma and in buccal mucosal cells. In the present study, an inverse correlation was found between increasing levels of β-carotene and a significant decrease of TBARS in buccal mucosal cells as shown in Fig. 3. TBARS levels in plasma were slighty reduced, but no significant difference could be determined.

Table 1. Dietary intake of α-tocopherol and β-carotene of the twelve subjects during the three study periods

(Mean values and standard deviations)

	1 week prior to study		During supplementation		Wash-out period	
	Mean	SD	Mean	SD	Mean	SD
β-Carotene (mg/d)	3·1	2·0	3·7	1·6	3·7	2·3
α-Tocopherol (mg/d)	16·2	4·0	15·2	6·0	14·9	4·4

The food analysis shown in Table 1 demonstrates that the daily intake of α-tocopherol and β-carotene integrated in food contributed approximately 10–15 % of the supplemented dose of the administered vitamins. No significant differences in the intake of food by the subjects between the three periods were found (Table 1).

Discussion

In recent years, several studies have been published concerning buccal mucosal cells as an indicator for the nutritional status (Badcock & Pinnock, 1990; Gilbert *et al.* 1990; Kaempf *et al.* 1994; Liede *et al.* 1998; Newcomb *et al.* 1990; Peng & Peng, 1992; Peng *et al.* 1993, 1994, 1995; Stich *et al.* 1986). Gilbert *et al.* (1990) as well as Newcomb *et al.* (1990) showed a large inter-individual variability, which indicates a dependency between the intake of β-carotene and the resulting concentration of β-carotene in buccal mucosal cells. Factors such as smoking or a lower resorption rate in low responders additionally contribute to these effects. In contrast, intra-individual

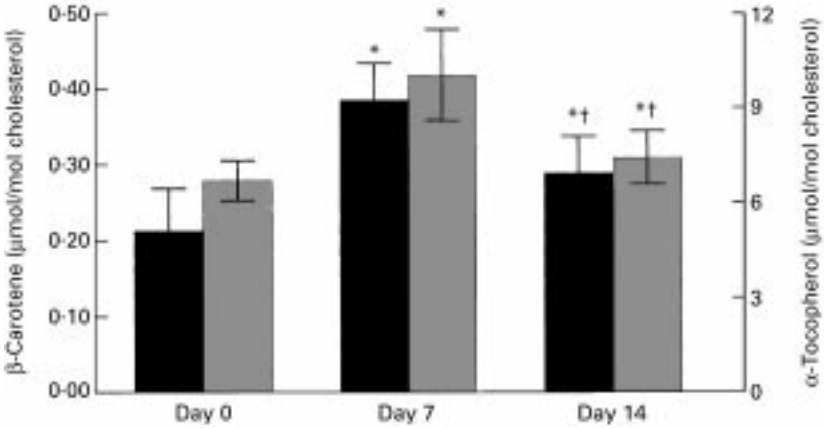

Fig. 1. Plasma level of β-carotene and α-tocopherol before (day 0), after supplementation (day 7) and after the wash-out period (day 14) in twelve subjects. For details of subjects and procedures, see p. 472. ■, β-carotene; ■, α-tocopherol. Values are means with standard deviations shown by vertical bars. Mean values were significantly different from those at day 0: *$P<0{\cdot}05$. Mean values were significantly different from those at day 7: †$P<0{\cdot}05$.

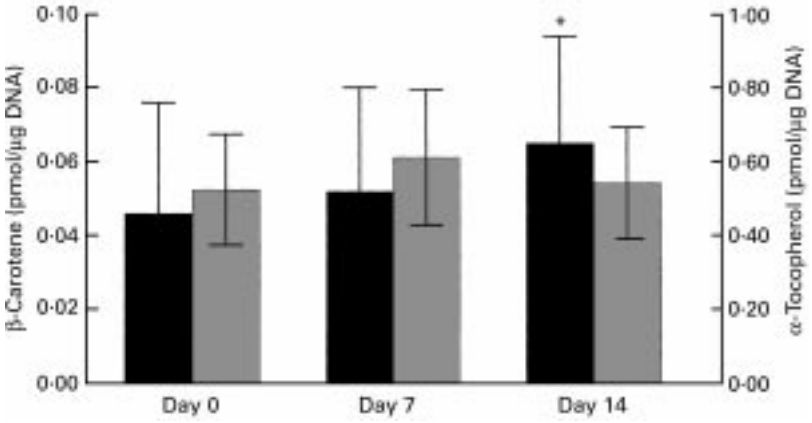

Fig. 2. Concentration of β-carotene and α-tocopherol in buccal mucosal cells before (day 0), after supplementation (day 7) and after the wash-out period (day 14) in twelve subjects. For details of subjects and procedures, see p. 472. ■, β-carotene; ■, α-tocopherol. Values are means with standard deviations shown by vertical bars. Mean value was significantly different from those at day 0 and day 7: *$P<0{\cdot}05$.

variability is small, making such a measurement suitable for evaluating the nutritional status (Peng *et al.* 1994).

The results of our present study showed an increase of β-carotene in plasma and buccal mucosal cells of each volunteer during supplementation. Remarkably, an increase of β-carotene in buccal mucosal cells could still be detected on day 14, after the wash-out phase. This can be explained by the fact that buccal mucosal cells which have accumulated the supplemented β-carotene need about 7 d to mature and move to the surface of the buccal mucosa. These results are in accordance with Prince & Frisoli (1993) who found an accumulation of β-carotene in the skin 2 weeks after supplementation.

In plasma the increase of the α-tocopherol level was comparable with the β-carotene-level; however, in buccal mucosal cells there was no significant increase from day 0 to day 7 or day 14. Different transport mechanisms compared with β-carotene, a possibly low dose of α-tocopherol or interactions between the two substances could be regarded as an explanation. For example Xu *et al.* (1992) demonstrated a decrease of α-tocopherol in plasma and skin after long-term supplementation with β-carotene.

As plasma values of β-carotene and α-tocopherol showed a rapid increase following supplementation and a sharp decrease after cessation of supplementation a good compliance by the volunteers could be assumed.

The oxidative stress of buccal mucosal cells and plasma was evaluated by the TBARS method. Our present study showed a significant decrease in TBARS in buccal mucosal cells between day 0 (start of supplementation) and day 14 (after the wash-out period). No significant variation with respect to TBARS was detected in plasma, in spite of higher variations in the concentration of antioxidants. It might be that blood antioxidants such as uric acid compensated for oxidative effects in the plasma of healthy volunteers in our short-term application (Nieto *et al.* 2000).

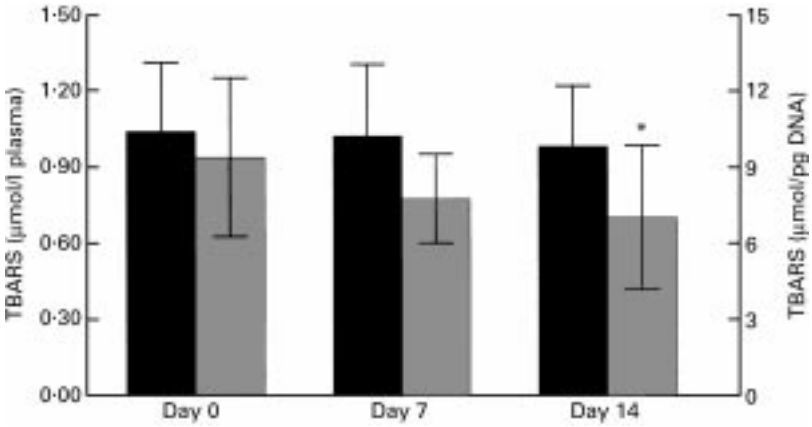

Fig. 3. Thiobarbituric acid-reactive substances (TBARS) in plasma and buccal mucosal cells before (day 0), after supplementation (day 7) and after the wash-out period (day 14) in twelve subjects. For details of subjects and procedures, see p. 472. ■, Plasma; ■, buccal mucosal cells. Values are means with standard deviations shown by vertical bars. Mean value was significantly different from those at day 0: *$P<0{\cdot}05$.

Several limitations regarding the measurement of TBARS have been reported (Janero, 1990), thus placing the physiological relevance of such measurements in doubt. However, in comparison with other studies we used a modified TBARS test (Jentzsch *et al.* 1996), which circumvents the formation of malondialdehyde during the incubation. With this modified test, Frank *et al.* (1998) have shown satisfying correlations of TBARS in cells and other variables for oxidative stress (like oxidative modifications of lipids and proteins detected by immunohistochemical methods). Because of a low amount of available cells, it was not possible to apply these methods in the present study.

In conclusion, supplementation with β-carotene and α-tocopherol causes a significant increase in the concentration of these antioxidants in plasma and partly in buccal mucosal cells, however, a possibly more important effect could be the reduced production of TBARS in the buccal mucosal cells. Hence, the measurement of oxidative stress via easily obtainable cells, e.g. buccal mucosal cells, may provide an accurate and easily available indication of oxidative stress in target tissues of an organism.

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