Carcinogenesis vol.18 no.9 pp.1847–1850, 1997

SHORT COMMUNICATION

Consumption of vegetables reduces genetic damage in humans: first results of a human intervention trial with carotenoid-rich foods

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A human intervention study with vegetable products has been performed in twenty three healthy, non smoking males aged 27–40. It was the aim of the study to assess whether consumption of vegetables containing different carotenoids could protect against DNA damage and oxidative DNA damage. The subjects consumed their normal diets, but abstained from vegetables high in carotenoids throughout the study period. After a 2 week depletion period, they received daily 330 ml tomato juice with 40 mg lycopene (weeks 3 and 4), 330 ml carrot juice with 22.3 mg β-carotene and 15.7 mg α-carotene (weeks 5 and 6), and 10 g dried spinach powder (in water or milk) with 11.3 mg lutein (weeks 7 and 8). Blood was collected weekly and DNA damage was detected in peripheral blood lymphocytes with the 'COMET' assay. Oxidised DNA bases were detected by including an incubation step with endonuclease III. The supplementation of the diet with tomato, carrot or spinach products resulted in a significant decrease in endogenous levels of strand breaks in lymphocyte DNA. Oxidative base damage was significantly reduced during the carrot juice intervention. These findings support the hypothesis that carotenoid containing plant products exert a cancer-protective effect via a decrease in oxidative and other damage to DNA in humans.

Epidemiological evidence suggests that a diet high in fruit and vegetables is associated with a decreased incidence of cancer, cardiovascular disease and maybe of other degenerative or age related diseases (1–3). One mechanism by which these plant foods could have protective effects is by exerting antioxidative activities (4). Next to the huge group of polyphenols, especially tocopherols, ascorbic acid and carotenoids have been associated with antioxidative properties (5). Many hundred carotenoids have been identified of which ~50 have vitamin A activity (6). In plants they serve the functions of accessory pigments in photosynthesis and photoprotection. The conjugated polyene structure of carotenoids allows the molecule to absorb light, to quench and to inactivate singlet oxygen and free radicals (7).

In humans, the antioxidative function of carotenoids has especially been investigated for the example of β-carotene and smoking related cancers. However, in the two large scale trials which have recently been completed, supplementation with capsules containing β-carotene (20 or 30 mg/day) with or without vitamins E or A resulted in an increased risk for lung cancer in smokers (8,9). These findings are in contrast to the numerous studies indicating an association between deficiency of these compounds or other vitamins, low fruit and vegetable intake and higher cancer incidences (10,11). Suggestions which have been put forth to explain these discrepancies include i) in smokers pro-oxidant activities of β-carotene prevail which leads to enhanced tumor progression, ii) the absorption, bioavailability and distribution of β-carotene is different when given as a single compound than as a vegetable or fruit and iii) additional carotenoids and other protectant plant components act together toward ensuring a protective effect, which cannot be simulated by intervention with supplements (12–14). Clearly more research is needed to assess the impact of antioxidant plant ingredients in healthy humans and especially to better understand the cancer preventive functions of whole fruits and vegetables (15).

Molecular biology approaches, utilizing biomarkers of DNA damage or biochemical parameters as indicators of carcinogenic risk and of antioxidant defense systems are becoming more and more available (16,17). They offer a focused and economical approach to answer specific questions pertaining to the protective potential of individual dietary antioxidants. Thus, Duthie *et al*. (1996) have shown that supplementation of the diet with a cocktail made from vitamin C (100 mg/day), vitamin E (280 mg/day) and β-carotene (25 mg/day) resulted in a highly significant decrease in endogenous oxidative base damage in the lymphocyte DNA of both smokers and non-smokers (18). This study was based on the use of a modified alkaline single cell gel electrophoresis assay ('Comet' assay) which specifically detects oxidation of pyrimidines in the DNA of human lymphocytes (19). The rationale of such an approach is that DNA damage can account for the genetic changes which occur at different stages in the progression of cancer (20). Therefore dietary factors that reduce the impact of attack by electrophilic intermediates of procarcinogens or by free radicals are likely to protect against cancer (21). Our recent studies using the 'Comet' assay in rats have shown it is a useful approach to demonstrate that dietary supplementation (lactulose or lactic acid bacteria) can efficiently protect against the activity of carcinogens (22,23). Now, we have followed up on these studies to test the hypothesis that whole plant products may protect against cancer-related genetic changes in the human.

We carried out a human intervention study with 23 healthy male volunteers (non smokers) aged 27–40 (33.8 ± 4.08, mean ± SD) to assess the antigenotoxic, antioxidative potencies of three vegetable products. The study was designed to assess physiological effects by nutritional components, and not pharmacological effects by single dietary supplements. Therefore it was our intention to study the consequences of nutrients under nutritional conditions (alternating vegetable intake). The juices from tomatoes, carrots as well as spinach powder were chosen on account of their respective contents of lycopene, α- or β-carotene and lutein. The juices were commercially canned varieties subjected to ultra-high temperature (Schoenberger, Magstadt, Germany). The spinach powder was obtained from washed and dried material (Völpel

GmbH, Königmoos, Germany). After a 2 week depletion period, the vegetable products were given with lunch consecutively for a 2 week period each. The daily intake was 330 ml tomato juice with 40 mg lycopene (weeks 3 and 4), 330 ml carrot juice with 22.3 mg β-carotene and 15.7 mg α-carotene (weeks 5 and 6), and 10 g dried spinach powder with 11.3 mg lutein, dissolved in water or milk (weeks 7 and 8). Blood was collected weekly from subjects before breakfast. Parameters of genetic change were determined in peripheral lymphocytes, isolated by gradient centrifugation (Histopaque 1077). We report here on the substantial protective effect of the three products on genetic damage in peripheral lymphocytes detected as decreased DNA strand breaks and reduced levels of oxidized DNA damage.

DNA damage was measured with the single cell microgel electrophoresis assay, also known as the 'Comet' test (24). With the original version of the assay, DNA strand breaks can efficiently be detected in single cells (25). By introducing an intermediate incubation step with lesion specific enzymes, the technique can be applied to detect oxidized DNA bases (19,26). Here we used our usual protocol of the comet assay and additionally the modification with endonuclease III, to detect levels of oxidized pyrimidine bases (18,27). Also, we monitored the resistance of the cells to external oxidative stress which can be monitored by treating them with hydrogen peroxide (H_2O_2). 20 µl of a lymphocyte suspension (2×10^5 cells) were distributed with 75 µl low melting point agarose on microscope slides and after 10 min covered with another layer of agarose. Three slides from each donor were placed on ice cold racks, treated with 50 µl physiological NaCl for 5 min, washed with lysis solution (100 mM Na_2EDTA, 1%Triton X 100, 2.5 M NaCl, 1% lauroyl sarcosin sodium salt, 10 mM TRIS, 10% DMSO) and then placed into a lysis bath for 2 h together with three 150 µM H_2O_2-treated slides. Three further slides were placed into the lysis solution for 1 h, washed 3 times with endonuclease buffer (40 mM HEPES-KOH, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/ml bovine serum albumin fraction V, pH 8) and then incubated with endonuclease III in buffer (1 µg/ml) sealed with a cover slip for 45 min at 37°C. Subsequently, all nine slides of one donor were placed into an electrophoresis chamber containing alkaline buffer (1 mM Na_2EDTA, 300 mM NaOH) for DNA unwinding. After 20 min, the current was switched on and electrophoresis carried out at 25 V, 300 mA for 40 min. This relatively long electrophoresis time was chosen to increase the basal levels of detectable DNA damage in the NaCl-control slides. The slides were removed from the alkaline buffer, placed on a tray and washed three times, 5 min with neutralisation buffer (0.4 mM Tris, pH 7.5). Subsequently the slides were stained with 100 µl ethidium bromide solution (20 µg/ml). All steps beginning with the isolated lymphocytes were conducted under red light. Thus for each donor and week, triplicate values for DNA strand breaks (NaCl-control slides), oxidative DNA damage (slides treated with endonuclease III), and sensitivity of cells against oxidative stress (H_2O_2-treated slides) were available for evaluation. Data were generated for at least 21 of the total participating 23 subjects each week, and missing values (weeks 1, 2, and 4) were due to the participant missing the vein puncture or to a technical failure. Evaluation was performed by microscopical analysis. Using the imaging software of Perceptive Instruments (Halstead, UK), 50 images were evaluated per slide and the percentage of fluorescence in the tail (TI, 'tail-intensity') was scored. The 'net tail intensity' indicates

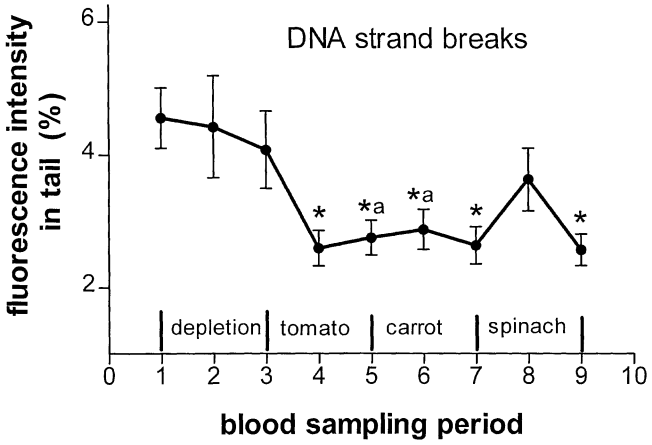

Fig. 1. Levels of DNA strand breaks in peripheral blood lymphocytes from humans receiving different vegetable products. The extent of DNA damage is indicated by the percentage of fluorescence in the comet tail ('tail intensity'), as indicated in the text. Results are shown as means ± SEM, n = 21–23 subjects, means of 3 slides per subject. *Statistically significant in comparison to sampling time 3, *[a]statistically significant in comparison to sampling time 1; two sided, paired Student's t-test, $P < 0.05$.

the damage due to oxidized DNA bases (values of slides with endonuclease III minus values from corresponding NaCl-control slides). We used the NaCl slides instead of a set of slides with endonuclease buffer as controls, which may mean that the levels of oxidized DNA bases may be slightly overestimated. In the worst case the overestimation may be 1–2% (difference between 'TI', NaCl and 'TI', endonuclease buffer), as we have observed with much more sensitive primary human colon cells.

Figure 1 shows the results of the NaCl-control slides which reflect the levels of endogenous DNA strand breaks during the 8 weeks of intervention. Before the beginning of the experiment and during the first two weeks of depletion (samples 1–3) the levels of DNA damage were significantly higher than during intervention with the vegetable products. In other words, the many compounds of tomato and carrot juice including lycopene, β-carotene, α-carotene and others are efficiently antigenotoxic *in vivo* in humans. For spinach, the effects are not as strong, since the first week of intervention with this vegetable did not significantly reduce the levels of strand breaks.

The carrot juice intervention also caused marked effects in reducing oxidized pyrimidine bases of the DNA. In contrast, dietary intervention with tomato juice or spinach did not reduce endogenous oxidative damage (Figure 2).

Finally, our results do not indicate that lymphocytes acquire a resistance against oxidative damage following consumption of vegetables. For this potential effect, the evaluation parameter '% comets' is probably more discriminatory than the parameter of 'tail intensity', since the response of lymphocytes to H_2O_2 challenge is heterogenous and results in a mixed population of images with comet and non-comet like structures (26). When subtracting the values of '% comets' (TI >6%, classes 1–4) for control slides from those treated with H_2O_2, no differences were seen (results not shown).

In summary, all three products seem capable of suppressing strand breaks. A decrease in base oxidation (a specific indicator of oxidative damage) is seen only during the carrot juice weeks, which suggests a particular efficiency of α- and β-carotene at quenching free radicals *in vivo*. The design of

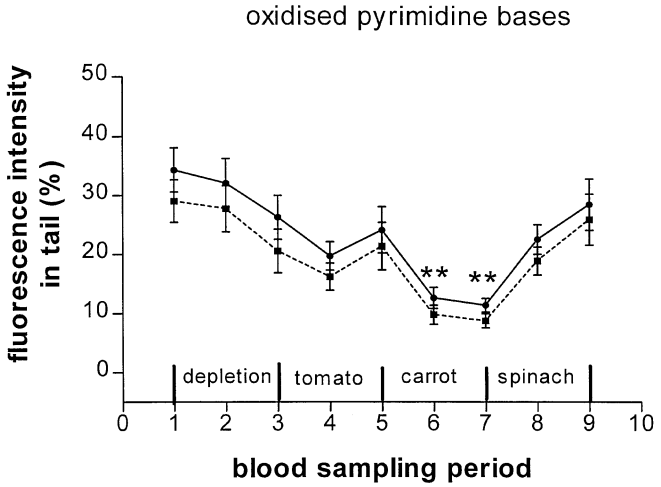

Fig. 2. Levels of total DNA damage (solid line) and oxidized pyrimidine bases in DNA (dotted line) of peripheral blood lymphocytes from humans receiving different vegetable products. The net yield of oxidative DNA is obtained by subtracting the percentage of fluorescence in the comet tail ('tail intensity') of the NaCl slides (means of 3 slides) from the corresponding values obtained from the slides treated with endonuclease III. Results are shown as means ± SEM, n = 21–23 subjects, means of 3 slides per subject. **Statistically significant in comparison to sampling time 3; two sided, paired Student's t-test, $P < 0.001$.

the experiment means that delayed effects of the earlier supplement cannot be excluded. Plasma antioxidant concentrations remain to be measured. However, our preliminary results show that carotenoids from the juices are not as persistant as would be expected from pharmacological studies with antioxidant supplements. The composition of the carotenoids in the serum are a close reflection of the carotenoid composition of the juices being consumed. At present, this aspect (analysis of 15 carotenoids in serum of each subject and week) and various other parameters indicative of antioxidative status in the blood are being measured together with biomarkers of bioavailability, genetic susceptibility and immune modulation and these results will be reported at a later date (Bub, Abrahamse, Müller, Pool-Zobel, Watzl and Rechkemmer, unpublished results).

We may conclude that the technique of microgel electrophoresis to detect DNA strand breaks coupled with the modification of an incubation step with lesion specific enzymes is a valuable approach to assess the impact of nutrition on endogenous genetic lesions. This protection was seen here in peripheral lymphocytes. But methods are also becoming available with which it should be possible to also analyze relevant tumor target tissues for such genetic changes during clinical or nutritional intervention trials (30,31). For now, however, we may state that these novel findings are support for the hypothesis that carotenoid containing plant products exert a cancer-protective effect via a decrease in oxidative and other damage to DNA. The potency of the protective effects varies with the type of products consumed and is thus probably dependent on the respective type and levels of antioxidant ingredients.

Acknowledgements

The authors are grateful to Dr Andrew Collins (Rowett Research Institute, Aberdeen, Scotland) for his generous gift of endonuclease III and for his valuable input in helping us establish the modified comet assay in our laboratory. We thankfully acknowledge the competent technical assistance of T.Gadau, M.Falk, S.T.Ji, M.Knoll, R.Lambertz, B.Mathony-Holzschuh, D.Oberreuther and C.Stoltz in performing this work and are indebted to all subjects recruited from the 'Forschungszentrum Karlsruhe' for contributing by continuously complying with the objectives of this study and willingly subjecting themselves to a weekly blood donation. We thank Walter Schoenenberger, Pflanzensaftwerk GmbH and Co. and Völpel GmbH and Co. KG for providing the vegetable products. The study was approved by the local ethics committee of the Landesärztekammer, Baden-Württemberg, Germany.

References

1. Block,G., Patterson,B. and Subar,A. (1992) Fruit vegetables, and cancer prevention: a review of the epidemiological evidence. *Nutr. Canc.*, **18**, 1–29.
2. Willett,W.C. (1994) Diet and health: what should we eat? *Science*, **264**, 532–537.
3. Ames,B.N., Gold,L.S. and Willett,W.C. (1995) The causes and prevention of cancer. *Proc. Natl Acad. Sci. USA*, **92**, 5258–5265.
4. Aruoma,O.I. (1994) Nutrition and health aspects of free radicals and antioxidants. *Fd Chem Toxicol.*, **32**, 671–683.
5. Bonorden,W.R. and Pariza,M.W. (1994) Antioxidant nutrients and protection from free radicals. In Kotsonis,F.N., Mackey,M. and Hielle,J. (eds.) *Nutritional Toxicology*, Raven Press Ltd, New York, pp. 19–48.
6. Isler,O. (1981) Foreword. In Bauernfeind,J.C. (ed.) *Carotenoids as Colorants and Vitamin A Precursors*, Academic Press, New York.
7. Krinsky,N.I. (1993) Actions of carotenoids in biological systems. *Ann. Rev. Nutr.*, **13**, 561–587.
8. The Alpha Tocopherol, Beta-Carotene Cancer Prevention Study group. (1994) The effect of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers. *N. Engl. J. Med.*, **330**, 1029–1035.
9. Omenn,G.S., Goodman,G.E., Thornquist,M., Grizzle,J., Rosenstock,L., Barnhart,S. *et al.* (1996) Effects of a combination of beta carotene and vitamin A on lung cancer and cardiovascular disease. *N. Engl. J. Med.*, **334**, 1150–1155.
10. Mayne,S.T. (1996) Beta-carotene, carotenoids, and disease prevention in humans. *FASEB J.*, **10**, 690–701.
11. Byers,T. and Guerrero,N. (1995) Epidemiological evidence for vitamin C and vitamin E in cancer prevention. *Am. J. Clin. Nutr.*, **62** (suppl), 1385S-1392S.
12. Mayne,S.T., Hanelman,G.J. and Beecher,G. (1996) Beta-Carotene and lung cancer promotion in heavy smokers—a plausible relationship? *J. Natl. Cancer Inst.*, **88**, 1513–1515.
13. Omenn,G.S., Goodman,G.E., Thornquist,M.D., Balmes,J., Cullen,M.R., Glass,A., Keogh,J.P., Meyskens,F.L., Valanis,B., Williams,J.H., Barnhart,S., Cherniak,M.G., Brodkin,C.A. and Hammar,S. (1996) Risk factors for lung cancer and for intervention effects in CARET, the beta-carotene and retinol efficacy trial. *J. Natl. Cancer Inst.*, **88**, 1550–1559.
14. Albanes,D., Heinonen,O.P., Taylor,P.R., Virtamo,J., Edwards,B.K., Rautalahti,M., Hartman,A.M., Palmgren,J., Freedman,L.S., Haapakoski,J., Barrett,M.J., Pietinen,P., Malila,N., Tala,E., Liippo,K., Salomaa,E.R., Tangrea,J.A., Teppo,L., Askin,F.B., Taskinen,E., Erozan,Y., Greenwald,P. and Huttunen,J.K. (1996) α-Tocopherol and β-carotene supplements and lung cancer incidence in the alpha-tocopherol, beta-carotene cancer prevention study: effects of base-line characteristics and study compliance. *J. Natl. Cancer Inst.*, **88**, 1560–1570.
15. Machlin,L.J. (1995) Critical assessment of the epidemiological data concerning the impact of antioxidant nutrients on cancer and cardiovascular disease. *Crit. Rev. Food Sci. Nutr.*, **35**, 41–50.
16. Szarka,C.E., Grana,G. and Engstrom,P. (1994) Chemoprevention of cancer. *Current Problems in Cancer*, **1**, 3–79.
17. Collins,A.R., Dusinská,M., Gedik,C.M. and Stetina,R. (1996) Oxidative damage to DNA: Do we have a reliable biomarker? *Environ. Hlth. Perspect.*, **104**, 465–469.
18. Duthie,S., Ma,A., Ross,M.A. and Collins,A.R. (1996) Antioxidant supplementation decreases oxidative DNA damage in human lymphocytes. *Cancer Res.*, **56**, 1291–1295.
19. Collins,A.R., Duthie,S.J. and Dobson,V.L. (1995) Direct enzymic detection of endogenous oxidative base damage in human lymphocyted DNA. *Carcinogenesis*, **14**, 1733–1735.
20. Fearon,E.R. and Vogelstein,B. (1990) A genetic model for colorectal tumorigenesis. *Cell*, **61**, 759–767.
21. Wattenberg,L.W. (1992) Inhibition of carcinogenesis by minor dietary constituents. *Cancer Res.*, **52** (Suppl.), 2085s–2091s.
22. Rowland,I.R., Bearne,C.A., Fischer,R. and Pool-Zobel,B.L. (1996) The effect of lactulose on DNA damage induced by 1,2-dimethylhydrazine in the colon of human-flora-associated rats. *Nutr. Canc.*, **26**, 38–47.

23. Pool-Zobel,B.L., Neudecker,C., Domizlaff,I., Ji,S., Schillinger,U., Rumney,C.J., Moretti,M., Villarini,M., Scassellatti-Sforzolini,G. and Rowland,I.R. (1996) *Lactobacillus*- and *Bifidobacterium*-mediated antigenotoxicity in colon cells of rats: prevention of carcinogen-induced damage *in vivo* and elucidation of involved mechanisms. *Nutr. Canc.*, **26**, 365–380.
24. Singh,N.P., McCoy,M.T., Tice,R.R. and Schneider,E.L. (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res.*, **175**, 184–191.
25. McKelvey,V.J., Green,M., Schmezer,P., Pool-Zobel,B.L., De Me′,M.P. and Collins,A.R. (1993) The single cell gelelectrophoresis (SCGE) assay (Comet assay): a European review. *Mutat. Res.*, **288**, 47–63.
26. Dusinská,M. and Collins,A.R. (1996) Detection of oxidised purines and UV-induced photoproducts in DNA of single cells, by inclusion of lesion-specific enzymes in the comet assay. *ATLA*, **24**, 405–411.
27. Pool-Zobel,B.L., Lotzmann,N., Knoll,M., Kuchenmeister,F., Lambertz,R., Leucht,U., Schröder,H.G. and Schmezer,P. (1994) Detection of genotoxic effects in human gastric and nasal mucosa cells isolated from biopsy samples. *Environ. Mol. Mutag.*, **24**, 23–45.
28. Holz,O., Jörres,R., Kästner,A., Krause,T. and Magnussen,H. (1995) Reproducibility of basal and induced DNA single-strand breaks detected by the single-cell gel electrophoresis assay in human peripheral mononuclear leukocytes. *Int. Arch. Occup. Environm. Hlth.*, **67**, 305–310.
29. Anderson,D., Yu,T.W., Phillips,B.J. and Schmezer,P. (1994) The effect of various antioxidants and other modifying agents on oxygen-radical-generated DNA damage in human lymphocytes in the COMET assay. *Mutat. Res.*, **307**, 261–271.
30. Pool-Zobel,B.L. and Leucht,U. (1997) Induction of DNA damage by risk factors of colon cancer in human colon cells derived from biopsies. *Mutat. Res.*, **375**, 105–115.
31. Pool-Zobel,B.L., Collins,A.R. and Rechkemmer,G. (1997) Levels of endogenous DNA damage and oxidised DNA bases in colon cells derived from biopsies of 50 non-colon cancer patients. *AACR*, **38**, 299 (Abstract).

Received on February 12, 1997; revised on June 4, 1997; accepted on June 4, 1997