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ORIGINAL CONTRIBUTION

Does a vegetarian diet influence genomic stability?

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Summary *Background* The vegetarian lifestyle is supposedly healthy, and differences between vegetarians and non-vegetarians in biomarkers related to diseases such as cancer might be expected. *Aim of the study* To investigate the possible role of different diets in maintaining genomic stability. *Methods* The vegetarian group, consisting of 24 volunteers (13 women and 11 men), were matched for age and sex with 24 volunteers (12 women and 12 men) with a traditional dietary habit. Among vegetarians there were 13 lacto-ovo-vegetarians (8 women, 5 men) with average length of vegetarian diet 10.8 years (ranging from 5 to 26) and 11 lacto-vegetarians (5 women, 6 men) with average length of vegetarian diet 8.2 years (ranging from 3 to15). All volunteers were non-smokers, non-consumers of alcohol and had similar education and patterns of physical activity. Chromosome aberrations, micronuclei and DNA damage (strand breaks, oxidised bases and H_2O_2-sensitivity) were examined in peripheral blood lymphocytes of vegetarians and non-vegetarians. Plasma antioxidant status was assessed with the FRAP assay. *Results* We did not find any differences in percentage of cells with chromosome aberrations or in the frequency of micronuclei between vegetarians and non-vegetarians or between lacto-ovo and lacto-vegetarians. There was no statistically significant difference in total antioxidant capacity between the groups. The group with traditional dietary habits had significantly higher levels of oxidative DNA damage (strand breaks and oxidised purines, $P = 0.005$) compared with vegetarians. A significant positive correlation between age and oxidative DNA damage (net FPG-sensitive sites) was found in non-vegetarians, while there was an opposite trend towards a *negative* association in vegetarians. On the other hand chromosome aberrations correlated with age in vegetarians ($r = 0.48$, $P = 0.017$) but not in non-vegetarians. *Conclusions* Our results indicate that a vegetarian diet can lead to a slight decrease in oxidative DNA damage in lymphocytes, but other markers of genetic stability are not affected. The lowest level of DNA damage was found in lymphocytes of lacto-vegetarians, (especially oxidised pyrimidines, $P = 0.0017$), suggesting that this diet provides some protection against oxidative stress.

Key words nutrition – vegetarianism – chromosome aberration – micronuclei – comet assay – FRAP

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Introduction

Vegetarianism is a complex life style that typically entails a health-conscious attitude not just to nutrition, but also to such things as alcohol consumption, smoking and exercise. Vegetarians can be divided into subgroups according to their specific dietary habits: lacto-ovo-vegetarians (LO) avoid refined (white) sugar, white flour, and other refined and chemically modified food products, but they consume milk, other dairy products and eggs; lacto-vegetarians (L) also consume milk and other dairy products, but exclude eggs from their diet; vegans eat only food of vegetable origin – nuts, legumes, grains,

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fruit, vegetable and honey; and vitarians consume only raw food.

Our previous investigations [1] have shown that vegetarians (the same groups as are the subject of the present report) consume substantially higher quantities of fruits and vegetables, and have significantly higher plasma levels of dietary antioxidants: vitamin C, β-carotene, and vitamin A, compared with non-vegetarians. A significantly higher molar ratio of vitamin E/cholesterol indicates a more effective protection especially of low-density lipoproteins against peroxidation. Vegetarians have higher plasma levels of selenium but similar levels of zinc and copper when compared to non-vegetarians. These results indicate that vegetarianism has a beneficial effect on antioxidative parameters, implying a possible reduction in risk of cardiovascular diseases and cancer.

On the other hand, vitamin D is absent in vegetable sources and there is a higher occurrence of deficiencies in iron, calcium, total proteins and vitamin B12 deficiency in vegetarians. Deficiencies of vitamins B12, B6, C, E, folate, niacin, or iron mimic radiation in causing single- and double-strand breaks and/or oxidative lesions in DNA [2]. Low levels of folic acid and vitamin B12 are associated with elevated chromosome damage rate and high concentrations of homocysteine in blood. It is apparent that elevated homocysteine and low vitamin B12 status are important risk factors for increased chromosome damage [3]; moreover, they are significantly correlated with increased micronucleus formation in lymphocytes [4].

Data obtained by MacGregor [5] demonstrate that dietary and nutritional factors can influence spontaneous rates of chromosomal damage in laboratory animals, and suggest strongly that some of these dietary factors may exert a quantitatively significant influence on spontaneous chromosomal damage frequencies in human populations. Results of Fenech and Rinaldi [6] do not support the hypothesis that vegetarians have a lower genetic damage rate than non-vegetarians. On the other hand, Gaziev et al. [7] showed that consumption of a mixture of antioxidant vitamins favours a decrease in the chromosome damage produced by endogenous and exogenous factors in human lymphocytes. Dhawan et al. [8], using the comet assay, revealed significant differences in the extent of DNA damage in smokers versus non-smokers as well as between the vegetarians and non-vegetarians in a normal healthy Indian population.

The aim of this study was to compare the effect of diet on formation of chromosome aberrations, micronuclei and oxidative DNA damage in peripheral lymphocytes in vegetarians and non-vegetarians.

Subjects and methods

The group of vegetarians consisted of 24 healthy adult volunteers (average age 40.0 ± 1.7, ranging from 20 to 62), 13 women and 11 men. The group of non-vegetarians consisted of 24 randomly selected healthy adult volunteers with traditional mixed diet (average age 41.2 ± 2.0, ranging from 20 to 69), 12 women and 12 men. The vegetarians included 13 LO-vegetarians (8 women, 5 men) and 11 L-vegetarians (5 women, 6 men). The average period of vegetarianism in the LO group was 10.8 years (ranging from 5 to 26), in the L group 8.2 years (ranging from 3 to 15). All probands lived in the same region (Bratislava and surroundings), had approximately the same patterns of physical activity, similar levels of education (high school and university), and all were non-smokers and non-consumers of alcohol.

Lymphocyte cultures

Samples of whole blood were taken from all subjects and conventional short-term lymphocyte cultures were made. Cultures were set up by adding 0.5 ml whole blood to 4.5 ml of RPMI medium with L-glutamine (Gibco) supplemented with 20 % foetal calf serum (Gibco) and antibiotics (penicillin and streptomycin). Lymphocytes were stimulated with 0.18 mg/ml phytohaemagglutinin (Murex) and incubated at 37 °C in 5 % CO_2. Two cultures of each sample were set up.

Chromosome aberrations

The cells were harvested at 48 h following stimulation; colchicine (Sigma), 0.75 μg/ml, was added 2 h before harvest. The cells were centrifuged and subjected to a hypotonic shock in 0.075 M KCl for 20 min, at 37 °C. The lymphocytes were fixed twice in methanol:acetic acid (3:1) and air-dried preparations were made. The slides were stained with 5 % aqueous Giemsa solution for 10 min.

A total of 100 well-spread metaphases per person were examined. All the basic chromosome abnormalities, chromatid and chromosome gaps, breaks and exchanges were recorded. Because of controversies over their classification, gaps were not included among aberrant cells. Chromosome damage was expressed as % of aberrant cells and number of breaks per cell.

Micronucleus test

Cytochalasin B (Sigma), final concentration 6 μg/ml, was added 44 h after the start of culture and at 72 h of incubation cells were centrifuged, resuspended in 0.075 M KCl and immediately centrifuged again and fixed twice

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with fixative (methanol: acetic acid, 3:1). The fixed cells were dropped onto slides, air dried and stained with 5 % aqueous Giemsa solution for 5 min. Cytochalasin B inhibits cytoplasmic cleavage without preventing mitosis. Thus cells that have divided are readily identified by the presence of two nuclei. MN analysis was performed on 2000 binucleated lymphocytes with preserved cytoplasm for each subject. MN were accepted according to the criteria of Fenech [11] if they were morphologically identical to, but smaller than, normal nuclei; had diameter between 1/16 and 1/3 of the main nuclei; were non-refractile; were not linked to the main nuclei via a nucleoplasmic bridge (though they might sometimes overlap the boundaries of the main nuclei).

Isolation of lymphocytes for the comet assay

The buffy coat after removal of plasma was diluted with RPMI 1640 medium with 10 % fetal bovine serum, layered over an equal volume of Lymphoprep (Nycomed, Oslo, Norway), and centrifuged at 700xg for 20 min at 20 °C. The layer above the Lymphoprep, containing lymphocytes, was removed, diluted with medium and centrifuged at 700xg for 15 min at 20 °C. Pelleted lymphocytes were suspended in PBS at $0.8x10^6$ ml and used for the comet assay.

Comet assay

The alkaline comet assay modified with lesion-specific enzymes was used for detection of strand breaks (SBs), oxidised purines and oxidised pyrimidines [10]. Briefly, fresh lymphocytes were embedded in duplicate agarose gels on microscope slides, lysed with 10 mM Tris-buffered 2.5 M NaCl, 1 % Triton X-100, 0.1 M EDTA, pH 10 at 4 °C for 1 h, washed with 40 mM HEPES-buffered 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/ml bovine serum albumin, pH 8, and incubated in this buffer with 50 µl of endonuclease III (for detection of oxidised pyrimidines) for 45 min, with 50 µl of formamidopyrimidine glycosylase (FPG) (for detection of oxidised purines, especially 8-oxo-guanine) for 30 min, or with neither enzyme. Slides were placed in 0.3 M NaOH, 1 mM EDTA for 40 min before electrophoresis in this solution at 25 V, 300 mA for 30 min. DNA loops containing breaks lose supercoiling and upon electrophoresis they are free to move out into a tail. The slides were neutralised with 0.4 M Tris-HCl, pH 7.5, and stained with 50 µl 4',6-diamidino-2-phenylindole (DAPI, 5 µg/ml). Comets were analysed by visual scoring of 100 randomly selected images per gel, classifying them into five categories representing increasing relative tail intensity and thus increasing degrees of damage. This method was calibrated by reference to computer image analysis based on fluorometric measurement of DNA intensities in head and tail [10, 11].

Sensitivity to hydrogen peroxide

Lymphocytes from each volunteer were treated with 50 µM hydrogen peroxide for 5 min on ice. After rinsing with PBS, the cells were placed in duplicate gels on microscopic slides and followed normal comet assay procedure without using FPG or endonuclease III (EndoIII). Controls without H_2O_2 provided an estimate of the background level of SBs.

Reference standard

A reference standard was used in each comet assay experiment consisting of aliquots of lymphocytes from a single blood collection sample.

Ferric reducing ability of plasma (FRAP)

The index of combined non-enzymic antioxidant capacity of plasma was measured spectrophotometrically according to Benzie and Strain [12]: ferric to ferrous ion reduction at low pH causes a coloured ferrous-tripyridyltriazine complex to form. FRAP values were obtained by comparing the absorbance change at 593 nm in test samples with solutions containing ferrous ions in known concentration.

Statistical analysis

To test for significant differences between groups we used the Mann-Whitney U-test, t-test, one-way analysis of variance and Bonferroni test for multiple comparisons, and χ^2-test. All tests were performed at significance level $\alpha = 0.05$. Pearson (for normal distributed data) or Spearman correlations (for non-normally distributed data) were used for analysing the possible association between markers.

Results

In this study 48 healthy adult volunteers were investigated; 24 vegetarians and 24 sex- and age-matched non-vegetarians. Among vegetarians there were 11 lacto-vegetarians and 13 lacto-ovo-vegetarians. The vegetarians and non-vegetarians, especially men, differ in BMI. Non-vegetarian men had a higher mean BMI (26.5 ± 4.01, $N = 12$) compared with vegetarian men (23.0 ± 1.09, $N = 11$; $P = 0.04$).

FRAP

The total antioxidant capacity of plasma measured by the FRAP assay was equal in both investigated groups, vegetarians (766.5 ± 39.0 μM) and non-vegetarians (817 ± 36.5 μM). There was no statistical difference comparing the two groups of vegetarians L (740.5 ± 42.8 μM) and LO (788.4 ± 63.2 μM) either.

Chromosome aberrations and micronuclei

Table 1 shows frequencies of cells with chromosome aberrations and frequencies of chromosome/chromatid breaks in both vegetarians and non-vegetarians. There were no significant differences in any parameters measured; aberration frequencies were within what is regarded as the normal range [13]. The same results were found in LO and L (Table 1).

Results of micronucleus test also show no differences between vegetarians and non-vegetarians or between LO and L (Table 2).

Oxidative DNA damage measured by the comet assay

DNA damage, including SBs, apurinic/apyrimidinic (AP) sites, oxidised purines and oxidised pyrimidines, was detected by the alkaline comet assay modified with lesion-specific enzymes, FPG and endonuclease III. The basic alkaline comet assay measures SBs and AP sites. The enzyme-modified assay measures oxidative damage as a combination of SBs, AP sites and oxidised bases – formamidopyrimidines and the oxidised purine 8-oxoguanine in the case of FPG, and oxidised pyrimidines in the case of endonuclease III. Subtraction of comet scores on incubation with buffer alone from comet scores for incubation with enzyme gives 'net enzyme-sensitive sites', representing oxidised bases.

Compared with vegetarians, non-vegetarians had higher levels of SBs, AP sites and FPG-sensitive sites (P = 0.035) (Fig. 1). There was no significant difference in net FPG-sensitive sites or net endonuclease III-sensitive sites, nor in SBs plus AP sites. There was no difference between the sexes in levels of DNA damage.

The two groups of vegetarians (L and LO) differed in the level of oxidised pyrimidines which was lower by nearly 40 % in L-vegetarians (P = 0.017) (Table 3).

In this study we also measured the sensitivity of lymphocytes to H_2O_2 as an indirect method for assessing the degree of antioxidant protection of cells against oxidative DNA damage. We did not find significant differences in sensitivity of lymphocytes to H_2O_2 either in vegetarians and non-vegetarians, or in the subgroups of vegetarians (L and LO) (Table 3).

Table 2 Micronuclei in lymphocytes of subjects with normal mixed diet and in vegetarians and their subgroups

Group	N	Number of analysed cells	% cells with micronucleus	Number of micronuclei/1000
Non-vegetarians	24	48000	0.38	4.5
Vegetarians All	24	48000	0.43	4.9
LO	13	26000	0.42	4.6
L	11	22000	0.44	5.2

LO lacto-ovo-vegetarians; *L* lacto-vegetarians; *N* number of subjects

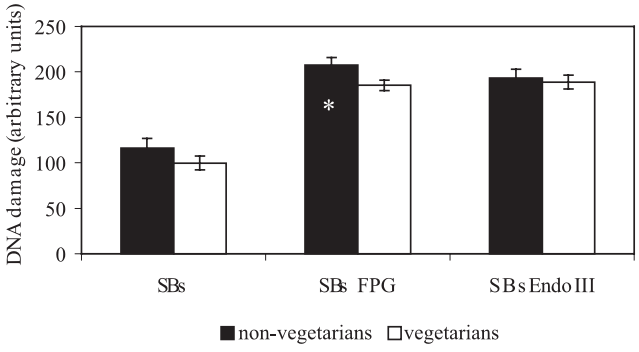

Fig. 1 Endogenous levels of DNA damage expressed as SBs, SBs + FPG-sensitive sites, and SBs + endonuclease III-sensitive sites in lymphocytes of non-vegetarians and vegetarians. *SBs* strand breaks alone; *SBsFPG* strand breaks together with FPG-sensitive sites (altered purines); *SBsEndoIII* strand breaks together with oxidised pyrimidines (endonuclease III sensitive sites). * P = 0.035

Correlations

Pearson (for normally distributed data – micronuclei, SBs, net FPG, net EndoIII, net H_2O_2, age) or Spearman

Table 1 Chromosome aberrations in lymphocytes of subjects with normal mixed diet and in vegetarians and their subgroups

Group	N	Number of analysed cells	% of aberrant cells	Chromatid breaks	Chromosome breaks	B/C	G/C
Non-vegetarians	24	2400	0.63	12	5	0.007	0.003
Vegetarians (All)	24	2400	0.63	10	8	0.008	0.007
LO	13	1300	0.62	7	4	0.008	0.009
L	11	1100	0.64	3	4	0.006	0.005

LO lacto-ovo-vegetarians; *L* lacto-vegetarians; *B/C* breaks per cell; *G/C* gaps per cell; *N* number of subjects

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Table 3 Oxidative DNA damage measured by the comet assay in lymphocytes from subjects with normal mixed diet and from vegetarians and their subgroups

Group	N	SBs	net FPG	net EndoIII	net H_2O_2
Non-vegetarians	24	116.3±10.5	92.9±9.7	79.7±9.6	161.9±14.6
Vegetarians All	24	99.8±7.7	86.1±7.3	89.6±8.8	181.0±10.8
LO	13	95.8±11.0	96.5±11.5	108.2±13.2	188.2±15.2
L	11	104.6±11.0	73.7±7.0	67.6±7.0*	172.5±15.6

LO lacto-ovo-vegetarians; *L* lacto-vegetarians; *SBs* strand breaks alone; *net FPG* net FPG-sensitive sites (oxidised purines); *net EndoIII* net-endonuclease III-sensitive sites (oxidised pyrimidines); *net H_2O_2* net strand breaks induced by H_2O_2; *N* number of subjects. * P = 0.017

(for non-normally distributed data – chromosome aberrations) correlation tests were used for analysing the possible association between studied biomarkers in groups of vegetarians and non-vegetarians (Table 4).

In both groups there was a correlation between frequency of micronuclei and age (Table 4). Chromosome aberrations correlated with age in vegetarians (r = 0.48, P = 0.017) but not in non-vegetarians (Table 4).

We found a significant positive correlation between age and oxidative DNA damage (net FPG-sensitive sites) in non-vegetarians. While no significant correlation was seen in vegetarians (Table 4), there was a trend towards a *negative* association between age and net FPG-sensitive sites (Fig. 2). A similar pattern (positive correlation in non-vegetarians, but inverse in vegetarians) was found between sensitivity of lymphocytes to H_2O_2 and age. However, the significance of these correlations was marginal in both non-vegetarians and vegetarians (Table 4).

BMI correlated positively with oxidised purines, oxidised pyrimidines and H_2O_2 sensitivity in non-vegetarians, whereas in vegetarians oxidised purines show a negative correlation with BMI. There is also an interesting negative correlation between BMI and SBs in non-vegetarians (Table 4).

Table 4 Correlations of age and BMI with DNA instability markers measured in lymphocytes of non-vegetarians and vegetarians

	Non-vegetarians		Vegetarians	
	Age	BMI	Age	BMI
Micronuclei	r = 0.57, P = 0.004		r = 0.50, P = 0.012	
Chrom. Aberration			r = 0.48, P = 0.017	
SBs		r = –0.51, P = 0.011		
net FPG	r = 0.42, P = 0.044	r = 0.58, P = 0.003		r = –0.42, P = 0.04
net EndoIII		r = 0.45, P = 0.029		
net H_2O_2	r = 0.40, P = 0.058	r = 0.65, P = 0.001	r = –0.4, P = 0.051	
Age				r = 0.60, P = 0.002

BMI body mass index; *SBs* strand breaks alone; *net FPG* net FPG-sensitive sites (oxidised purines); *net EndoIII* net endonuclease III-sensitive sites (oxidised pyrimidines); *net H_2O_2* net strand breaks induced by H_2O_2. *P* significance; *r* correlation coefficient. All data were evaluated using linear Pearson correlations (for normally distributed data); chromosome aberrations data were analysed using Spearman correlations (data were not normally distributed)

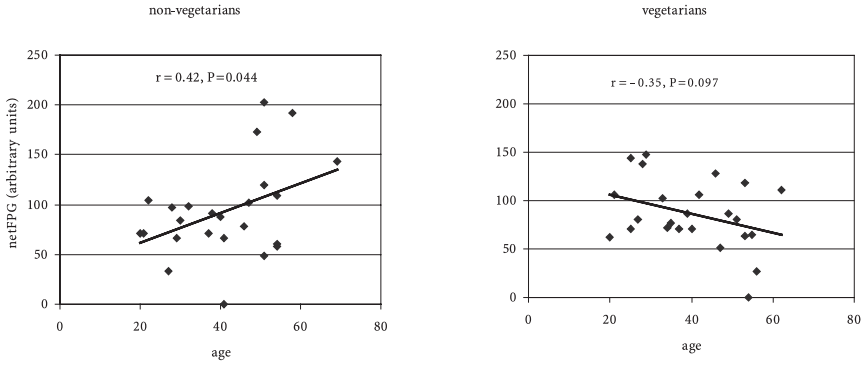

Fig. 2 Correlation of oxidised purines with age in non-vegetarians and vegetarians. *net FPG* net FPG-sensitive sites (oxidised purines). *P* significance; *r* correlation coefficient

Discussion

The typical vegetarian diet involves much higher consumption of fresh fruit, vegetables, cereal products, oil-bearing plants and nuts, vegetable oils and soya products compared with a traditional mixed diet. It is generally accepted that higher intake of antioxidants can protect against oxidative stress. In our group of 24 vegetarians the levels of vitamin C, vitamin E, vitamin A and β-carotene were significantly higher compared with 24 sex and age matched non-vegetarians [14]. The consumption of higher levels of micronutrients apparently did not affect levels of chromosome aberrations or micronuclei but slightly affected levels of endogenous oxidative DNA damage in the lymphocytes of these subjects. Oxidative stress, including DNA damage, oxidation of lipids, proteins and sugars, contributes to the cellular and sub-cellular changes which may be associated with chronic degenerative diseases such as cancer, cardiovascular diseases, cataracts and others, either as cause or consequence. In our study vegetarians had significantly lower levels of SBs plus FPG-sites and the subgroup of L-vegetarians had the lowest level of oxidised pyrimidines (Fig. 1, Table 3). The differences, however, are relatively small, and give only limited support to the hypothesis that higher consumption of fruit and vegetable protects against oxidative stress.

The lowest level of oxidative DNA damage seen in L-vegetarians suggests that lacto-vegetarians may have the greatest degree of protection against oxidative stress, although this is not reflected in their sensitivity to H_2O_2-induced damage.

The lack of a difference in micronucleus frequency between groups is not surprising. Similarly Fenech [6] did not find any difference between vegetarians and non-vegetarians in levels of micronuclei. His and our results together do not support the hypothesis of greater genomic stability in vegetarians.

Micronuclei are a sensitive marker of aging. Many studies show that the micronucleus frequency increases with age [15, 16]. Though our study consists of only 48 people, we also found a correlation between age and level of micronuclei in both vegetarians and non-vegetarians, this correlation being slightly stronger in non-vegetarians.

Chromosome aberrations are the only marker validated in prospective studies as predictive of cancer risk [17, 18]. The results of Ramsey et al. [19] support the hypothesis that stable chromosome aberrations show a greater accumulation with age than unstable aberrations and suggest that lifestyle factors contribute to the accumulation of cytogenetic damage. Rossner et al. [20] demonstrated an elevated spontaneous frequency of aberrant cells with age in 5430 control subjects. In this study we did find a positive correlation between % of aberrant cells and age – but only in vegetarians, and not in the group of subjects with normal mixed diet. Vegetarians and non-vegetarians do not differ in mean chromosome aberration frequencies.

In a previous study we supplemented middle-aged men who had traditional dietary habit with a mixture of vitamin C, vitamin E, β-carotene and selenium [21]. After three months supplementation we found a decrease in chromosome aberration in the supplemented group compared with the placebo group. However, this effect was seen only in smokers and there was no comparison with vegetarians.

A deficiency in vitamin B12 and folate contributes to increased DNA instability and therefore vegetarians who have lower levels of B12 might be at risk of genomic instability [3, 4]. On the other hand, a higher intake of vitamins and antioxidants from fruits and vegetable protects against oxidative stress. In our group of subjects with normal mixed diet, oxidative DNA damage increases with age but the opposite tendency was demonstrated in vegetarians.

Conclusion

In our study non-vegetarians had higher level of strand breaks and oxidised purines compared with vegetarians. The lowest level of DNA damage (especially oxidised pyrimidines) was found in lymphocytes of lacto-vegetarians, which may suggest that the lacto-vegetarian diet provides some protection against oxidative stress. There was also a significant positive correlation between age and oxidative DNA damage in non-vegetarians while the opposite tendency was seen in vegetarians. Chromosome aberrations correlated positively with age in vegetarians but not in non-vegetarians.

A vegetarian diet results in higher intake of vitamins and micronutrients which, although providing antioxidant defence, might lead to deficiency of other micronutrients involved in DNA metabolism and stability (such as vitamins belonging to B group). It seems that higher intake of vitamins can protect against genetic damage but only if all micronutrients responsible for maintenance of DNA stability are in balance.

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