Influence of diet on oxidative DNA damage, uracil misincorporation and DNA repair capability

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The contribution of diet to cancer ranges from 10 to 80%. The low ingestion of antioxidants and enzymatic cofactors involved in DNA repair and methylation reactions and the high ingestion of chemical additives present in the modern diet, associated with genetic factors, could lead to genomic instability and the hypomethylation of proto-oncogenes, thus contributing to development of genetic-related diseases such as cancer. The present study evaluated the influence of diet on the level of oxidative DNA damage, misincorporated uracil and DNA repair capability in peripheral blood lymphocytes from two groups of individuals with antagonist diets as follows: (i) 49 healthy individuals with a diet rich in organic products, whole grains, fruit and vegetables and poor in processed foods (Group I) and (ii) 56 healthy individuals with diet rich in processed foods and poor in fruit and vegetables (Group II). Oxidative DNA damage, uracil incorporation and DNA repair capability were assessed by the comet assay. The individuals in Group I presented lower levels of oxidative DNA damage (oxidized purines and pyrimidines) and lower levels of DNA damage induced by ex vivo treatment with hydrogen peroxide (H₂O₂) than those individuals in Group II. The analysis of our results suggests that a diet rich in organic products, integral grains, fruit and vegetables and poor in industrialized products can protect against oxidative DNA damage and DNA damage induced by H₂O₂.

Introduction

Diet plays an important role in preventing cancer and other genetic-related diseases, but the mechanisms involved are still not clear (1). It is estimated that 10–80% of cancer cases are related to dietary factors (2), and many chemical food additives have already been proven to be carcinogenic (3,4). However, not only the increased intake of such products but also decreased consumption of natural foods is a factor that contributes to the emergence of modern-life diseases. Several studies have shown, for instance, that alcohol, aflatoxin and salted foods (three diet constituents or contaminants), together with obesity and low intake of fruits and vegetables, are clearly associated with the increased incidence of cancer in humans (5,6).

Epidemiological studies have also shown that individuals who consume large amounts of fruits and vegetables rich in micronutrients with antioxidants properties, (such as vitamin C, vitamin E, carotenoids and flavonoids) show a lower incidence of lung, stomach, breast, colon, liver and pharynx cancer (7). These micronutrients can act as enzymatic cofactors in DNA maintenance, repair and methylation and in the process of apoptosis (8). Variations in the intake of micronutrients may be related to genomic instability, as detected by the increased incidence of DNA damage (genotoxicity), point mutation and chromosomal breakage or by alterations in chromosomal segregation and gene expression and also in the oxidative stress, necrosis and apoptosis rates (9). According to Fenech (10), genome damage caused by moderate micronutrient deficiency is equivalent to genome damage levels caused by exposure to significant doses of environmental genotoxins, such as chemical carcinogens and ultraviolet and ionizing radiation. Moreover, according to Ames (11), the deficiency of micronutrients, such as folic acid, vitamins B12, B6, C and E, niacin, iron and zinc can mimic the effect of radiation or chemical agents in the induction of DNA damage. The deficiency of folic acid in humans has been associated with megaloblastic anaemia, defects of the neural tube closure in newborns, heart disease and cancer development, especially of colon and rectum (12). In 2005, Fenech (13) observed that chromosomal damage in a culture of human lymphocytes caused by the reduction in the concentration of folic acid from 120 to 12 nmol/l was equivalent to that induced by acute exposure to ionizing radiation (0.2 Gy), a dose of radiation which is ~10-fold the annual exposure safety threshold for radiation workers. Folic acid and vitamin B12 play an important role in DNA metabolism. Thus, in conditions of folic acid deficiency, uracil is incorporated into DNA in the place of thymine (14). There is evidence showing that the excessive incorporation of uracil in DNA not only generates point mutations but can also cause single- and double-strand breaks, as well as lead to the formation of micronuclei (15,16).

Approximately 40 micronutrients, such as vitamins, essential minerals and other components are required in small quantities in the human diet for efficient metabolism. However, there is no consensus on the level of micronutrients necessary to prevent DNA damage in humans (17). The fundamental objective of these areas is to characterize ideal dietary intakes for preventing DNA damage and aberrant gene expression for genetic subgroups and for each individual because the amount of micronutrients that appear to be protective against genome damage varies greatly among food types, and a careful choice...
is needed to design dietary patterns optimized for genome health maintenance (10).

Several studies have also shown that many dietary components can act as enzymatic cofactors or substrates in the DNA repair and metabolic pathways (8,9,13,18,19). Ames and Wakimoto (8) have observed that Zn and Mg deficiencies may reduce those enzymatic activities.

Based on the information presented, this study aimed to evaluate the influence of diet on the level of oxidative DNA damage, incorporated uracil and DNA repair capability in peripheral blood lymphocytes of two groups of individuals with antagonist diets.

**Material and methods**

**Subject selection**

This study was approved by the Ethics Committee for Human Research of the Botucatu Medical School, São Paulo State University (UNESP), Botucatu, SP, Brazil. Informed consent was obtained from each volunteer.

A total of 132 healthy adult volunteers were interviewed. From these volunteers, a group of 27 individuals were excluded from the study due to technical problems or because they did not fit in the parameters established. Therefore, 105 healthy adult volunteers (mean age 35.6 ± 11.4, ranging from 19 to 66 years), 52 males and 53 females, were evaluated. All of the volunteers were non-smokers, were not abusing alcohol, were not using prescription or recreational drugs or any vitamin or mineral supplementation. These volunteers were distributed in two groups as follows: (i) Group I, 49 naturalistic individuals, living in the countryside, in a farm, with a lifestyle characterized by low level of urban stress, high intake of organic fruits and vegetables and low consumption of processed (industrialized) foods, such as cakes, margarine, ice cream, beverages, jellies, biscuits and sausages, among others, that are rich in dyes, flavourings, preservatives, stabilizers and acidulents and (ii) Group II, 56 individuals living in an urban area, with high ingestion of processed foods and low intake of fruits and vegetables.

Because this was our first work in the diet-related genotoxicity line of research, we decided to work with individuals who intake organic products (Group I) to avoid possible confounding factors influence our results, such as agrotoxic chemicals.

For evaluation of the dietary pattern, the estimated level of micronutrients ingested was calculated (data not shown), and for the group classification, a food frequency questionnaire adapted from Cardoso and Stocco (20) was applied. This quantitative questionnaire provided data, which were analysed by using the AvaNutri software package (Avanutri Informática Ltda, Rio de Janeiro, Brazil), to estimate the daily consumption of food and micronutrients. Moreover, three other dietary records were used as follows: one was applied at the weekend and the two other were applied during the week, to improve the calculation of the habitual dietary intake. Dietary recalls and the software analyses were conducted by a person trained (B.F.S.). Data obtained from questionnaires were analyzed using the SPSS 12.0 software. To be classified in Group I, the individuals had to consume organic fruits and vegetables equal to the 75th percentile and <25th percentile of each processed food. The individuals in Group II were those who consumed <25th percentile of organic fruits and vegetables and more than the 75th percentile of processed foods. Only the individuals adopting the respective diet pattern for at least 10 years were selected.

**Determination of oxidative DNA damage and level of uracil incorporation into DNA**

The alkaline comet assay (21), modified with lesion-specific enzymes, was used to detect single- and double-strand breaks, labile sites, oxidized purines and pyrimidines (22) and uracil (12). Briefly, 10 μl of the isolated lymphocyte suspension (23) (≥2 × 10^6 cells) were embedded into 0.5% low melting point agarose (Sigma) and spread on agarose-precotted microscope slides. Slides were immersed overnight in freshly prepared cold lysis solution [2.5 M NaCl, 100 mM ethylenediaminetetraacetic acid (EDTA), 10 mM Tris, 1% sodium salt N-lauryl sarcosine, pH 10.0, with 1% Triton X-100 and 10% dimethyl sulfoxide added fresh] at 4°C. After lysis, the slides were washed three times in enzyme buffer (Trevigen, Gaithersburg, MD, USA) and then incubated at 37°C for 45 min with 100 μl of endonuclease III (EndoIII, 1:1000; New England Biolabs Inc), 100 μl of formamidopyrimidine-DNA glycosylase (FPG, 1:1000; New England Biolabs Inc), 100 μl of uracil-DNA glycosylase (UDG, 1:1000; New England Biolabs Inc) or with enzyme buffer only. EndoIII recognizes oxidized pyrimidines (SBs EndoIII), while FPG identifies oxidized purines (SBs FPG), especially 8-oxo-guanine and UDG recognizes uracil (SBs UDG). Enzyme buffer only is used to identify SBs. Subsequently, the cells were exposed to alkali buffer (1 mM EDTA and 300 mM NaOH, pH 13.4) at 4°C, for 40 min to allow DNA unwinding and expression of alkali-labile sites. Electrophoresis was conducted in the same solution at 4°C, for 30 min, at 25 V and 300 mA. After electrophoresis, the slides were neutralized (0.4 M Tris, pH 7.5), stained with 50 μl SYBR Green (1:10000; Invitrogen) and analysed in a fluorescence microscope at ×400 magnification, using an image analysis system (Comet Assay II—Perceptive Instruments, Suffolk, UK).

One hundred randomly selected cells (50 from each of two replicate slides) were evaluated from each sample and the mean of the tail intensity (%DNA Tail) was determined. Tail Intensity, according to Comet Assay II—Perceptive instruments, is defined as ‘the sum of all intensity values in the tail region minus those which are part of the mirrored head region. The value is also expressed as a percentage of the total Comet or cell intensity’.

**DNA repair capability**

To analyse the influence of dietary patterns on the DNA repair capability, 200 μl of the freshly isolated lymphocytes were treated with 100 μl of H_2O_2 (100 μM), for 30 min, in ice, and analysis of the level of damage was evaluated. Then, after two washes in phosphate-buffered saline to remove H_2O_2, these aliquots were incubated at 37°C for 30 min, and DNA damage was checked again.

**Statistical analysis**

The data obtained in the Comet Assay had asymmetric distribution. Log transformations were applied to DNA damage distributions to help meet normality distribution assumptions. The statistical analysis consisted of applying the Student’s t-test to compare differences between the groups. In order to adjust data considering possible confounders, an analysis of variance was performed including age and gender as main factors (without interaction). Firstly, the statistical analysis was done distributing the individuals into two groups according to the age: 18–40 years and >40 years. In a second analysis, age was considered as covariate. The significance level adopted was 5% or a corresponding P-value.

**Results**

DNA damage, as single- and double-strand breaks and apurinic/apyrimidinic sites (SBs) can be evaluated by the comet formed under standard conditions. Endogenous formation of oxidized pyrimidines and purines are detected by enzymes EndoIII and FPG, respectively. The results showed no significant differences in the levels of SBs and misincorporated uracil in DNA between the two groups. However, the volunteers in Group I presented a lower level of oxidized purines and pyrimidines (P < 0.05) than individuals in Group II (Figure 1).

Regarding DNA repair capacity, no difference was detected between the two groups, although the individuals in Group I presented lower levels of DNA damage induced by H_2O_2 (Figure 2).

With respect to the level of micronutrients estimated through the food frequency questionnaire and the recall questionnaire, the individuals in Group I presented higher levels of vitamin A, vitamin B6, vitamin B12, vitamin C and folic acid than those in Group II. In addition, the individuals in Group II presented lower estimated intake level of vitamin A, vitamin B6, vitamin C and folic acid than the recommended dietary allowances (RDA) (Table I).

No relationship was detected between gender or age and the level of oxidative DNA damage and uracil misincorporation, in both groups (data not shown). But in Group II, the covariate age without division into age groups positively influenced the DNA repair capability.

**Discussion**

The development of cancer and other genetic-related diseases seems to be related to an interaction between genetic and environmental factors, such as cigarette smoking, urban and
industrial air pollution and dietary factors (24). Several studies have demonstrated that fruit, vegetables, olive oil, red wine and cereals, together with a low intake of fat from animal sources seem to be associated with lower risk for development of lung cancer, esophagus, stomach, colon and rectum cancer (25,26).

Fig. 1. Levels of DNA damage (tail intensity) expressed as SBs, SBs FPG, SBs EndoIII and SBs UDG in peripheral blood lymphocytes from Group I (n = 49) and Group II (n = 56) individuals. SBs, single- and double-strand breaks and alkali-labile sites; SBs FPG, SBs more FPG-sensitive sites (purine oxidized); SBs EndoIII, SBs more EndoIII-sensitive sites (pyrimidine oxidized) and SBs UDG, SBs more misincorporated uracil sites. Results are expressed in interquartile ranges within the box, with the median at the line, and the 5th and 95th percentiles at the whiskers. The Student’s t-test was used to compare differences between groups; *P < 0.05. The individuals in Group I presented lower levels of oxidized purines (SBs FPG) and pyrimidines (SBs EndoIII) than those individuals in Group II.

Fig. 2. Levels of DNA damage (tail Intensity) expressed as SBs H2O2 and SBs H2O2R in peripheral blood lymphocytes of individuals in Group I (n = 49) and Group II (n = 56); H2O2, strand breaks induced by treatment with H2O2; H2O2R, level of DNA damage 30 min after DNA repair of damage induced by treatment with H2O2. Results are expressed as mean ± SD. The Student’s t-test was used to compare differences between variables; *P < 0.05. Individuals in both groups did not present compromised DNA repair efficiency. Individuals in Group I presented lower levels of DNA damage induced by H2O2.

Table I. Level of micronutrients ingested by each group as estimated by the food frequency questionnaire and recall questionnaire and recommended dietary allowances (RDAs)

<table>
<thead>
<tr>
<th>Micronutrient</th>
<th>RDA*</th>
<th>Group</th>
<th>Mean</th>
<th>SD</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin A (RE)</td>
<td>900</td>
<td>1</td>
<td>2593.8</td>
<td>1065</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>349.9</td>
<td>440.7</td>
<td></td>
</tr>
<tr>
<td>Vitamin B2 (mg)</td>
<td>1.3</td>
<td>1</td>
<td>2.3</td>
<td>0.56</td>
<td>0.086</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>1.3</td>
<td>0.46</td>
<td></td>
</tr>
<tr>
<td>Vitamin B6 (mg)</td>
<td>1.3</td>
<td>1</td>
<td>1.9</td>
<td>0.37</td>
<td></td>
</tr>
<tr>
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<td></td>
<td>2</td>
<td>1.0</td>
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<td>0.036</td>
</tr>
<tr>
<td>Vitamin B12 (mcg)</td>
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<td>7.1</td>
<td>3.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>3.9</td>
<td>2.4</td>
<td>0.007</td>
</tr>
<tr>
<td>Vitamin C (mg)</td>
<td>90</td>
<td>1</td>
<td>515.1</td>
<td>340</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>34.4</td>
<td>104.9</td>
<td>0.0001</td>
</tr>
<tr>
<td>Folic acid (mcg)</td>
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<td>145.5</td>
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<tr>
<td></td>
<td></td>
<td>2</td>
<td>125.6</td>
<td>70.2</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

1 RE = 1 mcg of retinol. SD, standard deviation; P, P-value for the Student’s t-test.

*Based on the recommended dietary allowances (RDAs) of Food and Nutrition Board, Institute of Medicine, National Academies (USA) (2002).
Antioxidants and flavonoids could be responsible for such protective effect. However, some of these studies have reported only the effect of isolated nutrients and not whether there is a true relationship between them (synergistic effect) or the mechanisms involved in cancer chemoprevention, presenting only a correlation between certain nutrients/diets and carcinogenesis (27). Therefore, better understanding of possible pathways to modulate carcinogenesis and preventive interventions during these stages become very attractive.

Several biomarkers and methodologies have been used to elucidate the role of nutrition in carcinogenesis. The comet assay is a technique widely used for detecting genotoxicity. This assay has several advantages: it is economical, simple and fast because it requires a small number of cells (27), and it can provide information concerning antioxidant status (28). To ensure the correct analysis of the genotoxic effects induced by dietary factors, we adopted amendments to the protocol originally described by Singh et al. (21), which also allowed the detection of specific types of DNA damage, such as oxidative damage and uracil misincorporation besides DNA breaks induced after exposure of intact cells to reactive oxygen species (ROS). The most common ROS is H2O2. This compound can damage cellular proteins and lipids and form DNA adducts when present in high concentration, and this may contribute to carcinogenic activity (29).

Although some studies have not shown any significant association between diet and DNA damage, oxidative damage to DNA and chromosomal aberrations, such as between occupationally exposed men and non-occupationally polycyclic aromatic hydrocarbon-exposed workers (30) or between smokers and non-smokers (31), in the present study, we observed that the healthy individuals in Group I presented lower levels of oxidized purines and pyrimidines in peripheral blood lymphocytes. Moreover, a lower level of DNA damage was detected when the lymphocytes from these individuals were treated with H2O2 in vitro. Perhaps, these lower levels of DNA damage could be attributed to the micronutrients, vitamin C, vitamin E, carotenoids and flavonoids with antioxidant properties, present in the plants and fruit consumed by individuals in Group I. It is known that some micronutrients act as enzymatic cofactors, modulating several biological processes that are relevant for cancer, including biotransformation enzymes, oxidative damage to macromolecules, DNA adducts, DNA repair and methylation and apoptosis (8,30). Our results are in agreement with approximately half of the studies conducted before, when significant decrease of oxidized bases and/or protection towards ROS-mediated DNA damage were described, while only one-third of studies showed a decrease in DNA migration when alkaline Comet assay standard conditions were used (28,32). In a study using olive oil, reduced DNA damage was observed when the experiments were conducted in alkaline Comet assay standard conditions and with FPG. These effects were correlated with an increase in the plasma capacity antioxidants (33).

Another factor that could have influenced our results is the lifestyle of individuals in Group I. Their way of living is characterized by the absence of stress of modern life, with close contact with nature and organic dietary intake without chemical additives. Several studies have reported that lifestyle and environmental exposure, such as air pollution, diet, sunlight and exercise, influence the basal level of DNA damage in leucocytes (34).

On the other hand, the higher level of oxidative DNA damage detected in individuals in Group II could be related to many chemical additives, potentially genotoxics and carcinogens, present in processed foods that constituted their diet. Several chemical food additives have proven to be carcinogenic, and many of these can react with DNA (17). In addition, it is known that high ingestion of calories or certain foods, such as red meat, alcoholic beverages or coffee, may cause adverse effects, e.g., an increase in the DNA damage induced by heterocyclic aromatic amines (2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine), which could be explained by changes in the activities of activation and/or detoxification enzymes (35). Ferguson (17) has also shown that some of the chemical additives used for meat preservation (N-nitrous components), heterocyclic amines and polycyclic aromatic hydrocarbons, produced during the preparation of meat at high temperatures, may lead to colon and prostate cancer.

Folate deficiency upsets the balance of DNA precursors (36). Impaired methylation of dUMP, via depletion of methyl donor 5,10-methylene tetrahydrofolate, leads to increased levels of dUMP, low levels of dTTIP and misincorporation of uracil into DNA in place of thymidine, which is probably the major cause of spontaneous DNA damage (37). Normal cellular processes repair DNA, but as thymidine levels are suppressed repeated misincorporation and excision repair-mediated removal of deoxouridine may cause DNA double-strand breakage, chromosomal aberrations and, ultimately, malignant transformation (38). However, our results did not show differences between the levels of misincorporated uracil. Nevertheless, based on food frequency questionnaires, the individuals in Group I presented significantly higher levels of vitamin B6, B12 and folic acid than those individuals in Group II. Moreover, the individuals in Group II presented lower levels of vitamin A, B6, vitamin C and folate than the minimum recommended daily allowances (RDA), suggesting that other micronutrients and mechanisms can also be involved, e.g., polymorphisms of genes related to the process of folic acid metabolism.

By adding kiwifruit to the diet of healthy individuals as food supplement for three weeks, Collins et al. (1) observed an increased level of antioxidants in blood and increased DNA repair capability, suggesting that these effects could reduce the risk for developing cancer. However, little is known about the impact of micronutrient deficiency on DNA repair (39). In the present study, we did not find differences in DNA repair capability between the groups. Nevertheless, our study population consisted of only healthy volunteers, suggesting that the lower levels of micronutrient intake for individuals in Group II is enough to maintain DNA repair efficiency in healthy individuals.

In conclusion, our results provide evidence that diet rich in whole grains, fruit and vegetables, and poor in processed foods, associated with a healthy lifestyle can protect against oxidative DNA damage. Further, nutrigenomics evaluation on a larger number of individuals must be performed in order to also evaluate the influence of gene polymorphisms.

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