Inhibitory Effects of Lutein and Lycopene on Placental Glutathione S-Transferase–Positive Preneoplastic Lesions and DNA Strand Breakage Induced in Wistar Rats by the Resistant Hepatocyte Model of Hepatocarcinogenesis

Luciana Passos Toledo, Thomas Prates Ong, Ana Lúcia Galvão Pinho, Alceu Jordão Jr., Hélio Vanucchi, and Fernando Salvador Moreno

Abstract: Inhibitory effects of lutein (LUT) and lycopene (LYC) on hepatic preneoplastic lesions (PNLs) and DNA strand breakage induced in Wistar rats by the resistant hepatocyte (RH) model of hepatocarcinogenesis were investigated. Animals received by gavage during 8 consecutive weeks on alternate days 70 mg/kg body weight of LUT or LYC. Rats treated with only corn oil and submitted to this model were used as controls. At the end of the experiment, treatment of the animals with LUT or LYC resulted in an increase in the respective liver carotenoid concentrations (P < 0.05). Moreover, it tended to reduce the incidence, total number, and multiplicity of hepatocyte nodules compared with the control group, although the differences did not reach statistical significance. Animals treated with LUT or LYC presented also a lower number of hepatic placental glutathione S-transferase–positive (GST-P) PNLs (P < 0.05), which were smaller (P < 0.05) and occupied a smaller area of the liver section (P < 0.05). Finally, hepatic DNA strand breakage evaluated by the comet assay was lower (P < 0.05) in carotenoid-treated animals when compared with the control group. Therefore, the results indicate that LUT and LYC represent promising chemopreventive agents during hepatocarcinogenesis and whose anticarcinogenic actions could be related to a protection against DNA instability.

Introduction

Several epidemiological studies have consistently shown that high intake of fruits and vegetables presents a protective action against different cancer types (1). The important role of carotenoids in this anticarcinogenic action should be explored (2).

Carotenoids are a class of substances with over 600 constituents, widely distributed in fruits and vegetables. In vivo and in vitro protecting actions against some types of cancer were, for example, described for lycopene (LYC), present in tomatoes and its derivatives (3,4). However, regarding lutein (LUT), a carotenoid without provitamin A function such as LYC and present in green leafy vegetables, although cell culture and epidemiological investigations link it to cancer prevention or control (5–7), few studies have been conducted so far that demonstrate its anticarcinogenic potential in vivo (8,9). In general, carotenoids are being considered a potential class of chemopreventive agents (10,11).

Cancer chemoprevention consists of the pharmacological use of one or more substances with the purpose of delaying or even reverting the carcinogenic process before the emergence of malignancy (12). Therefore, great interest is dedicated to this potential option in the control of cancer because, in spite of the great advances in its diagnosis and treatment, mortality due to the disease continues to be high (13). This is particularly true in the case of liver cancer (14).

Almost 500,000 new liver cancer cases are estimated to occur each year, mainly in developing countries (15). In view of the limited treatment and negative prognosis for the disease, preventive control approaches, notably chemoprevention, have been emphasized (16).

Regarding LYC, although thought to be a promising chemopreventive agent for prostate cancer (17), concerning liver the few performed studies with the carotenoid led to disagreeing results (18,19). In addition, according to the International Agency for Research on Cancer, evidence regarding the chemopreventive ability of LUT and LYC in humans is still inadequate and is considered to be limited in animals (20).

Thus, in this study the chemopreventive activities of LUT and LYC, administered to Wistar rats submitted to the resistant hepatocyte (RH) model of hepatocarcinogenesis in a period comprising initiation and selection/promotion stages, were evaluated.

L. P. Toledo, T. P. Ong, A. L. Galvão Pinho, and F. S. Moreno are affiliated with the Laboratory of Diet, Nutrition and Cancer, Department of Food and Experimental Nutrition, Faculty of Pharmaceutical Sciences, University of São Paulo, SP, Brazil. A. Jordão Jr. and H. Vanucchi are affiliated with the Medical Clinic, Faculty of Medicine, University of São Paulo, Ribeirão Preto, SP, Brazil.
Neoplasms develop in steps termed initiation, promotion, and progression, each presenting different morphologic and molecular characteristics. Although initiation and progression involve genetic alterations in the affected cell, promotion involves reversible alteration in gene expression due to the action of a carcinogenesis-promoting agent, mostly of a chemical nature (21).

Although not all carcinogenesis models allow a distinction between the different steps of the process, in the RH model this is possible, and in addition the generation of preneoplastic and neoplastic lesions is described to occur in a synchronized form (22, 23). These characteristics render it ideal for the kinetic study of carcinogenesis as well as of chemoprevention.

The carcinogenic process is characterized by a continuous oxidative state in which reactive oxygen species (ROS) play an important role (24). These are able to damage biomolecules such as DNA and may result, for example, in important gene mutation such as in tumor suppressors and/or proto-oncogenes, therefore contributing to neoplasm formation (25). On the other hand, the high antioxidant potential of the LUT and LYC carotenoids is believed to represent an important feature of their hypothesized anticarcinogenic actions (4, 9).

We decided, therefore, to also evaluate in this study DNA strand breakage using the comet assay, considered to be an oxidative stress marker (26), in livers of animals submitted to the RH model and treated with LUT and LYC. This method is able to detect oxidation of pyrimidine bases, with the resulting break and relaxation of DNA loops of damaged cells, when submitted to electrophoresis (27).

Material and Methods

Animals and Experimental Protocol

Male Wistar rats from the colony of the Faculty of Pharmaceutical Sciences, initially weighing 40–45 g, maintained in cages of four each, at a constant temperature (22°C), with 12-h light–dark cycle, and receiving water and commercial diet (Purina, Campinas, SP, Brazil) ad libitum, were used.

For the study of the eventual chemopreventive activities of LUT and LYC during hepatocarcinogenesis, with the exception of 8 control rats not submitted to any experimental procedure [normal (N) group], 36 animals were divided into 3 experimental groups that received for 8 consecutive weeks and on alternate days by gavage corn oil (CO; 2.5 ml/kg body weight/day; Mazola®, CO group, controls), LUT [70 mg/kg body weight/day; purified LUT (94%) + zeaxanthin (5%) isolated from marigold flower extract; Food Composition Laboratory, Beltsville Human Nutrition Research Center, U.S. Department of Agriculture; LUT group], or LYC [70 mg/kg body weight/day; LYC (99%) purified from tomato oil resin containing 5% LYC (Lyc-O-Pen™ 5% Natural Tomato Extract; LycoRed, Natural Products Industries, Ltd., Beer-Sheeva, Israel; LYC group] dissolved in CO (2.5 ml/kg body weight/day). Two weeks after the beginning of the experiment all animals were submitted to the RH model adapted to Wistar rats (28). Hepatocyte initiation was obtained by the administration of a single intraperitoneal dose of diethylnitrosamine (DEN, 200 mg/kg body weight; Sigma) dissolved in 0.9% NaCl solution. After a recovery period of 2 wk, the initiated hepatocytes were selected/promoted by the administration of four single doses on consecutive days, by gavage, of 2-acetylaminofluorene (2-AAF, 20 mg/kg body weight/day; Sigma) dissolved in CO. Twenty-four hours after the last 2-AAF administration the animals were submitted to a potent mitogenic stimulus by a partial (70%) heptectomy (PH). Finally, 2 and 4 days after PH, two additional 2-AAF doses (20 mg/kg body weight/day) were administered. Six weeks after initiation with DEN, the animals were anesthetized and sacrificed by exsanguination. As expected, a Wistar rat mortality between 20 and 30% was observed in the experimental groups due to the RH model. The study was approved by the Faculty of Pharmaceutical Sciences animal review committee and conducted in accordance with NIH guidelines for the care and use of laboratory animals.

Morphology

After sacrifice, the liver was removed from each animal, weighed, and examined grossly on the surface and in cross-sections of 3 mm for the presence of nodular formations of varied sizes and a generally whitish or yellowish color different from the hepatic parenchyma. These nodular liver preneoplastic lesions (PNLs) were classified into two categories according to their diameter: “≤1 mm” and “≥2 mm.”

Representative fragments of each lobe were fixed in Carnoy’s solution (60% methanol, 30% chloroform, and 10% glacial acetic acid; Merck) for approximately 48 h and included in paraffin. Five-micron sections were hematoxylin-eosin (H&E) stained for histopathological examination, and the PNLs were classified according to the criteria established in the literature (29). Moreover, hepatocyte foci were considered PNLs smaller than one hepatic lobule, whereas hepatocyte nodules comprised spherical PNLs larger than one or more hepatic lobules (30).

Glutathione S-Transferase–Positive Immunohistochemistry and Morphometry

The liver sections were processed and submitted to the immunohistochemical reaction using the method of Hsu et al. (31). Basically, after removing the paraffin, the sections were immersed in a 1:10 methanolic hydrogen peroxide solution for 30 min to inactivate the endogenous peroxidase, followed by overnight incubation at 4°C with polyclonal anti-glutathione S-transferase–positive (GST-P) antibody (Pan Vera Corporation, Madison, WI) at a 1:60 dilution in a solution containing 0.5% sodium azide and fraction V bovine serum albumin (5%; Sigma). The sections were then incubated for 30 min with a secondary biotinylated anti-rabbit an-
tibody (Dako, Denmark), followed by streptavidine-biotin-peroxidase complex (Vectastain-ABC kit, Vector) application. The peroxidase binding sites were detected by incubation with 0.5% 3,3′-diaminobenzidine (Sigma) and 0.1% hydrogen peroxide dissolved in phosphate-buffered solution (PBS) for about 2 min at room temperature and counterstaining with hematoxylin.

A microscope (Olympus Optical, Tokyo, Japan) fitted with a video camera and connected to an image analysis system (Bioscan Optimas, Bioscan, Edmonds, WA) was used to quantify the areas of hepatic PNLS (foci/nodes) that were positive for GST-P. For this purpose, after calibration of the system with a Zeiss 2-mm ruler, each lesion was outlined with an electronic pen yielding immediately the area.

LUT and LYC Hepatic Concentrations

Hepatic LUT and LYC concentrations were determined by high-performance liquid chromatography in liver samples previously stored at −78°C, according to the methods described by Arnauld et al. (32) and Barua et al. (33). A Shimadzu LC 9A (Shimadzu, Japan) chromatograph with a multisolvent pumping system, UV-VIS detector, and reverse-phase column (CLC-ODS; 5 μm, 150 × 6 mm ID) was used. The mobile phase consisted of acetonitrile, dichloromethane, and methanol (70:20:10, v:v:v) at 2 ml/min flow. LUT and LYC were detected at 450 nm and identified and quantified, respectively, by comparison with retention time and peak areas of standards whose purity was previously determined by spectrophotometry.

Hepatic DNA Strand Breakage
(Single Cell Gel Electrophoresis, Comet Assay)

Hepatic DNA strand breakage was evaluated in liver samples previously stored at −78°C, using the comet assay essentially as described by Olive et al. (34). The tissues were smoothly homogenized in PBS (2.7 mM KCl, 8.1 mM Na2HPO4, 1.5 mM KH2PO4, and 0.14 M NaCl; pH 8.0), under refrigeration, and filtered. The isolated cells were then immobilized in a low-melting agarose (Sigma) matrix on a glass slide. Thereafter, these cells were lysed in a solution of TBE buffer (90 mM Tris, 90 mM H3BO3, and 2 mM Na2-EDTA, pH 8.4) and 2.5% SDS and submitted to electrophoresis in TBE buffer at 62 V for 2 min. The resulting comets were stained with silver nitrate. Normal rat liver tissues, chal-

 lenged or not with hydrogen peroxide (10% final concentration, 5 min at room temperature, and sonication), were used as positive and negative controls, respectively.

The length of the comets (35) was evaluated using a computerized image analysis system as previously described. One hundred nucleoids per animal were randomly analyzed.

Statistical Analysis

For statistical calculations the INSTAT program (GraphPad Software, version 2.01, ©1990–1992) was used. When indicated Fisher’s exact test was applied. One-way ANOVA, Duncan, and Student’s t-tests were used for results presenting normal distribution, and Kruskall-Wallis and Mann-Whitney’s tests were used when this did not occur. In all cases the level of significance was $P < 0.05$.

Results

Table 1 shows the average body weight, liver weight, and relative liver weight as well as liver LUT and LYC concentrations of the rats of each group at the end of the experiment. Neither body and liver weight nor relative liver weights showed statistically significant differences between the animals treated with LUT, LYC, or only CO (control group) during the initial phases (initiation and selection/promotion) of the RH model of hepatocarcinogenesis, indicating that LUT and LYC do not present a notable toxicity at the used dosages. As expected, compared with the CO-treated animals, administrations of LUT and LYC (70 mg/kg body weight) for 8 consecutive weeks on alternate days significantly increased ($P < 0.05$) the hepatic concentration of each carotenoid in the animals from LUT and LYC groups, respectively.

Table 2 presents the data of incidence, total number per group, average number per nodule-bearing liver (nodule multiplicity) as well as the size distribution of visible hepatocyte nodules. When compared with the controls (CO group), LUT- and LYC-treated rats tended to present a lower incidence, total number, and multiplicity of hepatocyte nodules. However, these differences did not reach statistical significance ($P > 0.05$). Concerning the distribution of hepatocyte nodules relative to size, no statistically significant differences were observed among the groups.

Table 3 shows the values obtained by morphometrical quantification of the number, mean area, and percentage of...

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Animals</th>
<th>Final Body Weight (g)</th>
<th>Liver Weight (g)</th>
<th>Relative Liver Weight (g/100 g body weight)</th>
<th>Lutein (μg/g)</th>
<th>Lycopene (μg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO</td>
<td>8</td>
<td>264 ± 11</td>
<td>12.9 ± 0.5</td>
<td>4.9 ± 0.2</td>
<td>1.0 ± 0.3</td>
<td>nd</td>
</tr>
<tr>
<td>LUT</td>
<td>10</td>
<td>272 ± 5</td>
<td>11.8 ± 0.3</td>
<td>4.4 ± 0.1</td>
<td>28.7 ± 0.2a</td>
<td>nd</td>
</tr>
<tr>
<td>LYC</td>
<td>8</td>
<td>270 ± 6</td>
<td>11.5 ± 0.6</td>
<td>4.3 ± 0.2</td>
<td>2.1 ± 0.3</td>
<td>25.8 ± 0.3b</td>
</tr>
</tbody>
</table>

a: Mean ± SEM; CO, corn oil; LUT, lutein; LYC, lycopene; nd, nondetectable.
b: Statistically significant difference compared with CO group ($P < 0.05$) according to Kruskal-Wallis test followed by Mann-Whitney’s test.
Table 2. Quantification of Visible Hepatocyte Nodules of Rats Treated with CO, LUT, or LYC and Submitted to the RH Model of Hepatocarcinogenesis

<table>
<thead>
<tr>
<th>Group</th>
<th>Rats with Nodules</th>
<th>Incidence of Nodules (%)</th>
<th>Total Number of Nodules</th>
<th>Multiplicity</th>
<th>% of Nodules by Size (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO</td>
<td>7/8</td>
<td>88</td>
<td>258</td>
<td>37 ± 13</td>
<td>86 / 14</td>
</tr>
<tr>
<td>LUT</td>
<td>7/10</td>
<td>70</td>
<td>132</td>
<td>19 ± 10</td>
<td>94 / 6</td>
</tr>
<tr>
<td>LYC</td>
<td>4/8</td>
<td>50</td>
<td>38</td>
<td>10 ± 3</td>
<td>97 / 3</td>
</tr>
</tbody>
</table>

a: Abbreviations are as follows: CO, corn oil (control group); LUT, lutein; LYC, lycopene.
b: Mean number of nodules per animals with nodules. Mean ± SE.
c: Difference not statistically significant compared with CO group (P < 0.05), according to Fisher’s exact test.
d: Difference not statistically significant compared with CO group (P < 0.05), according to Kruskall-Wallis and Mann-Whitney’s tests.

Table 3. Morphometric Analysis of GST-P PNLs of Rats Treated with CO, LUT, or LYC and Submitted to the RH Model of Hepatocarcinogenesis

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Rats</th>
<th>Number of PNLs per cm²</th>
<th>Area of PNL (mm²)</th>
<th>% Area of PNL/Total Liver Section</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO</td>
<td>8</td>
<td>14 ± 5</td>
<td>0.40 ± 0.11</td>
<td>5.0 ± 2.6</td>
</tr>
<tr>
<td>LUT</td>
<td>10</td>
<td>9 ± 7</td>
<td>0.19 ± 0.06</td>
<td>1.5 ± 2.0</td>
</tr>
<tr>
<td>LYC</td>
<td>8</td>
<td>8 ± 8</td>
<td>0.15 ± 0.06</td>
<td>1.0 ± 1.3</td>
</tr>
</tbody>
</table>

a: Abbreviations are as follows: CO, corn oil (control group); GST-P, placental glutathione-S-transferase; LUT, lutein; LYC, lycopene; PNL, preneoplastic lesions (foci/nodules).
b: Mean ± SEM.
c: Statistically significant difference compared to CO group (P < 0.05), according to one-way ANOVA test followed by Duncan’s multiple comparison test.

The histological section area occupied by GST-P PNLs of Wistar rats treated for 8 consecutive weeks with LUT, LYC, or CO (controls) and submitted to the RH model. GST-P is a marker of foci and nodules that can demonstrate more numbers and larger sizes of these putative PNLs than other markers in various rat liver carcinogenesis studies and thus has been believed to be a very useful marker in the quantification of intensity of both initiating and promoting events (36,37). Similarly to the results of the macroscopic analysis, animals treated with LUT or LYC presented a lower number of GST-P PNLs (P < 0.05), which were smaller (P < 0.05) and occupied a smaller area of the liver section (P < 0.05) when compared with the CO group (control group).

Histopathological examination of H&E-stained liver sections revealed the presence of multiple mixed (clear and acidophilic) (17) hepatocyte foci and nodules in the CO group, whereas in the LUT and LYC groups their number was smaller. In addition, in the CO group an intense proliferation of oval cells was observed from the portal space toward the foci. In the case of the LUT and LYC groups, proliferation of these cells was slight.

Table 4. Lengths of “Comets” of Normal Rat Livers Challenged or Not with Hydrogen Peroxide as Well as of Livers of Rats Treated with CO, LUT, or LYC and Submitted to the RH Model of Hepatocarcinogenesis

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Rats</th>
<th>Length of Comets (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>8</td>
<td>84.9 ± 3.0</td>
</tr>
<tr>
<td>N(HP)</td>
<td>8</td>
<td>200.9 ± 11.9</td>
</tr>
<tr>
<td>CO</td>
<td>8</td>
<td>147.4 ± 9.0</td>
</tr>
<tr>
<td>LUT</td>
<td>10</td>
<td>94.7 ± 5.3</td>
</tr>
<tr>
<td>LYC</td>
<td>8</td>
<td>101.2 ± 5.9</td>
</tr>
</tbody>
</table>

a: Mean ± SEM.
b: Positive comet assay controls.
c: Negative comet assay controls.
d: Abbreviations are as follows: CO, corn oil (control group); LUT, lutein; LYC, lycopene; N, normal group; N(HP), normal group livers challenged with hydrogen peroxide.
e: Statistically significant difference compared with the N group (P < 0.05) according to one-way ANOVA test followed by Student’s t-test.
f: Statistically significant difference compared with the CO group (P < 0.05) according to one-way ANOVA test followed by Student’s t-test.

LUT- and LYC-treated animals presented smaller comets (P < 0.05) than those observed in the CO group animals. Figure 1 illustrates examples of observed comets.

Discussion

LUT and LYC are two non–vitamin A active carotenoids present in many fruits and vegetables as well as in human serum, where they are abundant. LUT and LYC possess excep-
tionally high antioxidant activity compared with other carotenoids and may therefore be useful in chemoprevention of cancer (10).

In Western countries the estimated daily intake of LUT and LYC ranges between 0.57 and 4.40 mg and 0.55 and 9.38 mg, respectively (38,39). Compared with normal human intake, LUT and LYC dosages (70 mg/kg body weight on alternate days) used in the present study can be considered supranutritional.

Information on LUT and LYC absorption, metabolism, and tissular distribution as well as possible mechanisms of their anticarcinogenic action is scarce (4,9,40). To further study these issues, research evaluating, for example, different carotenoid doses and concentration in the different tissues is needed (41).

Therefore, we decided to also evaluate hepatic LUT and LYC concentrations in the animals submitted to the RH model and treated with the carotenoids (LUT and LYC groups) or with only CO (control group). The increases of LUT and LYC hepatic concentrations observed in the animals of LUT and LYC groups, respectively, are in agreement with previous studies in which supplementation with LUT (40) or LYC (42) in the diet of normal rats, in concentrations similar to those used in our study, resulted in effective carotenoid absorption and deposition in the liver of the animals. Also liver LUT and LYC concentrations in this study reached levels seen in human liver samples (9,43). These data are in agreement with the suggestion that, because rats absorb carotenoids less efficiently than humans, it is necessary to determine the amount of dietary carotenoids required to achieve tissue concentrations similar to those of humans (42).

There are several models for the study of some aspects of liver cancer development with chemicals. In many of these models the growth of the hepatocytes in foci is very slow and generally asynchronous, taking many weeks or even a few months to form visible hepatocyte nodules. In the RH model this is accomplished in a rapid and synchronous fashion so that nodules are seen grossly within a week or 10 days after the selection pressure is applied. The RH model is based on the concept that 2-AAF generates a differential mitoinhibitory effect on the noninitiated hepatocytes while permitting the initiated hepatocytes (the resistant ones) to respond to liver cell–proliferative stimulus such as that induced by PH (22). Among the various protocols of rat hepatocarcinogenesis, the RH model is well characterized and is particularly adapted to compare effects of compounds potentially able to modulate an ongoing carcinogenic process (44).

In our study we evaluated the chemopreventive activities of LUT and LYC administered to Wistar rats for 8 consecutive weeks in a period comprising the initiation and selection/promotion phases of the RH model of hepatocarcinogenesis.

Although no statistically significant differences were observed among groups, treatment with LUT or LYC tended to reduce the incidence, total number, and multiplicity of visible hepatocyte nodules. In addition, animals treated with LUT or LYC presented a lower number of hepatic GST-P PNLs (foci/nodules), which were smaller and occupied a smaller area of the liver section.

There is a large body of evidence demonstrating that hepatocarcinogenesis is a multistage process in which the emergence of hepatocellular carcinoma in rats, and possibly in humans, is preceded by the appearance of foci and nodules of hepatocytes in the liver (45). Together our results indicate a chemopreventive activity of LUT and LYC when administered to Wistar rats during the initiation and selection/promotion phases of the RH model.

The size of GST-P PNLs in rats is generally described as being a reflex of the magnitude of hepatocarcinogenesis pro-
motion, and their number relates to the intensity of the initiation process (46). That the number and size of these lesions were smaller in the LUT and LYC groups when compared with the CO group suggests that the protective effects of these carotenoids observed in this study may have occurred during both the initiation and promotion phases.

To our knowledge, these are the first reports on specifically inhibitory LUT effects on hepatocarcinogenesis. Antimutagenic LUT effects (47) would play an important role in the initial phases of the carcinogenic process. On the other hand, it is described that promoting agents act by stimulating cell proliferation and inhibiting apoptosis so, that in the end, initiated cells would present a higher tendency to progress to a neoplasia (48). The ability of LUT to stimulate apoptosis specifically in transformed mammary and not in normal cells (5) in this case seems to be a relevant feature of its anticarcinogenic action in the postinitiation period. It was also observed that LUT administration to mice previously initiated with 1,2-dimethylhydrazine resulted in inhibitory effects on the size and number of aberrant crypts that, according to the authors of the study (49), were associated with inhibition of cell proliferation.

In the case of LYC, former studies in which the carotenoid was tested in animals submitted to different hepatocarcinogenesis models presented different results. Similarly to what was observed in the present investigation, one study showed anticarcinogenic activity in rats submitted to the RH model when LYC (5% LYC oleoresin from tomato) was administered in a semiliquid purified diet (0.03% LYC final concentration) at the initiation phase (18). On the other hand, in a model of spontaneous liver cancer, where Long-Evans Cinnamon rats were chronically fed a prepared diet containing 0.005% (wt/wt) LYC (LYC-enriched tomato oleoresin containing 13% LYC), no inhibitory effects were observed both on initiation and promotion, so that hepatocellular carcinoma incidence was equal both in LYC-treated and control animals. In this case, the carotenoid's antioxidant ability is believed to have not been sufficient to result in inhibitory effects on hepatocarcinogenesis (19).

Oxidative stress is described to play an important role in the process of hepatocarcinogenesis (50). The occurrence of intense lipid peroxidation has been observed already in the initial phases of the process (51), which could be associated with superoxide dismutase depletion (52). In addition, in DEN-induced rat hepatomas a general collapse of the enzymatic antioxidant system was observed (53). Because they are able to damage important biomolecules such as DNA, ROS may cause mutations in important genes, such as tumor suppressor and/or proto-oncogenes, and thus stimulate the formation of neoplasms (25). The importance of oxidative damage to DNA in hepatocarcinogenesis should be emphasized (54).

On the other hand, although it is believed that the high antioxidant potential of LUT and LYC would be responsible for a great part of their anticarcinogenic actions, there are surprisingly few studies evaluating the ability of these substances to inhibit DNA damage (55). It has been described that in humans the consumption of tomato products may reduce the susceptibility of lymphocyte DNA to oxidative damage, as measured by the comet assay (56).

With the purpose of verifying if the chemopreventive activities observed with LUT and LYC in this study would be eventually related to inhibition of DNA instability, we decided here to use the comet assay. This method is able to evaluate breaks in the DNA strands and oxidized bases due to ROS action. This assay has been used to evaluate DNA strand breakage, considered to be a biomarker of the oxidative status. In vivo damage (that is, damage arising from endogenous causes, such as the ROS released from mitochondria during normal respiration, modulated by endogenous and dietary antioxidants) can be measured, or lymphocytes, for example, can be treated ex vivo with a DNA-damaging agent to examine the ability of the cells to resist attack. Studies using the comet assay have provided important information about antioxidant carotenoid effects (26,56).

In this study, we opted for staining the formed comets with silver nitrate because of the advantages of the method, for example, to allow the permanent record of the experiment and independent verification of the results as well as to avoid problems associated with fluorescence such as decay. In addition, staining with silver allows the comets to be analyzed using a simple light microscope instead of expensive and complex equipment such as fluorescence microscopes (57).

That in the present study CO group animals presented comets with increased lengths compared with those observed in the N group (normal rats) indicates that DNA strand breakage seems to constitute an early event during hepatocarcinogenesis, which could play an important role in hepatic preneoplastic foci development by triggering an imbalance between high cell proliferation and relatively low apoptosis in the liver (58). Also, LUT- and LYC-treated animals presented smaller comets than those observed in the CO group animals, indicating that the carotenoids were able to inhibit DNA strand breakage when administrated to Wistar rats submitted to the RH model. In a study where healthy individuals received supplementation with tomato and carrot juice as well as spinach powder, a reduction in lymphocyte DNA damage was observed as evaluated by the comet assay. According to the authors (27), these data suggest that the protective effect against cancer of foods rich in carotenoids, such as LUT, LYC, and β-carotene, would involve reduction in oxidative DNA damage. However, in a study where individuals received specific LUT and LYC supplementation, no significant effect was observed regarding oxidative lymphocyte DNA damage (59). Considering the lack of evidence about a correlation between oxidative DNA status of lymphocytes and that of tissues, the need for the evaluation of DNA damage specifically in tissues at risk for cancer development was recently emphasized (60).

That we evaluated DNA strand breakage in the livers of animals and also that we observed an increase in liver LUT and LYC levels after supplementation with these carotenoids suggest that both LUT and LYC effectively protected against hepatic DNA instability and inhibited the development of PNLs. Accumulation of LYC and LUT in the liver of animals
constitutes an important aspect related to the observed protecting actions considering that there are descriptions that hepatic concentrations of these carotenoids are quite reduced in patients with cirrhosis (61), which is thought to be a risk for the development of hepatocarcinoma (62).

The data of the present study show that oral administration of LUT or LYC inhibited GST-P PNLs and DNA strand breakage in Wistar rats during hepatocarcinogenesis. These results provide support for current epidemiological and experimental evidence that LUT and LYC act as effective cancer chemopreventive agents. Moreover, they suggest that a protection against DNA instability could play a role in carotenoid chemopreventive action.

Acknowledgments and Notes

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