Mixed Tocopherols Inhibit N-methyl-N-Nitrosourea-Induced Mammary Tumor Growth in Rats

Nanjoo Suh, Shoby Paul, Hong Jin Lee, Yan Ji, Mao-Jung Lee, Chung S. Yang, Bandaru S. Reddy, and Harold L. Newmark

Abstract: Tocopherols are present in significant amounts in vegetable oils used in human foods. The most prevalent tocopherols in foods are the α, β, γ, and δ variants with (RRR) stereochemistry. Tocopherols are lipophilic phenolic antioxidants, produced by plants. In the United States, γ-tocopherol is the most prominent dietary tocopherol due to its high amount in the dominant commercially produced vegetable oils such as soybean, corn, and cottonseed. In this report, experiments were designed to study the inhibitory effect of mixed tocopherols against N-methyl-N-nitrosourea-induced mammary tumor growth in female Sprague-Dawley rats. Beginning at 21 days of age, rats were treated with a single intraperitoneal injection of 50 mg/kg body weight of N-methyl-N-nitrosourea. One wk later, the rats were fed experimental diets containing 0 or 0.1% mixed tocopherols containing over 50% γ-tocopherol. At 9 wk after N-methyl-N-nitrosourea treatment, all rats were evaluated for inhibition of mammary tumor growth and proliferating cell nuclear antigen. Dietary administration of mixed tocopherols significantly suppressed mammary tumor growth (P < 0.05) and proliferating cell nuclear antigen (P < 0.01) and also moderately suppressed tumor multiplicity. The treatment increased the serum levels of γ- and δ-tocopherols without affecting the body weight. The results of this study suggest that mixed tocopherols may be safe and effective agents for the prevention of breast cancer.

Introduction

Tocopherols are an important group of lipophilic phenolic antioxidants produced by plants as radical scavengers (i.e., chain-breaking antioxidants) and therefore present in significant amounts in vegetable oils used in human foods. The most important tocopherols in foods are the α, β, γ, and δ variants, which are natural (RRR) tocopherols differing only in the number and location of the methyl groups on the phenol ring (part of the total chromanol ring system). In the United States, α-tocopherol and particularly γ-tocopherol are the most prominent dietary tocopherols due to their higher amounts in the dominant commercially produced vegetable oils such as soybean, corn, and cottonseed (1).

From a chemical viewpoint, α-tocopherol, with methyl groups in both positions adjacent to the phenolic group, is a hindered phenol, that is poorly available for reaction, and it is thus a weaker antioxidant than γ-tocopherol with one less methyl group (Figure 1), when tested as an antioxidant in vitro. The antioxidant activity of the tocopherols differ from their vitamin E activity as a fertility maintenance agent in rodents, where γ-tocopherol has about one-tenth the apparent biological activity of α-tocopherol (1,2) but is known as a far more effective anti-oxidant when tested in in vitro studies.

In many epidemiology studies, dietary intake of total (mixed) tocopherols in food has been assumed to be “vitamin E.” The few intervention studies with pure α-tocopherol or its more stable acetate ester have largely proved negative in protection against cardiovascular disease or cancer in humans (3). In contrast, a nested case-control study of serum levels of antioxidant nutrients and prostate cancer risk over a 15-yr period indicated a strong inverse dose-response association with γ-tocopherol and prostate cancer risk but no significant trend for α-tocopherol (vitamin E) (4).

Several reports indicate that γ-tocopherol is an effective inhibitor of cyclo-oxygenase (COX) enzyme transcription, proinflammatory eicosanoid formation, and inflammation damage (5–8), but α-tocopherol was relatively inactive in these studies. Although an extensive literature has been published on the potential health benefits of α-tocopherol, little is known about γ-tocopherol. γ-Tocopherol has recently received more research attention based on findings from in vitro and animal studies indicating that it has potent antiinflammatory and antioxidant properties (9). The antiinflammatory and COX enzyme inhibitory activity suggested to us that γ-tocopherol may be an effective agent for inhibition of cancer.

In a previous study, we used readily available mixed tocopherols (containing over 50% γ-tocopherol) added at 0.1%
to AIN-76A standardized semipurified laboratory diet to produce a significant inhibition of azoxymethane-induced aberrant crypt foci (ACF) in the colon of rats, a recognized early biomarker of colon cancer risk (10). We now report that mixed tocopherols also inhibit mammary tumor growth in rats.

Materials and Methods

Diets

Semipurified modified AIN-76A diet was obtained from Research Diets Laboratory (New Brunswick, NJ) and used unchanged as the control diet. The test diet was prepared by adding 1,000 ppm (0.1%) of mixed tocopherols to AIN-76A diet. The mixed tocopherols were supplied by the Cognis Corporation (Kankakee, IL), and contained about 58% \( \gamma \)-tocopherol, 21% \( \delta \)-tocopherol, 12% \( \alpha \)-tocopherol, and insignificant \( \beta \)-tocopherol (about 1.5%). The mixed tocopherols used are commercially available, produced as a byproduct in the refining of edible vegetable oils (e.g., deodorization processes etc.). Pure \( \gamma \)-tocopherol is available from laboratory supply sources only at very high cost, is very sensitive to oxidation on handling, and at present is not available in quantities necessary for laboratory rodent dietary studies. The diets were stored in sealed containers at 4°C, and the food cups were replenished with fresh diets twice weekly.

In Table 1, estimates are given of the amounts of the 4 tocopherols in each feed as prepared in standardized AIN-76A, from the corn oil, the AIN-76A vitamin mix (control diet), and the effect of adding 0.1% of the mixed tocopherols. The \( \gamma \)-tocopherol increases about 20-fold, whereas the \( \alpha \)-tocopherol (vitamin E) only increases about threefold. The \( \delta \)-tocopherol is magnified over 100-fold, but little is currently known of its effect in COX enzymes or antiinflammatory activity except in some cell culture studies (11–13). At this time, pure or highly concentrated \( \delta \)-tocopherol is not available in sufficient quantities to perform rodent dietary studies. However, we are pursuing potential sources to obtain some pure or concentrated \( \gamma \)-tocopherol to perform such studies.

Animals and Experimental Procedure

Female Sprague-Dawley rats were obtained from Taconic Farms (Germantown, NY). Rats (21 ± 1 days old) were treated with a single intraperitoneal injection of the carcinogen N-methyl-N-nitrosourea (NMU) (50 mg/kg body weight). One week after N-methyl-N-nitrosourea injection, rats were fed AIN-76A control diet or AIN-76A diet containing mixed tocopherols (0.1% of the diet). Tumors were palpated weekly. Nine weeks after N-methyl-N-nitrosourea injection, the rats were sacrificed and tumors were counted at autopsy. All animal studies were performed in accordance with an institutionally approved protocol.

Immunohistochemistry

Mammary tumors from each group were harvested at autopsy and fixed in 10% formalin for 24 h. They were sectioned into segments, paraffin embedded, and cut into 4-\( \mu \)m-thick tissue sections. The slides were incubated overnight at room temperature with antibody to proliferating cell nuclear antigen (PCNA; 1:1,000 diluted, BD Pharmin Gen, San Diego, CA). The slides were incubated with biotinylated secondary antibody and then with avidin/biotinylated

Table 1. Estimated Tocopherols in AIN-76A Feed and With Added 0.1% Mixed Tocopherols (mg/kg Feed)

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet amount (%)</th>
<th>( \alpha )</th>
<th>( \beta )</th>
<th>( \gamma )</th>
<th>( \delta )</th>
<th>Type of ( \alpha )-tocopherol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn oil( ^a )</td>
<td>5</td>
<td>5.6</td>
<td>0.25</td>
<td>30.1</td>
<td>0.9</td>
<td>RRR (0.67 mg = 1 I.U.)( ^d )</td>
</tr>
<tr>
<td>AIN-76A vitamin mix( ^b,c )</td>
<td>1</td>
<td>50</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>All rac ( \alpha )-tocopherol acetate (1.0 mg = 1 I.U.)( ^d )</td>
</tr>
<tr>
<td>Total AIN-76A diet</td>
<td>—</td>
<td>55.6</td>
<td>0.25</td>
<td>30.1</td>
<td>0.9</td>
<td>Tocopherols in control AIN-76A feed</td>
</tr>
<tr>
<td>0.1% mixed tocopherols (1 gram)</td>
<td>0.1</td>
<td>120</td>
<td>15</td>
<td>580</td>
<td>210</td>
<td>Tocopherols in AIN-76A diet with added 0.1% mixed tocopherols (e.g., test diet)</td>
</tr>
<tr>
<td>Total AIN-76A plus 0.1% mixed Tocopherols</td>
<td>—</td>
<td>175.6</td>
<td>15.25</td>
<td>610.1</td>
<td>210.9</td>
<td>Tocopherols in AIN-76A diet with added 0.1% mixed tocopherols (e.g., test diet)</td>
</tr>
</tbody>
</table>


\( ^d \): Type of \( \alpha \)-tocopherol: RRR (0.67 mg = 1 I.U.), All rac.
peroxidase complex for 30 min at room temperature (Vector Labs, Burlingame, CA). The slides were then incubated with 3'-diaminobenzamine substrate, and the sections were counterstained with Modified Harris hematoxylin. The images were taken randomly at ×400 using a Zeiss AxiosCam HRc camera fitted to a Zeiss Axioskope 2 Plus microscope (Thornwood, NY). A positive reaction is noted by a reddish-brown precipitate in the nucleus for PCNA.

Analysis of Tocopherol Levels in Rat Serum

Rat serum was collected at autopsy, and serum tocopherol levels (α-, δ-, or γ-tocopherol) were measured by a method modified from a previously described procedure (14). In brief, fat-soluble vitamins were extracted from 150 µl of plasma with ethanol and hexane and then dissolved in a mixture of ethanol and acetonitrile. An high-performance liquid chromatography system was developed using a Supelcosil LC18 column, 5 µm (4.6 × 150 mm; Bellefonte, PA) and ethanol:acetonitrile (45:55) as the mobile phase. A Waters 490 multiwavelength detector (Waters-Millipore, Milford, MA) was used to detect absorbance at 292 nm (α-, γ-, δ-tocopherol) and 325 nm (retinol). Reference samples of pure α-, γ- and δ-tocopherol as well as retinol were obtained from the Centers for Disease Control and Prevention (Atlanta, GA). The structures of α-, β-, γ-, and δ-tocopherol are shown in Fig. 1.

Statistical Analysis

Statistical significance for average tumor burden and average body weight was evaluated using the Student’s t-test. The PCNA labeling index (PI) was calculated as the [(number of positive cells)/(total number of cells)] × 100 for each field, which is averaged to get the PI for each section. The significance of treatment between the groups was analyzed by the Student’s t-test.

Results

Efficacy of Mixed Tocopherols on Mammary Tumorigenesis and on Body Weights in Rats

Body weights of animals fed the experimental diet containing 0.1% mixed tocopherols were comparable to those fed the control diet throughout the study, indicating that the dose of 0.1% mixed tocopherols used did not cause any overt toxicity. There was no significant difference in body weights at the end of the 9-wk experimental period (Table 2). The average tumor burden (tumor weight) per rat was reduced by about 44% (P < 0.05) by 0.1% mixed tocopherols in the AIN-76A diet during the 9-wk test feeding period (Table 2). Administration of the mixed tocopherols reduced tumor multiplicity per rat by 42%, but the change was not statistically significant (Table 2).

PCNA Staining of Mammary Tumors and Cell Counting

The PCNA was evaluated as a marker for cell proliferation in mammary tumors. Three independent mammary tumors from the control group or the mixed-tocopherols-fed group are shown (Fig. 2). The PCNA staining of the mammary tumors was much stronger in N-methyl-N-nitrosourea-treated rats fed the control diet (Fig. 2A) than in the mixed tocopherol-fed group (Fig. 2B). The PCNA labeling index is also shown in Fig. 2. The percentage of PCNA positive cells in the mammary tumors in the control group were 67.3% ± 4.6%, whereas the percentage of PCNA positive cells in tumors from the mixed-tocopherol-fed group was 44.3% ± 4.8%. The PCNA labeling indexes in the 2 groups were significantly different (P < 0.01).

Analysis of Mixed Tocopherols in the Serum From Rats

We fed AIN-76A control diet or 0.1% mixed tocopherols in AIN-76A diet throughout the 9-wk study. To determine whether the tocopherol diets were bioavailable in experimental animals, we collected blood samples at autopsy and determined the serum levels of retinol and α-, γ- and δ-tocopherol (Table 3). Serum levels of retinol and α-tocopherol in rats fed with 0.1% mixed tocopherols were comparable to those fed the control diet. However, rats fed with 0.1% mixed tocopherols had much higher levels of both γ-tocopherol and δ-tocopherol than the control group (Table 3). The level of δ-tocopherol was not detectable in the control group fed the AIN-76A diet. The serum level of β-tocopherol was very low in the control group and did not change in the group fed 0.1% mixed tocopherols (data not shown) because the mixed tocopherols only contained a negligible amount of β-tocopherol.

Table 2. Mixed Tocopherols Inhibit Mammary Tumorigenesis in Sprague-Dawley Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Average Body Weight (g)</th>
<th>Average Tumor Burden (g)</th>
<th>Average No. of Tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIN-76A control</td>
<td>223 ± 7.6</td>
<td>15.6 ± 2.2</td>
<td>4.2 ± 0.4</td>
</tr>
<tr>
<td>AIN-76A plus 0.1% mixed toco</td>
<td>223 ± 3.9</td>
<td>8.8 ± 3.1d</td>
<td>2.9 ± 0.5</td>
</tr>
</tbody>
</table>

a: All rats (21 ± 1 days old) were given an ip injection of 50 mg N-methyl-N-nitrosourea per kilogram body weight 1 wk before starting the feeding of mixed tocopherols. Rats were autopsied 9 wk after injection of NMU.
b: Mean ± standard error of measurement (n = 12).
c: Average tumor burden; average weight in grams of all tumors in each rat at autopsy.
d: Significantly different from control by the Student’s t-test.

References


Figure 2. Proliferating cell nuclear antigen (PCNA) staining of mammary tumors and cell counting. Three representative sections of mammary tumor samples from the control group (A) or mixed tocopherol-fed group (B) are shown. The PCNA positive cells are stained brown in the nucleus, whereas the negative cells, having taken the hematoyxin stain, are blue colored. Independent sections of the mammary tumors from 3 rats were stained, and approximately 800 cells were counted from each section. The PCNA labeling index (PI) was calculated as the [(number of positive cells)/(total number of epithelial cells)] × 100 for each field. These PI values for the different mammary tumors from the animals belonging to same group were then averaged. Statistical significance of treatment between the groups was analyzed by the Student's t-test (*, P < 0.01).

Discussion

This study was part of an ongoing investigation of the potential antitumor effects of γ-tocopherol, a common component of the US human diet derived largely from vegetable oils. In this study, commercially available mixed tocopherols containing over 50% γ-tocopherol as well as smaller amounts of α- and δ-tocopherols fed at 0.1% of the diet to rats resulted in inhibition of mammary tumorigenesis in an animal model of breast cancer. We suggest that the γ-tocopherol is the major acting antitumorigenic agent, or the combination of tocopherols synergistically inhibit mammary tumorigenesis. The 0.1% addition of mixed tocopherols to AIN-76A diet, which significantly reduced mammary tumor growth, also reduced multiplicity (Table 2) but not significantly. A possibly explanation may be the dose level (0.1%) of mixed tocopherols used. Actually, this dose level was adopted from our previous study in reduction of colon ACF in rats (10).

Table 3. Analysis of Tocopherol Levels in the Serum From Sprague-Dawley Female Ratsa

<table>
<thead>
<tr>
<th>Group</th>
<th>Retinol (µg/dL)b</th>
<th>α-Tocopherol (µg/dL)b</th>
<th>δ-Tocopherol (µg/dL)b</th>
<th>γ-Tocopherol (µg/dL)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIN-76A control</td>
<td>34.3 ± 1.9</td>
<td>1482 ± 97</td>
<td>0.0 ± 0.0</td>
<td>39.2 ± 3.6</td>
</tr>
<tr>
<td>AIN-76A plus 0.1% mixed tocopherols</td>
<td>34.6 ± 1.5</td>
<td>1663 ± 63</td>
<td>68.5 ± 4.9</td>
<td>146.5 ± 10.2c</td>
</tr>
</tbody>
</table>

a: All rats (21 ± 1 days old) were given an ip injection of 50 mg N-methyl-N-nitrosourea per kilogram body weight 1 wk before starting the feeding of mixed tocopherols. Rats were fed with control or mixed tocopherol containing diet for 9 wk. Serum samples were collected at autopsy.
b: Mean ± standard error of the measurement (n = 12).
c: Significantly different from control by the Student’s t-test, P < 0.001.
The high safety of the dietary mixed tocopherols suggests that we pursue higher levels added to the AIN-76A feed in future studies, which are currently being planned.

Several nonsteroidal antiinflammatory drugs (NSAIDS) have demonstrated significant tumor inhibitory activity in human epidemiology studies (15,16), in a placebo controlled human colon polyp recurrence study (17), and in several laboratory animal studies (18–20). NSAIDS function largely by inhibiting COX enzymes whose activities produce the active inflammatory agents (e.g., prostaglandins, etc.). γ-Tocopherol is a dietary inhibitor of COX enzymes (5–8) and thus also active as an antiinflammatory agent (7,8,10). This report and our previous report (10) serve to establish a dietary source of γ-tocopherol as a potentially useful and possibly safer COX enzyme inhibitor, at low levels of intake, as a cancer chemopreventive agent.

Gould and colleagues (21) reported earlier on the chemopreventive effects of 2 forms of vitamin E, α-tocopherol and tocotrienol, in 2 chemically induced rat mammary-tumor models (21). When mammary tumors were induced by 7,12-dimethylbenz(a)anthracene, only tocotrienol treatment significantly increased tumor latency. When tumors were induced by N-methyl-N-nitrosourea, neither analog of vitamin E modified latency nor mammary tumor growth (21). In our study, the mixed tocopherols inhibited mammary tumor growth and perhaps tumorigenesis when induced by N-methyl-N-nitrosourea, neither analog of vitamin E modified latency nor mammary tumor growth (21). Our results, together with those by Gould and his colleagues (21), suggest that γ-tocopherol and/or δ-tocopherol, not α-tocopherol, may have chemopreventive activity against mammary tumorigenesis.

Other recent studies indicate that the α-tocopherol derivatives α-tocopheryl succinate and α-tocopheryloxyacetic acid, but not α-tocopherol, suppressed primary tumor growth and dramatically reduced spontaneous metastatic spread to the lung in prophylactic and therapeutic settings (22–24). Using MDA-MB-435-FL-GFP human breast cancer xenografts in immunodeficient mice, liposomal-formulated α-tocopheryloxyacetic acid administered as an aerosol and celecoxib fed at 500 or 1,250 mg/kg diet significantly reduced tumor volume and lung metastases (25). Although many studies with α-tocopherol derivatives have been reported (26–28), our study reports for the first time the efficacy of a mixture of natural dietary tocopherols, mainly γ-tocopherol, for inhibition of mammary tumor growth and tumorigenesis.

Our findings represent a rational extension of the antiinflammatory and COX enzyme modulation by γ-tocopherol previously discussed (5–8). A review of γ-tocopherol in biology and medicine (29) suggests several possible mechanisms for its antitumor activity. Further studies are needed to properly establish γ-tocopherol alone or in combination as a useful tumor inhibitor as well as to determine the effects of selected types of mixed tocopherols as a practical and economic dietary source with antitumor activity. There is also a need to properly ascertain the activity and mechanisms of action of γ-tocopherol.

A recent publication (30) of a study using mixed tocopherols (500 mg mixed tocopherols containing 60% γ-tocopherol) in type 2 diabetic human subjects showed analogous high levels of serum γ-tocopherol similar to what is shown in Table 3. The dose of mixed tocopherols used (500 mg per day) is approximately 0.1% of a 2,000 kcal daily diet, so a rough correlation of control and supplemented (or fortified) serum levels in humans and in rats in this study are strongly suggested. On the basis of data presented here, we believe that mixed tocopherols, particularly γ-tocopherol, may have potential chemopreventive activity against breast cancer.

Acknowledgments and Notes

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