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## $\delta$ - and $\gamma$ -Tocopherols, but not $\alpha$ -Tocopherol, Inhibit Colon Carcinogenesis in Azoxymethane-Treated F344 Rats

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### Abstract

The cancer preventive activity of vitamin E has been extensively discussed, but the activities of specific forms of tocopherols have not received sufficient attention. Herein, we compared the activities of  $\delta$ -tocopherol ( $\delta$ -T),  $\gamma$ -T and  $\alpha$ -T in a colon carcinogenesis model. Male F344 rats, 7 weeks old, were given 2 weekly subcutaneous injections of azoxymethane (AOM) each at a dose of 15 mg/kg body weight. Starting 1 week before the AOM injection, the animals were maintained on a modified AIN76A diet, or the same diet containing 0.2% of  $\delta$ -T,  $\gamma$ -T,  $\alpha$ -T or a  $\gamma$ -T-rich mixture of tocopherols ( $\gamma$ -TmT), until the termination of the experiment at 8 weeks after the second AOM injection.  $\delta$ -T treatment showed the strongest inhibitory effect, decreasing the numbers of aberrant crypt foci by 62%.  $\gamma$ -T and  $\gamma$ -TmT were also effective, but  $\alpha$ -T was not. Immunohistochemical analysis showed that  $\delta$ -T and  $\gamma$ -T treatments reduced the levels of 4-hydroxynonenal and nitrotyrosine and the expression of cyclin D1 in the colon, preserved the expression of PPAR- $\gamma$ , and decreased the serum levels of prostaglandin E2 and 8-isoprostane. Supplementation with 0.2%  $\delta$ -T,  $\gamma$ -T or  $\alpha$ -T increased the respective levels of tocopherols and their side-chain degradation metabolites in the serum and colon tissues. Rather high concentrations of  $\delta$ -T and  $\gamma$ -T and their metabolites were found in colon tissues. Our study provides the first evidence for the much higher cancer preventive activity of  $\delta$ -T and  $\gamma$ -T than  $\alpha$ -T in a chemically-induced colon carcinogenesis model. It further suggests that  $\delta$ -T is more effective than  $\gamma$ -T.

### Keywords

Tocopherols; tocopherol metabolites; colon carcinogenesis; ACF

### Introduction

Tocopherols, the major forms of vitamin E, contain a chromanol ring system and a phytyl chain of 16 carbons. Depending on the number and position of the methyl groups on the ring, tocopherols exist as  $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\delta$ -tocopherols ( $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\delta$ -T, respectively)

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(Fig. 1). Because of their activity in the trapping of reactive oxygen and nitrogen species (RONS), tocopherols are important biological antioxidants. Among the 4 tocopherols,  $\gamma$ -T is most abundant in the human diet (1), but  $\alpha$ -T is the major form of vitamin E found in blood and tissues. Because of the higher levels of  $\alpha$ -T in the body and its superior activity over other tocopherols in the classic fertility restoration assay,  $\alpha$ -T has been regarded as “the” vitamin E, and most studies on vitamin E have focused on  $\alpha$ -T.

The effects of tocopherols on human cancers have been investigated in many studies, and the results are generally inconsistent (reviewed in (2)). Whereas many studies have shown an inverse association between vitamin E intake and cancer risk (2), some large-scale human trials with high doses of  $\alpha$ -T, such as the Selenium and Vitamin E Cancer Prevention Trial (SELECT), failed to demonstrate the expected cancer preventive effect (3). One possible explanation for the negative results is that supplementation of a nutrient to individuals who already have adequate intake may not produce any beneficial effect. Another interpretation is that  $\gamma$ - and  $\delta$ -tocopherols in our diet, rather than  $\alpha$ -T, lower the cancer risk. Many studies have demonstrated that  $\gamma$ -T is more effective than  $\alpha$ -T in trapping reactive nitrogen species, in generating anti-inflammatory metabolites from arachidonic acid, and in inhibiting cancer cell growth and inducing apoptosis (4–12). In addition, high doses of  $\alpha$ -T have been shown to decrease the blood and tissue levels of  $\gamma$ -T (3, 13–15).

There have been a large number of animal studies on the cancer preventive effects of  $\alpha$ -T, and the results are inconsistent (2). For example, among 10 studies on the effect of  $\alpha$ -T and its synthetic analogs on colon tumorigenesis and aberrant crypt foci (ACF) formation, only 1 showed a preventive effect, 1 showed an enhancement effect, and 8 showed no effect (2). On the other hand, we and others have recently shown the inhibition of colon, mammary, lung and prostate carcinogenesis by a  $\gamma$ -T-rich mixture of tocopherols ( $\gamma$ -TmT) (12, 16–19).  $\gamma$ -TmT is a by-product in the refining of vegetable oil and contains 57%  $\gamma$ -T, 24%  $\delta$ -T, 13%  $\alpha$ -T, and 1.5%  $\beta$ -T. Because of the higher level of  $\gamma$ -T in  $\gamma$ -TmT, it is possible that  $\gamma$ -T contributes to most of the cancer preventive activity; however,  $\delta$ -T may also make an important contribution. Recently, our research group showed that 0.17 or 0.3%  $\delta$ -T in the diet significantly inhibited lung xenograft tumor growth in mice, and the activity of  $\delta$ -T was higher than that of  $\gamma$ -T; whereas  $\alpha$ -T was ineffective (20). Nevertheless, the cancer preventive activity of  $\delta$ -T remains to be determined in an animal carcinogenesis model.

In the present work, we investigated the inhibitory activities of  $\delta$ -T,  $\gamma$ -T,  $\alpha$ -T, and  $\gamma$ -TmT on the formation of ACF in azoxymethane (AOM)-treated F344 rats. ACF were first reported by Bird and colleagues in AOM-treated mice as pre-cancerous lesions in the colon (21). In humans, ACF are commonly observed in familial adenomatous polyposis patients and in sporadic colorectal cancer patients (22, 23). ACF have since been accepted as pre-neoplastic lesions of the colon. Newmark *et al.* have shown the inhibition of AOM-induced ACF in rats by 0.1%  $\gamma$ -TmT (16). In this model, 8 weeks after the AOM injection, all the rats developed a large number of ACF. The large number of ACF in this model provides the statistical power needed for multiple group comparisons on the relative inhibitory activities of different forms of tocopherols. Our work provides the first evidence for the higher cancer preventive activity of  $\delta$ -T than  $\gamma$ -T, and the lack of activity of  $\alpha$ -T, in a chemically induced-model for colon carcinogenesis.

## Materials and Methods

### Tocopherols and other chemicals

$\gamma$ -TmT, containing 57%  $\gamma$ -T, 24%  $\delta$ -T, 13%  $\alpha$ -T, and 1.5%  $\beta$ -T, was from Cognis Corporation.  $\gamma$ -T was purified from  $\gamma$ -TmT using an automated flash chromatographic system (Teledyne ISCO CombiFlash<sup>®</sup> Companion<sup>®</sup> XL) with RediSep Rf Gold high

performance flash silica gel column (20–40  $\mu\text{M}$  in particle size) to 97% purity, with no detectable  $\alpha$ -T and  $\delta$ -T. d- $\alpha$ -forms of  $\delta$ -tocopherol (with 94%  $\delta$ -T, 5.5%  $\gamma$ -T, and 0.5%  $\alpha$ -T) and  $\alpha$ -tocopherol (with 69.7%  $\alpha$ -T, 2.6%  $\gamma$ -T and 0.2%  $\delta$ -T) were from Sigma-Aldrich. Other reagents were of the highest grade available commercially.

### Treatment of animals

Animal experiments were conducted under protocol no. 02–027 approved by the Institutional Animal Care and Use Committee at Rutgers University. Male F344 rats were purchased from Charles River Laboratories. All experimental diets were prepared by Research Diets, Inc. and stored in sealed bags at 4°C, under which conditions tocopherols are stable for at least 6 months. Animals were randomly allocated into the control and the experimental groups and housed in plastic cages (3 per cage). The animal room was controlled at  $20 \pm 2^\circ\text{C}$ ,  $50 \pm 10\%$  humidity and a 12 h light/dark cycle. Animals had free access to food and water at all times.

At 7 weeks of age, rats were given 2 weekly subcutaneous injections of AOM (Midwestern Research Institute) at a dose of 15 mg/kg body weight. Starting 1 week before the injection, all rats were fed the basal (referred to as AIN76m) or experimental diets. The AIN76m diet was modified from the AIN76A diet by using stripped corn oil (to remove tocopherols) and adding 33 IU  $\alpha$ -tocopheryl acetate per kg diet to meet the nutritional requirement. For the experimental groups, the AIN76m diet was supplemented with 0.2% of  $\alpha$ -T,  $\gamma$ -T,  $\delta$ -T or  $\gamma$ -TmT. The rats in the negative control group were given saline instead of AOM and maintained on the AIN76m diet. Body weight and food consumption were monitored weekly. At 8 weeks after the second AOM injection, the animals were sacrificed by  $\text{CO}_2$  asphyxiation. After laparotomy, the large intestine from the cecum to the anus was longitudinally opened and fixed in 10% buffered formalin for 24 h. The liver and spleen were harvested and stored at  $-80^\circ\text{C}$ .

### Identification of ACF and histological analysis

The formalin-fixed colonic tissues were cut into 6 cm segments and stained in 0.2% methylene blue solution for 1.5–2 min. Then the total number of ACF and the number of aberrant crypts (ACs) in each focus were counted under a Zeiss Axioskop 2 plus microscope (Carl Zeiss Microscopy, LLC). ACF were identified with the following morphological characteristics: (i) enlarged and elevated crypts compared to normal mucosa and (ii) increased pericryptal space and irregular lumens, according to previously established criteria (24).

From 5 rats per group, colonic tissues with multi-ACF containing 4 or more ACs were collected for histological analyses. ACF containing 4 or more ACs were marked with permanent ink and dissected from the middle and the distal parts of the colon, where most ACF occurred. After being de-stained with 80% ethanol, the tissues were paraffin-embedded for cross sectioning at 4  $\mu\text{m}$  in thickness. For histological evaluation, sections 5, 10, 20, and 30 were stained with hematoxylin and eosin, which allowed the visualization of ACF at different section levels. Based on the nuclear-to-cytoplasmic ratio, cell polarity, chromatin pattern, mitotic figures and mucin secretion, ACF were classified into 3 categories: (i) ACF with hyperplasia (no dysplasia); (ii) ACF with low-grade dysplasia (elongated, slightly crowded and pseudostratified nuclei, but polarity well preserved; normal or slightly reduced number of goblet cells); and (iii) ACF with high-grade dysplasia (elongated, crowded and pseudostratified nuclei and a markedly increased nucleus-to-cytoplasm ratio; significantly reduced number of goblet cells; back-to-back glands and markedly decreased interglandular stroma) (24).

## Immunohistochemical (IHC) analysis

A standard avidin–biotin–peroxidase complex method as described previously was employed (20). In brief, the deparaffinized sections on slides were heated in a microwave oven for antigen retrieval. Endogenous peroxidase was quenched using 3% H<sub>2</sub>O<sub>2</sub>. The slides were incubated with a primary antibody overnight at 4 °C. Biotinylated secondary antibody and Vectastain Elite ABC reagent (Vector Laboratories, Inc.) were then applied, using 3, 3'-diaminobenzidine as the chromogen. Sections were then counterstained with hematoxylin.

Antibodies against 4-hydroxynonenal (4-HNE) and nitrotyrosine were from Japan Institute for the Control of Aging and Millipore Corp., respectively. For these markers, more than 5 areas on each slide were chosen for picture taking with a Zeiss AxioCam camera under the Zeiss Axioskop 2 plus microscope (Carl Zeiss Microscopy, LLC). Positive stainings in the pictures were then quantified with ImagePro Plus 5.1 software (Media Cybernetics). The number of crypts in each picture was counted and the positive stainings were shown as intensity/crypt for 4-HNE (stained in the membrane and the cytoplasm) and nitrotyrosine (stained mainly in the cytoplasm).

The results of the IHC analysis with antibodies against peroxisome proliferator-activated receptor- $\gamma$  (PPAR- $\gamma$ ) and cyclin D1 (Cell Signaling), and against retinoid X receptor  $\alpha$  (RXR $\alpha$ ) (Santa Cruz Biotechnology) were quantified with an Aperio ScanScope scanner (Aperio Technologies, Inc.). ACF with 4 or more ACs and areas with normal colon crypts were selected for counting of the positively and negatively stained nuclei. The percentage of positive nuclear staining was calculated by the ImageScope software (Aperio Technologies, Inc.).

## Analysis of tocopherols and their metabolites

For these analyses, a modified procedure from our previous method were used (20). The serum sample (20  $\mu$ L) was mixed with 130  $\mu$ L of 0.1% ascorbic acid and 150  $\mu$ L of ethanol. The colon tissue sample (40 mg) was homogenized in a mixture of 160  $\mu$ L of 0.1% ascorbic acid and 200  $\mu$ L of ethanol using a bead ruptor homogenizer. The mixture or homogenate was extracted twice with 1 mL of hexane, and the supernatant was dried in a Savant Speedvac SC110 centrifugal vacuum concentrator (Thermo Scientific). The residue was reconstituted in 100  $\mu$ L of methanol and injected onto HPLC. Tocopherols were separated on an HPLC system equipped with a Supelcosil C18 reversed-phase column (150  $\times$  4.6 mm I.D.; 5  $\mu$ m particle size) and eluted with a mobile phase consisting of solvent A (acetonitrile/methanol/water, 280/40/680, v/v/v, containing 30 mM of lithium acetate and 0.3% acetic acid) and solvent B (acetonitrile/methanol/water, 840/130/30, v/v/v, containing 30 mM of lithium acetate and 0.3% acetic acid). The gradient cycle consisted of an initial 84% A and 16% B at a flow rate of 0.6 mL/min. The linear gradient was changed progressively by increasing to 85% B at 13 min with a flow rate of 0.7 mL/min, to 88% B at 20 min at 0.8 mL/min, to 94% B at 22 min at 1.0 mL/min, and to 100% B at 32 min at 1.2 mL/min. After running at 100% B at 1.2 mL/min until 56 min, solvent B was reduced to 16% at 56.1 min at 1.1 mL/min and maintained at 16% until 65 min. Then, the flow rate was changed to 0.6 mL/min for the next run. The eluent was monitored with an ESA 5600A Coulochem electrode array system (CEAS, ESA, Inc.) with potential settings at 400, 500, 600, and 700 mV. The tocopherol concentrations in the serum and tissues were determined by comparison with the peak heights of the standard serum.

For the analysis of total amounts (conjugated and unconjugated forms) of tocopherol metabolites, 100  $\mu$ L of serum sample was mixed with 10  $\mu$ L of ascorbic acid (10 mg/mL), 100  $\mu$ L of deionized water, 200  $\mu$ L of phosphate-buffered saline, and 1 mL of methanol. The colon tissue sample (40 mg) was homogenized in a mixture of 160  $\mu$ L of 0.1% ascorbic

acid and 200  $\mu\text{L}$  of methanol as above, and then mixed with 1 mL of methanol. The serum mixture or homogenate mixture was vortexed for 4 min and centrifuged. The supernatant was dried as above and redissolved in a mixture of 10  $\mu\text{L}$  of ascorbic acid (10 mg/mL), 100  $\mu\text{L}$  of water, 100  $\mu\text{L}$  of 0.23 M sodium acetate, 26 U of sulfatase, and 600 U of  $\beta$ -glucuronidase. After overnight incubation at 37  $^{\circ}\text{C}$ , samples were acidified to pH 3–4 with 15  $\mu\text{L}$  of acetic acid and then extracted twice with 1 mL of ethyl acetate. The dried ethyl acetate extracts were reconstituted in 100  $\mu\text{L}$  of 70% aqueous methanol, and the total tocopherol metabolites were analyzed by HPLC, as described above.

### Enzyme immunoassay (EIA)

The levels of prostaglandin E2 (PGE2), leukotriene B4 (LTB4) and 8-isoprostane were determined as previously described (12). In brief, serum samples were mixed with ethyl acetate, shaken for 30 min on a table top orbital shaker and then centrifuged. The supernatants were transferred to fresh tubes and dried using a centrifugal vacuum concentrator. The dried samples were then reconstituted in EIA buffer, and the levels of PGE2, LTB4 and 8-isoprostane were determined using EIA kits from Cayman Chemical, following the manufacturer's protocol.

### Statistical analysis

The differences on ACF multiplicity and intensity of IHC staining among groups were determined by one-way ANOVA followed by Duncan's post-hoc test. Effects of the treatments on the number of ACF in different categories were analyzed by chi-square test. The serum and colon levels of tocopherol were compared by one-way ANOVA followed by Dunnett's post-hoc test. The significance level used for all the tests was  $\alpha = 0.05$ .

## Result

### Effects of dietary tocopherol treatment on general health

The tocopherol-fed rats looked healthy throughout the experiment and no signs of toxicity were observed. All animals had a steady body weight gain during the treatment. Starting from the day after the second AOM injection, the average body weight of the positive control group (with AOM injections) was slightly lower than that of the negative control group (with no AOM injection) (data not shown). The body weights of all the different tocopherol diet groups were in-between the positive and the negative groups and did not show significant differences. There was no significant difference among all groups in the weights of liver and spleen at the time of sacrifice.

### Dietary $\delta$ -T and $\gamma$ -T treatments inhibited ACF formation

Colonic ACF were identified and counted under a light microscope after methylene blue staining. Figure 2A shows 2 typical ACF, 1 with 3 ACs and the other with 5 ACs. Compared with the positive control group, all tocopherol treatment groups had significantly lower total numbers of ACF per rat, except the 0.2%  $\alpha$ -T group, (Table 1, Fig. 2B). All groups had significantly reduced total numbers of AC per rat. The 0.2%  $\delta$ -T group showed the strongest inhibitory effect and decreased the total numbers of ACF and AC (per rat) by 62.3% and 62.9%, respectively, as compared to the control group.  $\gamma$ T and  $\gamma$ -TmT had similar potency in lowering the numbers of ACF and AC. The  $\gamma$ -TmT\* group, which started receiving the  $\gamma$ -TmT diet 1 day after the second AOM injection, had lower inhibitory effect against ACF formation than the  $\gamma$ -TmT group. This result suggests that tocopherols also exerted a protective effect during the carcinogen treatment period. Based on the number of AC in each focus, ACF were subsequently categorized into foci with 1, 2, 3 and 4 or more ACs.  $\delta$ -T,  $\gamma$ -T and  $\gamma$ -TmT treatments decreased the number of foci in all 4 categories, whereas  $\alpha$ -T treatment did not (Table 1).



### Dietary $\delta$ -T decreased the formation of dysplastic ACF

A total of 38–44 ACF with 4 or more ACs from each group were examined histologically and classified into ACF with hyperplasia, low-grade dysplasia and high-grade dysplasia (Fig. 2C–F and Table 2), according to the criteria described in “Materials and Methods”. Chi-square test was used to determine the relationship between the tocopherol treatments and the percentage of each ACF category in each group. The percentages of both low-grade and high-grade dysplastic ACF appeared to be decreased in all tocopherol-treated groups; however only the  $\delta$ -T group showed a significant effect, decreasing the high-grade dysplastic ACF by 70%.

### Dietary tocopherols inhibited the formation of 4-HNE and nitrotyrosine

4-HNE is a product of lipid peroxidation (25), and was located in the membrane and cytosol by IHC (Fig. 2G–I). All tocopherol treatment groups had significantly lower 4-HNE staining than the control group. The  $\delta$ -T treatment was most effective, decreasing the level of positive staining by 56.9%, followed by  $\gamma$ -T with a 39.9% reduction.

The effect of different tocopherols on nitrosative stress in the colon was studied by determining the levels of nitrotyrosine with IHC (Fig. 2J–L). Nitrotyrosine was observed in normal colon crypts as well as in ACF. Compared with the negative control, the level of nitrotyrosine staining in the colon of the positive control group increased by 4.5 fold (data not shown). Treatments with  $\gamma$ -T,  $\delta$ -T, and  $\gamma$ -TmT significantly decreased the level of nitrotyrosine by about 60%, whereas  $\alpha$ -T did not produce a significant effect.

### Dietary tocopherols regulated the level of PPAR- $\gamma$ and RXR $\alpha$ in ACF

PPAR- $\gamma$  is a member of the nuclear hormone receptor PPAR superfamily (26, 27). Because it has been reported that tocopherols increased the level of PPAR- $\gamma$  in human colon cancer cells (28), the effects of tocopherol treatment on the expression of PPAR- $\gamma$  was investigated with IHC. PPAR- $\gamma$  was expressed in the normal crypts of the negative control group as well as of the animals treated with AOM, and the percentage of positively stained nuclei was in the range of 46.4% to 51.3% (data not shown). The percentages of cells with PPAR- $\gamma$  nuclear staining in ACF with 4 or more ACs were quantified (Fig. 3A–C). Compared with normal crypts, nuclear staining of PPAR- $\gamma$  in ACF of the positive control group was markedly decreased (from 47% to 10.6%, Fig. 3A). Interestingly, the decrease of PPAR- $\gamma$  levels in ACF was significantly attenuated by  $\gamma$ -T,  $\delta$ -T, and  $\gamma$ -TmT treatment (Fig. 3B and C).  $\gamma$ -T and  $\gamma$ -TmT also significantly prevented the decrease of PPAR- $\gamma$  levels in dysplastic ACF (data not shown). The effect of 0.2%  $\alpha$ -T on PPAR- $\gamma$  level was insignificant.

PPAR- $\gamma$  exerts its action through forming a heterodimer with RXRs (29, 30). To assess the role of RXR $\alpha$  in ACF formation, we analyzed the expression of RXR $\alpha$  in ACF with 4 or more ACs (Fig. 3D–F). RXR $\alpha$  was highly expressed in the normal colon crypts of the negative control group (46.3% of positive nuclear staining, data not shown). The nuclear staining in the ACF of the positive control rats was reduced to 20.4%, and  $\delta$ -T significantly elevated the positive staining cells to 31.4% in ACF (Fig. 3D–F). Compared to the negative control group, the nuclear staining of RXR $\alpha$  in the normal crypts of the positive control group was significantly reduced to 33.2% (data not shown).  $\gamma$ -T and  $\delta$ -T supplementations partially (nonsignificantly) restored the staining in the normal crypts; whereas the  $\alpha$ -T and  $\gamma$ -TmT groups showed the same staining as that of the positive control group (data not shown).

### Dietary tocopherols inhibited cyclin D1 expression in ACF

Aberrant  $\beta$ -catenin expression is a common feature of AOM-induced rat colon tumor and human colorectal cancer (31, 32). In this study, we observed cell membrane and cytoplasmic

staining of  $\beta$ -catenin in most ACF, but nuclear staining only in a few dysplastic ACF in some samples (data not shown). The number of cells with nuclear staining was lower than what we observed previously (24). The effect of tocopherols on the localization of  $\beta$ -catenin was not apparent. Since cyclin D1 is a downstream protein of  $\beta$ -catenin signaling involved in cell proliferation and overexpressed in many human cancers including colon cancer (33, 34), we investigated its expression in AOM-induced ACF with 4 or more ACs (Fig. 3G–I). In the positive control group, 38.5% of cells in ACF showed positive nuclear staining; whereas in the  $\delta$ -T and  $\gamma$ -TmT groups, the positive staining was significantly reduced to around 28%. Even though the inhibition was moderate, these results suggest that the down-regulation of cyclin D1 by  $\delta$ -T and  $\gamma$ -TmT contributed to the inhibition of cell growth in ACF. We also studied the effects of tocopherols on cell proliferation by examining the level of Ki67. Compared with the positive control,  $\delta$ -T significantly decreased the percentage of positively stained nuclei in ACF ( $p < 0.05$  by t-test), whereas other forms of tocopherols showed no effect (data not shown).

### Dietary tocopherols decreased the levels of PGE2 and 8-isoprostane in serum

We and others have demonstrated that  $\gamma$ -TmT and  $\gamma$ -T inhibited inflammation (11, 12, 18, 35, 36). Because all the colons were fixed for ACF counting, we instead examined the effects of tocopherol treatments on the PGE2, LTB4 and 8-isoprostane levels in the serum. As shown in Figure 3J, AOM treatment significantly increased the serum level of PGE2; dietary tocopherols appeared to suppress the increase, but only the effect of  $\gamma$ -T was statistically significant.  $\delta$ -T and  $\gamma$ -T significantly suppressed the increase of the serum level of 8-isoprostane by AOM (Fig. 3K). AOM did not induce the LTB4 level in the serum; although  $\gamma$ -T,  $\delta$ -T and  $\gamma$ -TmT had a tendency to reduce LTB4, the effect was not statistically significant (data not shown).

### Levels of different forms of tocopherols and their metabolites in the serum and colon tissues

Because all the colon tissues in the experiment were utilized for ACF counting, we conducted a short-term experiment to determine the colon levels of tocopherols and their metabolites (Table 3). In this experiment, male F344 rats received one dose of AOM (15 mg/kg). They were maintained on either the AIN76m diet or the AIN76m diet containing 0.2% of  $\alpha$ -T,  $\gamma$ -T or  $\delta$ -T for a total of 10 days, starting 1 week before the AOM injection until the termination of the experiment. Because the AIN76m diet was formulated by using stripped corn oil (to remove tocopherols) and adding 33 IU tocopheryl acetate per kg diet, the levels of  $\gamma$ -T,  $\delta$ -T and their metabolites of the positive control group (on the AIN76m diet) in the serum and colon tissues (Table 3) were either very low or nondetectable. The serum level of  $\alpha$ -T was increased to 30.7  $\mu$ M by the 0.2%  $\alpha$ -T, but decreased by the 0.2%  $\gamma$ -T treatment ( $p < 0.05$ ). With supplementation, the  $\gamma$ -T level reached 21.2  $\mu$ M, and the  $\delta$ -T level was increased to 6.7  $\mu$ M; the latter was significantly lower than the former. The colon levels of  $\alpha$ -T in different groups followed a pattern similar to that of serum  $\alpha$ -T. For example, the colon level of  $\alpha$ -T was also decreased by supplementation with  $\gamma$ -T. If we assume a tissue concentration of nmol/g is equivalent to  $\mu$ M, then the colon  $\alpha$ -T levels were higher than the serum  $\alpha$ -T level. In the supplemented groups, colon  $\gamma$ -T and  $\delta$ -T were comparable to or higher than the corresponding serum levels.

$\delta$ -T and  $\gamma$ -T were extensively metabolized via side-chain degradation. In the serum,  $\delta$ -CEHC was the major short-chain metabolite (highest concentration of 23.3  $\mu$ M) and  $\gamma$ -CEHC levels also reached 6.1  $\mu$ M with supplementation. Comparable levels of  $\gamma$ -CEHC and lower levels of  $\delta$ -CEHC were observed in colon tissues. The levels of CMBHCs were much lower in the serum and colon tissues. Medium- and long-chain metabolites of  $\delta$ -T and  $\gamma$ -T, including carboxydimethyloctyl hydroxychroman (CDMOHC), carboxydimethyldecyl

hydroxychromans (CDMDHC), and carboxyl tocopherol, were also detectable in the serum and colon tissues, but their levels were low ( $< 1\mu\text{M}$ ; data not shown). On the other hand,  $\alpha$ -T was not extensively metabolized. The serum and colon tissue levels of  $\alpha$ -CEHC and  $\alpha$ -CMBHC were approximately 1.6 and 0.02  $\mu\text{M}$  (nmol/g), respectively, and the levels of longer-chain metabolites were even lower (data not shown). In the serum, most tocopherol metabolites (79–90%) existed in conjugated forms; however, in the colon tissues most tocopherols metabolites (85–90%) were in the free form (data not shown). The serum levels of  $\alpha$ -T,  $\gamma$ -T,  $\delta$ -T and their metabolites of the 10-week experiment (in which ACF were produced) followed the same pattern as the 10-day experiment, except that the  $\alpha$ -T level was higher and the  $\gamma$ -T,  $\gamma$ -CEHC and  $\delta$ -CEHC levels were lower in the corresponding groups that received  $\alpha$ -T,  $\gamma$ -T, and  $\delta$ -T supplementations (data not shown).

## Discussion

Consistent with many previous reports (2), the present study shows that dietary  $\alpha$ T does not inhibit colon carcinogenesis. On the other hand, dietary  $\delta$ -T and  $\gamma$ -T supplementations are very effective in inhibiting AOM-induced ACF formation.  $\delta$ -T showed the strongest inhibitory effect on both the total numbers of ACF and the percentage of dysplastic ACF. To our knowledge, this is the first demonstration that  $\delta$ -T has the higher cancer preventive activity than other forms of tocopherols in a model for carcinogenesis.

In this study, we modified the AIN-76A diet by using stripped corn oil (to remove tocopherols), and adding  $\alpha$ -tocopheryl acetate (33 IU/kg) to meet the nutritional need. This provides a clean dietary system to study the activities of  $\delta$ -T and  $\gamma$ -T. Because of the large number of ACF that can be produced in the rat colon following AOM treatment, this model is a convenient experimental system that provides the statistical power for multi-group comparisons among  $\delta$ -T-,  $\gamma$ -T,  $\alpha$ -T and  $\gamma$ -TmT. We realize that ACF can only be considered as precancerous lesions, and dysplastic ACF may be closely related to the future development of carcinomas. Additional studies are needed to study the inhibition of carcinoma formation by  $\delta$ -T- and  $\gamma$ -T in the future. It should be noted that the  $\delta$ -T preparation used in this study contained 5.5%  $\gamma$ -T, and this preparation had higher inhibitory activity than pure  $\gamma$ -T and  $\gamma$ -TmT (with a  $\gamma$ -T to  $\delta$ -T ratio of 57 to 24). Whether  $\delta$ -T is still more active in cancer prevention under other experimental conditions remains to be determined. In practical applications, however, the readily available  $\gamma$ -TmT may be a better agent to use. This mixture contains different forms of tocopherols at a ratio similar to those in the vegetable oils commonly consumed in the United States.

To test the hypothesis that tocopherols trap RONS in our experimental system, we examined the levels of 4-HNE and nitrotyrosine in colon tissues. 4-HNE is a product of liperoxidation and possesses cytotoxic, mutagenic, and genotoxic properties (37). Corresponding to its strongest inhibitory effect on ACF formation,  $\delta$ -T had the strongest effect in decreasing 4-HNE levels, whereas the effect of  $\alpha$ -T was the weakest (Fig. 2G–I). The low inhibitory activity of  $\alpha$ -T on 4-HNE formation is in agreement with previous reports that  $\alpha$ -T had no or little effect on 4-HNE levels in human urine (38) or on 4-HNE levels in oxidized low density lipoproteins from human serum (39). Nitrotyrosine is a product formed between reactive nitrogen species and tyrosines in proteins and has been used as a biomarker of nitrosative stress (18, 20). The present observed inhibition of nitrotyrosine by tocopherols (Fig. 2J–L) is consistent with our previous result in a lung cancer cell xenograft model in mice (20). The results also agree with our previous finding that  $\gamma$ -TmT treatment decreased nitrotyrosine levels in the colon of AOM/DSS-treated mice (12, 20).

Compared with normal crypts, the staining of PPAR- $\gamma$  in ACF was markedly decreased, and treatment with  $\delta$ -T,  $\gamma$ -T and  $\gamma$ -TmT partially prevented this decrease (Fig. 3A–C). PPAR- $\gamma$



is a nuclear receptor that, upon activation by binding to ligands, regulates the transcription of downstream target genes which are involved in cell growth, differentiation and apoptosis; thus, it plays an important role in tumorigenesis (26, 27). Previous investigations have shown that tocopherols increased the mRNA and protein levels of PPAR- $\gamma$  in human SW 480 colon cancer cells, with  $\gamma$ -T having higher activity over  $\alpha$ -T (28). In the NCTC 2544 human keratinocytes cell line, all 4 forms of tocopherols increased transcriptional activity of PPAR- $\gamma$ , and  $\gamma$ -T had the strongest activity (40). Recently, we found that  $\delta$ -T,  $\gamma$ -T and  $\gamma$ -TmT activated PPAR- $\gamma$  transcription in estrogen receptor-positive breast cancer cell lines MCF-7 and T47D, and  $\delta$ -T was the most active one (17). PPAR- $\gamma$  exerts its action through the formation of a heterodimer with RXRs (29, 30). Dysfunctional RXRs were associated with carcinogenesis in multiple organ sites (41–44). Consistent with the effect on PPAR- $\gamma$  expression in ACF, we found that  $\delta$ -T partially, but significantly, prevented the loss of RXR $\alpha$  (Fig. 3F). These results suggest that upregulation of PPAR- $\gamma$  and RXR $\alpha$  may be one of the mechanisms for the cancer preventive action of tocopherols.

As expected, the serum levels of  $\alpha$ -T,  $\gamma$ -T and  $\delta$ -T were increased by treatment with respective tocopherols (Table 3). After supplementation, the serum levels follow the ranking order of  $\alpha$ -T (30.7  $\mu$ M),  $\gamma$ -T (21.2  $\mu$ M), and  $\delta$ -T (6.7  $\mu$ M). These levels are consistent with the concept that  $\alpha$ -T transfer protein, which transports tocopherols from the liver to the blood, has affinities to  $\alpha$ -T >  $\gamma$ -T >  $\delta$ -T (45). However, the observed levels of  $\gamma$ -T and  $\delta$ -T are higher than those in corresponding experiments in mice ((20) and unpublished results), possibly due to a species difference. It is interesting to note that supplementation with  $\gamma$ -T decreased the serum levels of  $\alpha$ -T. This result agrees with the concept that  $\gamma$ -T competes with  $\alpha$ -T for the  $\alpha$ -T transfer protein (3, 13–15, 46). In theory,  $\alpha$ -T supplementation should effectively lower the serum  $\gamma$ -T level; however, this was not observed because 1) the baseline level of  $\gamma$ -T (rats on the AIN76m diet) was very low, and 2) the  $\alpha$ -T preparation for supplement contained 2.6%  $\gamma$ -T as a contaminant. The colon levels of  $\alpha$ -T and  $\gamma$ -T were comparable to those in the serum, and the colon  $\delta$ -T level was higher. As discussed previously (20, 47, 48),  $\gamma$ -T and  $\delta$ -T are more extensively metabolized through the  $\omega$ -oxidation and  $\beta$ -oxidation pathway. In this study, rather high  $\delta$ - and  $\gamma$ -CEHCs were found in the serum and colon, and the serum levels of  $\delta$ -CEHC was much higher than  $\gamma$ -CEHC (Table 3). These metabolites, retaining the intact chromanol ring structure but less lipophilic, could also trap RONS in the cytosol and contribute to the cancer preventive activity of tocopherols.

In summary, we found that 0.2%  $\gamma$ -T,  $\delta$ -T, and  $\gamma$ -TmT in the diet significantly decreased the total numbers of ACF in AOM rats, whereas  $\alpha$ -T was not effective.  $\delta$ -T showed the strongest inhibitory activity and also decreased the percentage of dysplastic ACF. The inhibitory activities are associated with trapping of RONS as well as other actions of  $\delta$ -T,  $\gamma$ -T and their metabolites. This is the first report showing that  $\delta$ -T had a higher cancer preventive activity than  $\gamma$ -T in a model for carcinogenesis. These and our previous results (12, 16–20, 49) point out the importance of using  $\delta$ -T,  $\gamma$ -T or related mixtures in cancer prevention studies. This point is especially worth noting in the light of the SELECT results showing that supplementation of high doses of  $\alpha$ -tocopherol acetate increased prostate cancer risk (3, 50).

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## Abbreviations

<b>AC</b>	aberrant crypt
<b>ACF</b>	aberrant crypt foci
<b>AOM</b>	azoxymethane
<b>CDMDHC</b>	carboxydimethyldecyl hydroxychroman
<b>CDMOHC</b>	carboxydimethyloctyl hydroxychroman
<b>CEHC</b>	carboxyethyl hydroxychroman
<b>CMBHC</b>	carboxymethylbutyl hydroxychroman
<b>EIA</b>	enzyme immunoassay
<b>4-HNE</b>	4-hydroxynonenal
<b>IHC</b>	immunohistochemistry
<b>LTB4</b>	leukotriene B4
<b>PGE2</b>	prostaglandin E2 (PGE2)
<b>PPAR-<math>\gamma</math></b>	peroxisome proliferator-activated receptor- $\gamma$
<b>RONS</b>	reactive oxygen and nitrogen species
<b>RXR<math>\alpha</math></b>	retinoid X receptor
<b><math>\alpha</math>-T</b>	$\alpha$ -tocopherol
<b><math>\gamma</math>-T</b>	$\gamma$ -tocopherol
<b><math>\delta</math>-T</b>	$\delta$ -tocopherol
<b><math>\gamma</math>-TmT</b>	$\gamma$ -tocopherol-rich mixture of tocopherols

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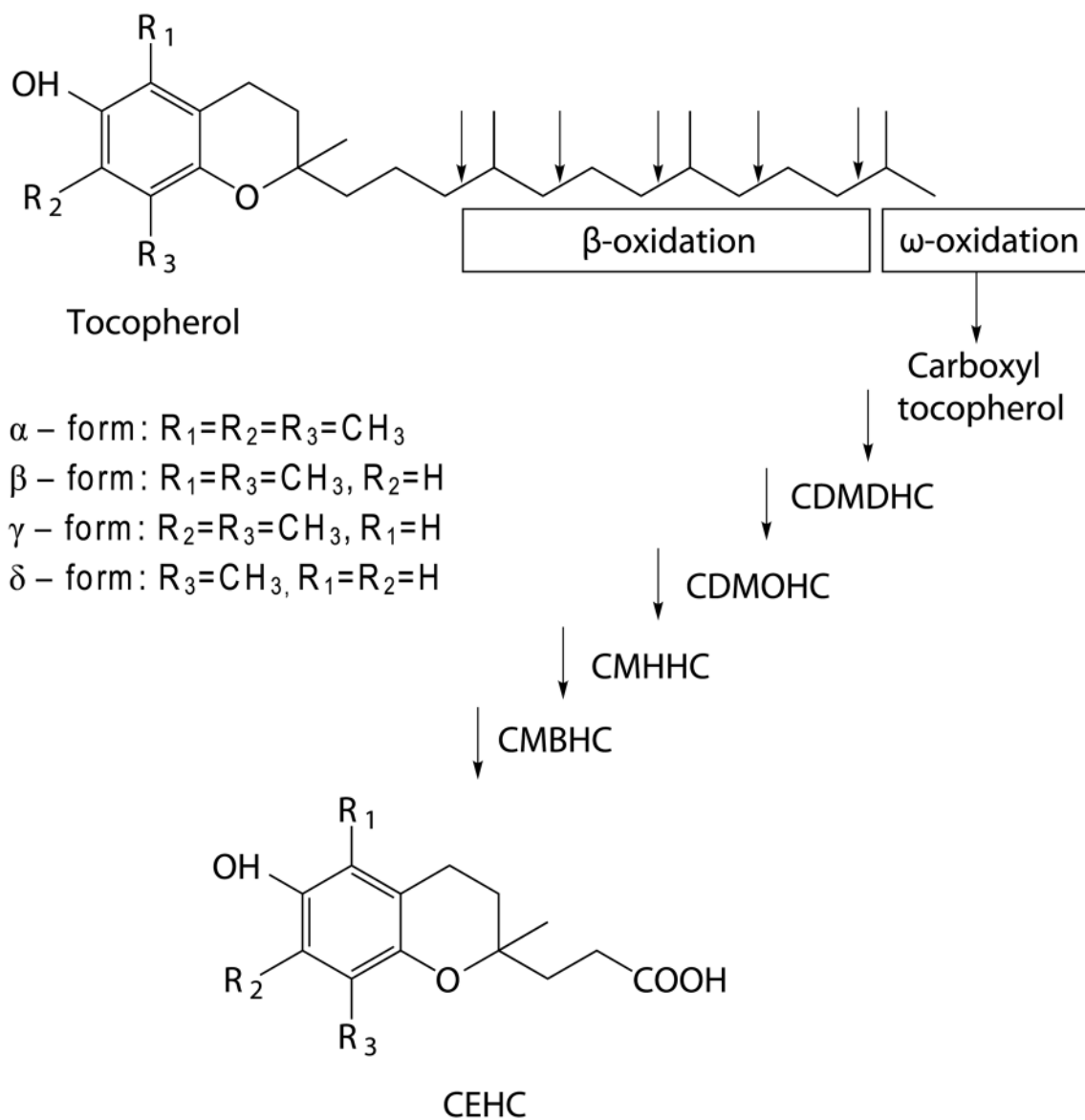
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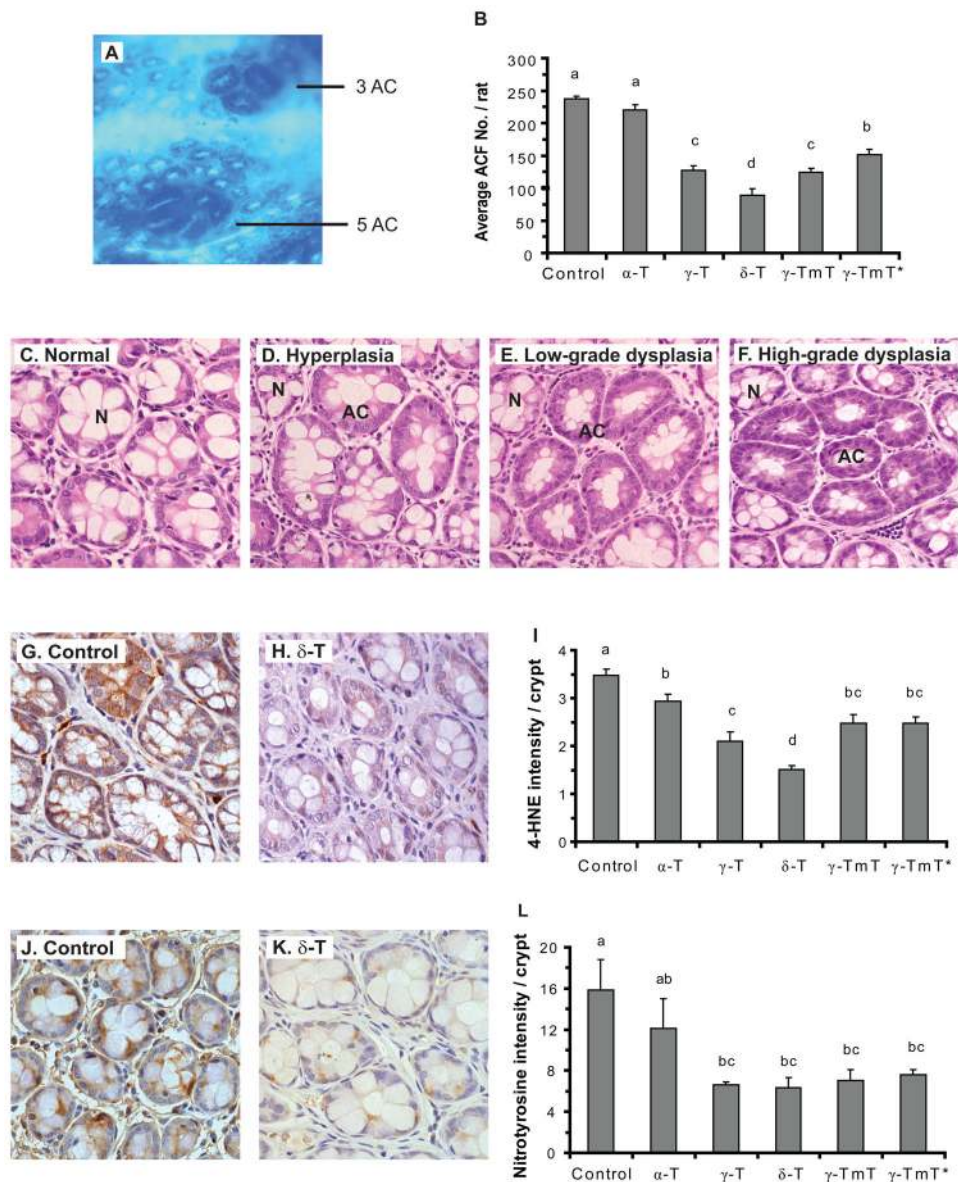
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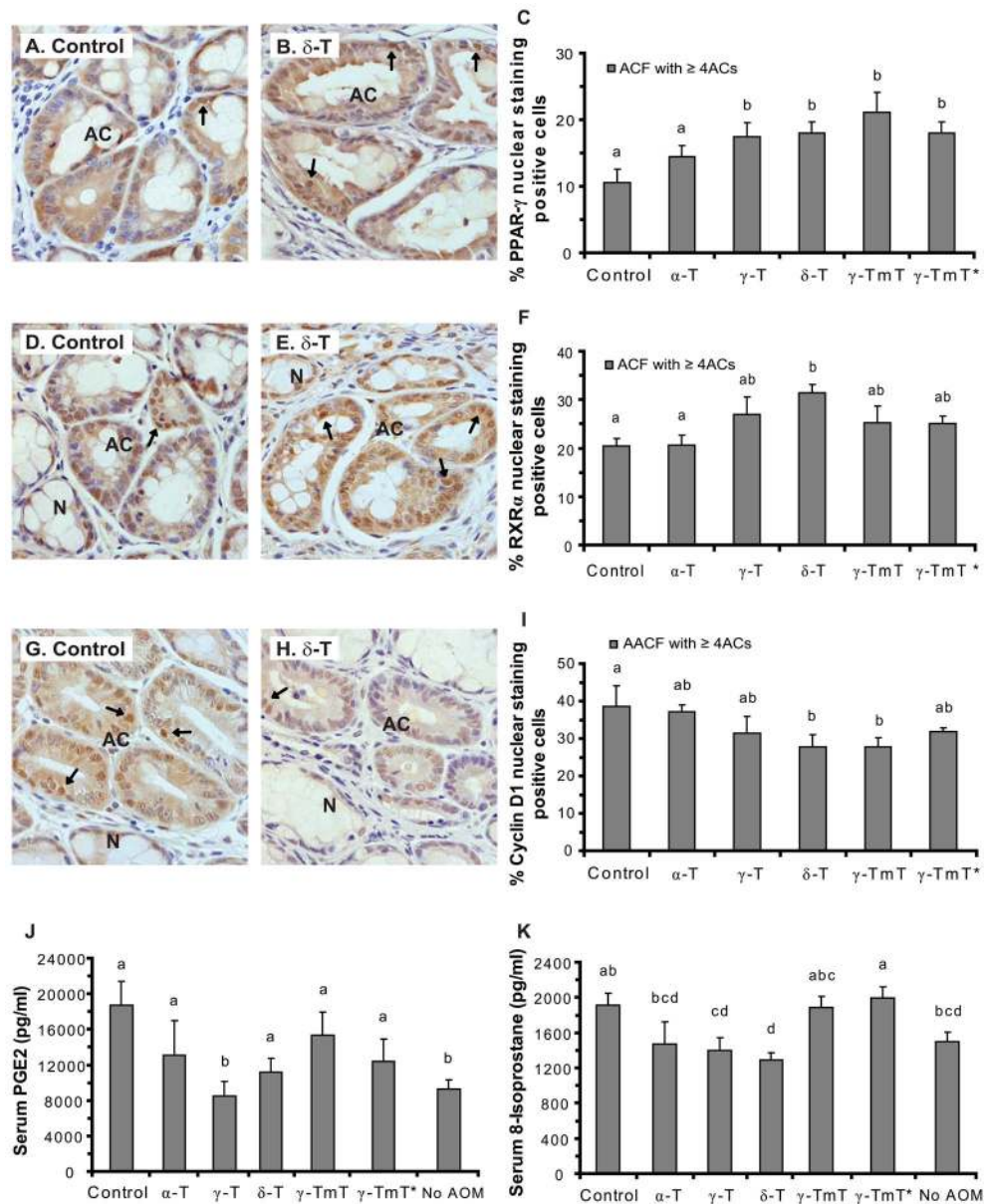




**Fig. 1.** Structure of tocopherols and the metabolic pathways. The structures are illustrated as the general chromanol ring system of tocopherols, and the positions of the methyl group or hydrogen for each individual tocopherol. The arrows indicate the sequential side chain degradation and the resulting metabolites.



**Fig. 2.** Histological characterization of ACF, and the effects of tocopherol treatment on ACF formation, 4-HNE levels and nitrotyrosine levels in the colon. A, AOM-induced colorectal ACF stained with 0.2% methylene blue. B, effects of tocopherol treatments (0.2% in the diet) on ACF formation, shown as mean of ACF numbers/rat  $\pm$  SE (n = 10). Examples of histological diagram shown are C, normal crypts; D, an ACF with hyperplasia; E, an ACF with low-grade dysplasia; F, an ACF with high-grade dysplasia. G-L, effects of dietary tocopherols on the levels of 4-HNE (G-I) and nitrotyrosine (J-L). Examples of 4-HNE membrane and cytosolic staining (brown colored) and nitrotyrosine cytosol staining (brown colored) in the colon crypts of the positive control group (G and J), and in the colon crypts of the 0.2%  $\delta$ -T group (H and K), are shown. I and L, effects of different tocopherol treatments (0.2% in the diet) on the levels of 4-HNE (I) and nitrotyrosine (L), are shown as mean of intensity/crypt  $\pm$  SE (n = 5). ANOVA-Duncan's post-hoc test was conducted. Different letters designate significant difference (p<0.05).  $\gamma$ -TmT\*, rats switched to 0.2%  $\gamma$ -TmT diet one day after the second AOM treatment. N, normal crypt; AC, aberrant crypt.



**Fig. 3.** Effects of dietary tocopherols on the levels of PPAR- $\gamma$  (A–C), RXR $\alpha$  (D–F) and cyclin D1 (G–I) in the colon, and the levels of PGE2 (J) and 8-isoprostane (K) in the serum. Examples of PPAR- $\gamma$ , RXR $\alpha$  and cyclin D1 nuclear staining (brown colored) in an ACF of the positive control group (A, D and G), and in an ACF of the 0.2%  $\delta$ -T group (B, E and H) are shown. C, F and I, effects of dietary tocopherol treatments (0.2% in the diet) on the levels of PPAR- $\gamma$  (C), RXR $\alpha$  (F), and cyclin D1 (I) in the ACF (with 4 or more ACs), are shown as mean of the percentage of nuclear staining positive cells  $\pm$  SE (n = 5). The effects of dietary tocopherols on the levels of PGE2 (J) and 8-isoprostane (K) in the serum were determined with EIA assay and shown as mean  $\pm$  SE (n = 10 except for the negative control group for which n = 6). ANOVA-Duncan's post-hoc test was conducted. Different letters designate significant difference (p < 0.05). Arrows indicate nuclear staining of PPAR- $\gamma$  in A and B, RXR $\alpha$  in D and E, and cyclin D1 in G and H. N, normal crypt; AC, aberrant crypt.

**Table 1**

Effect of dietary tocopherol treatments on ACF formation in AOM-treated F344 rats

Group	Total ACF No./rat	Total AC No./rat	Number of foci containing			
			1 AC	2 AC	3 AC	≥4 AC
Control	237.0 ± 13.3 <sup>a</sup>	554.5 ± 64.4 <sup>a</sup>	82.4 ± 19.2 <sup>ab</sup>	72.7 ± 15.0 <sup>a</sup>	50.7 ± 10.8 <sup>a</sup>	31.2 ± 9.0 <sup>a</sup>
0.2% α-T	220.7 ± 25.5 <sup>a</sup>	466.3 ± 94.2 <sup>b</sup>	89.7 ± 20.8 <sup>a</sup>	70.0 ± 18.9 <sup>a</sup>	38.1 ± 11.5 <sup>ab</sup>	26.3 ± 8.0 <sup>a</sup>
0.2% γ-T	126.8 ± 24.8 <sup>c</sup>	291.9 ± 60.0 <sup>c</sup>	38.8 ± 9.5 <sup>c</sup>	43.1 ± 10.0 <sup>bc</sup>	28.6 ± 8.6 <sup>c</sup>	16.3 ± 4.7 <sup>b</sup>
0.2% δ-T	89.3 ± 29.2 <sup>d</sup>	205.5 ± 69.1 <sup>d</sup>	23.9 ± 8.2 <sup>c</sup>	31.9 ± 12.2 <sup>c</sup>	21.0 ± 8.7 <sup>c</sup>	12.5 ± 6.2 <sup>b</sup>
0.2% γ-TmT	124.0 ± 20.0 <sup>c</sup>	274.7 ± 60.1 <sup>c</sup>	49.8 ± 11.5 <sup>c</sup>	44 ± 4.4 <sup>b</sup>	24.8 ± 7.0 <sup>c</sup>	13.6 ± 2.9 <sup>b</sup>
0.2% γ-TmT*	151.1 ± 28.2 <sup>b</sup>	304.3 ± 67.7 <sup>c</sup>	67.8 ± 25.2 <sup>b</sup>	41.7 ± 12.3 <sup>bc</sup>	26.2 ± 11.3 <sup>c</sup>	15.4 ± 6.4 <sup>b</sup>

Note: Values are mean ± SD. n = 10. Different superscripts (a, b, c, d) indicate significant statistical difference by one-way ANOVA followed by Duncan's post-hoc test. γ-TmT\*: rats switched to diet containing 0.2% γ-TmT one day after the second AOM injection.

**Table 2**

Effect of dietary tocopherol treatment on histology of ACF with 4 or more ACs.

Group	Total ACF analyzed	Hyperplasia (%)	Low-grade dysplasia (%)	High-grade dysplasia (%)	<i>p</i> <sup>†</sup>
Control	43	16 (37.2%)	13 (30.2%)	14 (32.6%)	
0.2% $\alpha$ -T	41	18 (43.9%)	12 (29.3%)	11 (26.8%)	0.79
0.2% $\gamma$ -T	43	25 (58.1%)	10 (23.3%)	8 (18.6%)	0.14
0.2% $\delta$ -T	38	27 (71.1%)	7 (18.4%)	4 (10.5%)	<b>0.01<sup>a</sup></b>
0.2% $\gamma$ -TmT	40	23 (57.5%)	10 (25.0%)	7 (17.5%)	0.14
0.2% $\gamma$ -TmT*	44	21 (47.7%)	12 (27.3%)	9 (20.5%)	0.41

Note:

<sup>†</sup>, Chi-square test was used to analyze the difference between the positive control and treatment groups.<sup>a</sup>The percentage of ACF with dysplasia was significantly lower than the positive control ( $p < 0.01$ ). $\gamma$ -TmT\*: rats were switched to diet containing 0.2%  $\gamma$ -TmT one day after the second AOM treatment.



Table 3

Serum and colon levels of total tocopherols and their metabolites

Group	$\alpha$ -T	$\gamma$ -T	$\delta$ -T	$\gamma$ -CEHC	$\delta$ -CEHC	$\gamma$ -CMBHC	$\delta$ -CMBHC	$\gamma$ -CDMDHC	$\delta$ -CDMDHC
<i>Serum Levels (<math>\mu</math>M)</i>									
Control	14.39 $\pm$ 6.13	0.09 $\pm$ 0.02	0.02 $\pm$ 0.01	0.01 $\pm$ 0.00	0.13 $\pm$ 0.02	0.02 $\pm$ 0.00	0	0	0
0.2% $\alpha$ -T	30.65 $\pm$ 6.28 <sup>‡</sup>	0.16 $\pm$ 0.06	0.09 $\pm$ 0.03	0.04 $\pm$ 0.01	0.32 $\pm$ 0.03	0	0	0	0
0.2% $\gamma$ -T	4.51 $\pm$ 4.35 <sup>‡</sup>	21.20 $\pm$ 9.56 <sup>‡</sup>	0.02 $\pm$ 0.00	6.07 $\pm$ 1.77 <sup>‡</sup>	0.14 $\pm$ 0.00	0.12 $\pm$ 0.04 <sup>‡</sup>	0	0.10 $\pm$ 0.04 <sup>‡</sup>	0
0.2% $\delta$ -T	7.74 $\pm$ 4.58	1.17 $\pm$ 0.71	6.69 $\pm$ 3.63 <sup>‡</sup>	0.49 $\pm$ 0.21	23.32 $\pm$ 5.29 <sup>‡</sup>	0.02 $\pm$ 0.00	0.23 $\pm$ 0.08 <sup>‡</sup>	0	0.70 $\pm$ 0.01 <sup>‡</sup>
<i>Colon Levels (nmol/g)</i>									
Control	18.85 $\pm$ 2.80	0.38 $\pm$ 0.39	0.02 $\pm$ 0.01	0.05 $\pm$ 0.04	0.12 $\pm$ 0.10	0	0	0	0.15 $\pm$ 0.07
0.2% $\alpha$ -T	34.83 $\pm$ 1.92 <sup>‡</sup>	0.47 $\pm$ 0.32	0.04 $\pm$ 0.02	0.15 $\pm$ 0.20	1.29 $\pm$ 1.42	0.13 $\pm$ 0.20	0	0	0.21 $\pm$ 0.04
0.2% $\gamma$ -T	13.44 $\pm$ 1.52 <sup>‡</sup>	21.49 $\pm$ 3.26 <sup>‡</sup>	0.05 $\pm$ 0.06	6.91 $\pm$ 7.62 <sup>‡</sup>	0.16 $\pm$ 0.07	0.65 $\pm$ 1.01	0	0.05 $\pm$ 0.03 <sup>‡</sup>	0.17 $\pm$ 0.05
0.2% $\delta$ -T	18.05 $\pm$ 0.88	1.52 $\pm$ 0.71	16.91 $\pm$ 7.11 <sup>‡</sup>	0.37 $\pm$ 0.34	7.60 $\pm$ 4.95 <sup>‡</sup>	0.04 $\pm$ 0.04	0.36 $\pm$ 0.27 <sup>‡</sup>	0.05 $\pm$ 0.02 <sup>‡</sup>	0.37 $\pm$ 0.06 <sup>‡</sup>

Note: The samples were from the short term experiment. Values are mean  $\pm$  SD. n = 5.<sup>‡</sup>The value is different from that of the AOM-treated positive control group (p < 0.05 by the ANOVA-Dunnett's test).<sup>‡</sup>p < 0.01 by the ANOVA-Dunnett's test.