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Effect of γ-Tocotrienol and α-Tocopherol on Blood Glutathione and Tumor Marker Enzymes during Chemical Hepatocarcinogenesis in the Rat

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Summary The protective effect of two types of vitamin E (α-tocopherol and γ-tocotrienol) in rats treated with diethylnitrosamine (DEN) and 2-acetylaminofluorene (AAF) were studied by determination of plasma alkaline phosphatase (ALP), plasma and liver microsomal γ-glutamyl transpeptidase (GGT) activities, and blood glutathione (GSH). Rats treated with DEN/AAF had significantly elevated plasma and microsomal GGT, plasma ALP activities, and blood GSH levels compared with the normal controls ($p<0.05$). Supplementation with vitamin E of normal controls did not affect the enzyme activities or blood GSH. In rats treated with DEN/AAF, vitamin E supplementation attenuated GGT and ALP activities and blood GSH levels. The optimum dose required for highest attenuation of the tumor marker enzyme activities was 34 mg/kg diet for α-tocopherol and 30 mg/kg diet for γ-tocotrienol. Higher doses of the vitamin did not show further attenuation in the level of the tumor marker enzyme activities.

Key Words: γ-tocotrienol, α-tocopherol, γ-glutamyl transpeptidase, alkaline phosphatase, hepatocarcinogenesis

Vitamin E has been reported to demonstrate a protective effect in chemical carcinogenesis [1]. It has been suggested that vitamin E protects the cell against

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carcinogenesis by scavenging and inhibiting the formation of free radicals or by increasing cellular immune responses [2–6]. Of the two major forms of vitamin E, γ-tocotrienol has been shown to have a higher anti-tumor activity than α-tocopherol in several transplantable murine tumors [7]. It was suggested that γ-tocotrienol is capable of reducing the severity of chemical hepatocarcinogenesis in the rat [8]. Long-term administration of γ-tocotrienol to rats with experimentally induced cancer resulted in attenuation of tumor marker enzyme activities, providing additional evidence that γ-tocotrienol is able to reduce the severity of carcinogenesis [9].

Carcinogenesis has been monitored by examination of gross morphology and histological and ultrastructural study, complemented with the determination of marker enzyme activities, namely, those of γ-glutamyl transpeptidase (GGT, EC 2.3.2.2), uridine diphosphate glucuronyltransferase (EC 2.4.1.18), and glutathione S-transferase (EC 2.5.1.18) [10–12]. Alkaline phosphatase (ALP, EC 3.1.3.1) has also been reported to be useful as a tumor marker enzyme [13]. Histochemical methods have also been used, in which GGT was selected as a marker [14]. The severity of carcinogenesis was determined by evaluating the extent of the GGT-positive foci. Chemical hepatocarcinogenesis as evaluated by histochemical methods has been shown to be affected differently by differing doses of α-tocopherol; the extent of GGT-positive foci was enhanced by diets containing 0.36 to 0.72% vitamin E, but this was not seen in a diet containing 1.5% of the vitamin.

We report the effect of different doses of α-tocopherol and γ-tocotrienol on chemical hepatocarcinogenesis induced by DEN and AAF in the rat. Hepatocarcinogenesis was evaluated by the determination of plasma ALP, plasma and liver GGT activities, as well as blood GSH.

MATERIALS AND METHODS

Chemicals. A basal diet of rat chow was purchased from Gold Coin Co., Klang, Malaysia. 2-Acetylaminofluorene (AAF), γ-glutamyl carboxynitroanilide, glycylglycine, *p*-nitrophenol phosphate, α-*dl*-tocopherol acetate, diethanolamine, 5-5′-dithiobis(2-nitrobenzoic acid), and all other reagents used were the highest grade commercially available (Sigma Chemical Co., St. Louis, MO). The γ-tocotrienol-enriched fraction (75% γ-tocotrienol, 25% α- and β-tocotrienol) used in this study was supplied by The Palm Oil Research Institute of Malaysia, Bangi, Malaysia.

Animal treatment. One hundred ninety-two male *Rattus norwegicus*, 120–160 g, 7–8 weeks old, were caged individually and maintained on normal or treated rat chow and water *ad libitum* for the duration of the experiment. The rats were divided into 14 groups consisting of the control, vitamin E treatment at two doses of $2E$ and $10E$, where $E=15$ and 17 mg/kg diet for γ-tocotrienol and α-tocopherol, respectively, DEN/AAF treatment, and finally DEN/AAF treatment

supplemented with either γ-tocotrienol or α-tocopherol at doses of $1E$, $2E$, $4E$, and $10E$.

Hepatocarcinogenesis was induced according to the method described by Solt and Farber [15] but without partial hepatectomy. DEN was injected once intraperitoneally at 200 mg/kg body weight followed by a recovery period of 2 weeks on a basal diet. The rats were then fed 0.02% (w/w) AAF-treated rat chow for another 2 weeks. Vitamin E supplementation was started 2 weeks after the DEN injection. The supplementation was continued until the time of sacrifice.

The rats were killed by cervical dislocation at 4 and 8 weeks from the DEN injection. Blood was taken immediately from the heart and placed into heparinized tubes; and 20-μl aliquots were taken for determination of GSH. The heparinized blood was then centrifuged at $800 \times g$ for 10 min after which the plasma was pipetted out and assayed for enzyme activity. The plasma preparation was carried out at 0–4°C.

Rat liver microsomal fraction was prepared as described by Speier and Wattenberg [16]. Rat liver was homogenized in 1.15% KCl (ratio of KCl to tissue, 3 : 1 v/w) with an Ultra Turrax homogenizer (Janker and Kunkel, Staufen, FRG) for 10 min and centrifuged at $10{,}000 \times g$ at 4°C for 20 min in a Sorvall RC-5B superspeed centrifuge. The supernatant was then centrifuged at $105{,}000 \times g$ at 4°C for 60 min in a Beckman L60 centrifuge. The resulting microsomal fraction was stored at −70°C before assay of enzyme activity.

Determination of glutathione, γ-glutamyl transpeptidase, and alkaline phosphatase. GSH estimation was carried out according to the method of Ellman [17]. γ-Glutamyl transpeptidase was assayed as previously described [8]. Protein concentration in the microsomal fraction was determined by the method of Bradford [18]. Alkaline phosphatase activity was assayed by the method of Jahan and Butterworth [19]. Plasma enzyme activities were expressed as IU/liter plasma. Liver microsomal γ-glutamyl transpeptidase activity was expressed as IU/g protein. Blood glutathione was expressed as a micromolar concentration.

Statistical analysis. The results obtained were analyzed by ANOVA and Student's t-test. A value of $p < 0.05$ was considered as significant.

RESULTS

Treatment with α-tocopherol or γ-tocotrienol had no effect on either plasma or microsomal GGT activities (Tables 1, 2). DEN/AAF treatment increased GGT activity in both the plasma and liver microsomes when compared with that of the control ($p < 0.001$). Plasma and microsomal GGT activities were attenuated by γ-tocotrienol supplementation in DEN/AAF-treated rats. α-Tocopherol supplementation to the DEN/AAF treated rats also showed a similar pattern for plasma and microsomal GGT activities ($p < 0.001$). The different doses of vitamin E used did not seem to affect the plasma and liver GGT activities. However, there

was some indication of $2E$ being the optimum dose as seen from the level of GGT activities.

ALP also showed a similar pattern as GGT activities with γ-tocotrienol, α-tocopherol, and DEN/AAF administration (Table 3). When the different doses of vitamin E were considered, no difference in ALP activities was observed for either type of vitamin E. While the supplementation of DEN/AAF-treated rats with γ-tocotrienol caused lowering of ALP activity as early as 4 weeks, α-tocopherol supplementation was significantly lowered only after 8 weeks.

There was no difference in blood GSH level between the control and groups supplemented with γ-tocotrienol or α-tocopherol (Table 4), except with $10E$ γ-tocotrienol, which increased it. Treatment with DEN/AAF caused a significant

Table 1. Comparison of plasma γ-glutamyl transpeptidase activities between control and treated rats supplemented with γ-tocotrienol or α-tocopherol.

Treatment	4 weeks	8 weeks		
Normal	6.3±0.3	6.0±0.4		
DEN/AAF	14.2±0.9*	13.4±0.9*		
	γ-Tocotrienol		α-Tocopherol	
	4 weeks	8 weeks	4 weeks	8 weeks
Normal+$2E$	6.7±0.4	5.7±0.5	4.8±0.3	4.9±0.4
Normal+$10E$	7.5±0.5	6.6±0.7	5.3±0.6	5.8±0.6
DEN/AAF+$1E$	11.3±1.0*	9.2±0.6*+	10.5±1.0*	8.9±0.9*+
DEN/AAF+$2E$	7.6±1.2+	9.2±0.6*+	10.7±1.8*	8.8±1.0*+
DEN/AAF+$4E$	10.3±1.1*+	9.9±0.5*+	11.6±0.7*	8.1±0.5*+
DEN/AAF+$10E$	10.1±1.2*+	10.4±0.4*+	10.5±1.5*	7.7±0.2*+

Enzyme unit expressed as IU/liter plasma. Values shown are mean±SEM. Significance range $^*p<0.01$–0.002, compared with normal control; $^+p<0.05$–0.002, compared with DEN/AAF control.

Table 2. Comparison of liver microsomal γ-glutamyl transpeptidase activities between control and treated rats supplemented with γ-tocotrienol or α-tocopherol.

Treatment	4 weeks	8 weeks		
Normal	4.1±0.2	4.2±0.3		
DEN/AAF	8.6±1.0*	6.9±0.5*		
	γ-Tocotrienol		α-Tocopherol	
	4 weeks	8 weeks	4 weeks	8 weeks
Normal+$2E$	4.7±0.6	4.4±0.8	4.7±0.7	4.6±0.3
Normal+$10E$	4.3±0.6	4.1±0.3	4.9±0.3	4.7±0.5
DEN/AAF+$1E$	6.1±0.6*	5.8±0.4*+	6.4±0.7*	6.1±0.4*
DEN/AAF+$2E$	5.0±0.7+	5.9±0.4*+	5.6±0.6*	5.4±0.4*+
DEN/AAF+$4E$	5.3±0.4*+	5.4±0.1*+	6.1±0.8*	5.2±0.3*+
DEN/AAF+$10E$	5.4±0.5*+	5.1±0.2*+	7.8±0.5*	5.1±0.3*+

Enzyme unit expressed as IU/mg protein. Values shown are mean±SEM. Significance range $^*p<0.05$–0.001, compared with normal control; $^+p<0.02$–0.002, compared with DEN/AAF control.

Table 3. Comparison of plasma alkaline phosphatase activities between control and treated rats supplemented with γ-tocotrienol or α-tocopherol.

Treatment	4 weeks	8 weeks		
Normal	229.3±20.3	254.2±25.7		
DEN/AAF	507.4±22.6*	560.0±44.3*		
	γ-Tocotrienol		α-Tocopherol	
	4 weeks	8 weeks	4 weeks	8 weeks
Normal+2E	296.7±32.6	306.7±32.0	309.4±30.6	218.5±38.8
Normal+10E	238.4±43.4	214.1±14.0	297.3±39.7	283.8±14.6
DEN/AAF+1E	404.7±27.1*+	250.2±23.2+	467.3±35.1*	341.2±7.9*
DEN/AAF+2E	404.5±36.6*+	319.0±14.3+	497.1±51.2*	284.1±31.5+
DEN/AAF+4E	386.9±34.0*+	316.4±20.1*+	470.2±52.6*	300.6±14.1+
DEN/AAF+10E	391.1±29.6*+	366.1±20.0*	493.3±39.4*	354.3±17.5+

Enzyme unit expressed as IU/liter plasma. Values shown are mean±SEM. Significance range *$p<0.02$–0.001, compared with normal control; +$p<0.05$–0.001, compared with DEN/AAF control.

Table 4. Comparison of blood glutathione concentration between control and treated rats supplemented with γ-tocotrienol or α-tocopherol.

Treatment	4 weeks	8 weeks		
Normal	4.8±0.3	5.5±0.2		
DEN/AAF	8.5±0.5*	7.4±0.3*		
	γ-Tocotrienol		α-Tocopherol	
	4 weeks	8 weeks	4 weeks	8 weeks
Normal+2E	4.6±0.2	5.8±0.4	4.8±0.2	4.4±0.2
Normal+10E	5.8±0.2*	6.1±0.2	5.9±0.2	5.9±0.1
DEN/AAF+1E	6.0±0.3*+	6.5±0.3*	4.9±0.3+	5.3±0.1+
DEN/AAF+2E	7.6±0.4*	4.8±0.5+	5.9±0.1+	5.9±0.2+
DEN/AAF+4E	7.8±0.4*	5.4±0.5+	6.1±0.2*+	6.1±0.3+
DEN/AAF+10E	8.0±0.5*	4.6±0.2+	6.0±0.3+	6.4±0.2*

Blood glutathione concentration expressed as μM. Values shown are mean±SEM. Significance range *$p<0.05$–0.001, compared with normal control; +$p<0.01$–0.001, compared with DEN/AAF control.

increase in GSH level at both 4 and 8 weeks when compared with the control level. After 4 weeks, the DEN/AAF-treated rats supplemented with γ-tocotrienol had significantly elevated GSH values ($p<0.01$) that were equivalent to those of the DEN/AAF-treated rats. This was observed for all doses except the lowest dose used. However, after 8 weeks, GSH levels in the DEN/AAF-treated animals supplemented with γ-tocotrienol were lower than those in the DEN/AAF-treated rats at all the doses used except for dose 1E ($p<0.05$–$p<0.001$). α-Tocopherol supplementation of DEN/AAF-treated rats resulted in GSH levels similar to those for the γ-tocotrienol-supplemented DEN/AAF-treated rats, except that the differences were obvious from 4 weeks of treatment ($p<0.05$–$p<0.001$).

DISCUSSION

The protective effect of tocopherols against chemical carcinogenesis has been extensively studied in various animals models with different routes of vitamin E administration [14, 20]. While tocotrienols have been reported to inhibit the growth of several transplantable murine tumors, particularly sarcoma 180, Ehrlich carcinoma, IMC carcinoma and Meth A fibrosarcoma, the tocopherols had no such effect [7]. We reported previously that plasma and microsomal GGT activities were increased with carcinogen treatment, whereas γ-tocotrienol supplementation moderated the increase in these activities [8]. In humans, plasma GGT determinations have also been reported to correlate closely with the clinical status; i.e., significantly elevated GGT levels corresponded to disease progression and death. Patients with low enzyme levels were found to be free from disease [21, 22]. In a more recent report, GGT isoenzymes were determined as a potential specific marker for primary or metastatic liver neoplasia [23]. The results of the present study showed a similar pattern of enzyme activities in both the plasma and liver microsomes. Increases in plasma GGT activities could be due to an overflow from the neoplastic cells in the liver and could be considered a more accurate measure of the extent of the carcinogenic process.

The determination of ALP as a marker of neoplasia has not been used as extensively as GGT. However, there have been reports of the diagnostic use of ALP and its isoenzymes for liver cancer in humans [24]. Elevation in ALP enzyme activities has also been reported in lung cancer, seminoma, and ovarian cancer in women [13]. So far, the determination of ALP in animals with experimentally induced hepatocarcinogenesis has not been reported.

The different doses of vitamin E used in this study did not show great variation in terms of their effect on the severity as assessed by measurement of the ALP and GGT levels. However, it seemed that the optimum dose for supplementation of vitamin E was 30 mg for γ-tocotrienol and 34 mg for α-tocopherol. The lowest dose used in the present study (15 mg γ-tocotrienol, 17 mg α-tocopherol) was insufficient for maximum protection while higher doses above 30 mg γ-tocotrienol or 34 mg α-tocopherol did not show further attenuation of the marker enzyme activities.

The detoxification of carcinogens, which are in nature strong electrophiles, depends on the rate of reaction and concentration of GSH. It has been suggested that GSH may be important as a free radical trap in antimutagenesis and anticarcinogenesis, since GSH readily donates a hydrogen atom to free radicals, notably hydroxy and carbon radicals [25]. The results obtained in the present study showed that both γ-tocotrienol and α-tocopherol, when used alone, did not alter the blood GSH level. The increase in GSH level due to the DEN/AAF treatment is expected since GSH levels have been shown to increase in the presence of foreign compounds including carcinogens [26]. Tissue GSH has been shown to

increase 1.5–2.5-fold in hepatocyte nodules after exposure to carcinogens.

In conclusion, γ-tocotrienol and α-tocopherol supplementation are protective in the early stages of hepatocarcinogenesis. The optimum dose appears to be 30 mg/kg for γ-tocotrienol and 34 mg/kg for α-tocopherol.

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REFERENCES

1. Knekt, P. (1991): Role of vitamin E in the prophylaxis of cancer. *Ann. Med.*, **23**, 3–12.
2. Tappel, A.L. (1972): Vitamin E and free radical peroxidation of lipids. *Ann. NY Acad. Sci.*, **203**, 12–28.
3. Tengerdy, R.P. (1990): The role of vitamin E in immune response and disease resistance. *Ann. NY Acad. Sci.*, **487**, 24–33.
4. Packer, L. (1991): Protective role of vitamin E in biological systems. *Am. J. Clin. Nutr.*, **53**, 1050S–1055S.
5. Moriguchi, S., Kobayashi, N., and Kishino, Y. (1990): High dietary intakes of vitamin E and cellular immune functions in rats. *J. Nutr.*, **120**, 1096–1102.
6. Bendich, A., Gabriel, E., and Machlin, L.J. (1986): Dietary vitamin E requirement for optimum immune responses in rats. *J. Nutr.*, **116**, 675–681.
7. Komiyama, K., Iizuka, K., Yamaoka, M., Watanabe, H., Tsuchiya, N., and Umezawa, I. (1989): Studies on the biological activity of tocotrienols. *Chem. Pharm. Bull.*, **37**, 1369–1371.
8. Wan Ngah, W.Z., Jarien, Z., San, M.M., Marzuki, A., Md Top, A., Shamaan, N.A., and Khalid, B.A.K. (1991): Effect of tocotrienols on hepatocarcinogenesis induced by 2-acetylaminofluorene in rats. *Am. J. Clin. Nutr.*, **53**, 1076S–1081S.
9. Rahmat, A., Wan Ngah, W.Z., Shamaan, N.A., Md Top, A.G., and Khalid, B.A.K. (1993): Long-term administration of tocotrienols and tumor-marker enzyme activities during hepatocarcinogenesis in rats. *Nutrition*, **9**, 229–232.
10. Yamamoto, K., Yokose, Y., and Nakajima, A. (1988): Comparative histochemical investigation of the γ-glutamyl transpeptidase during N-nitrobis(2-hydroxypropyl)amine induced lung carcinogenesis in rats. *Carcinogenesis*, **9**, 399–404.
11. Sato, K., Kitahara, A., Satoh, K., Ishikawa, T., Tatematsu, M., and Ito, N. (1984): The placental form of glutathione S-transferase as a new marker protein for preneoplasia in rat chemical hepatocarcinogenesis. *Gann*, **75**, 199–202.
12. Yin, Z., Sato, K., Tsuda, H., and Ito, N. (1982): Changes in activities of uridine diphosphate-glucuronyltransferase during chemical hepatocarcinogenesis. *Gann*, **73**, 239–248.
13. Fishman, W.H. (1987): Clinical and biological significance of an isoenzyme tumor marker-PLAP. *Clin. Biochem.*, **20**, 387–392.
14. Ura, H., Denda, A., Yokose, Y., Tsutsumi, M., and Konishi, Y. (1987): Effect of vitamin E on the induction and evolution of enzyme-altered foci in the liver and rats treated with diethylnitrosamine. *Carcinogenesis*, **8**, 1595–1600.
15. Solt, D., and Farber, E. (1976): New principle for the analysis of chemical carcinogens. *Nature*, **263**, 701–703.
16. Speier, C.L., and Wattenberg, L.W. (1975): Alterations in microsomal metabolism of benzo(*a*)pyrene in mice fed butylated hydroxylanisole. *J. Natl. Cancer Inst.*, **55**, 469–472.
17. Ellman, G.L. (1959): Tissue sulfhydryl groups. *Arch. Biochem. Biophys.*, **82**, 70–77.
18. Bradford, M.M. (1976): A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–

254.

19. Jahan, M., and Butterworth, P.J. (1986): Alkaline phosphatase of chick kidney. *Enzyme*, **35**, 61–69.

20. Birt, D.F. (1986): Update on the effects of vitamin A, C and E and selenium on carcinogenesis. *Proc. Soc. Exp. Biol. Med.*, **183**, 311–320.

21. Murray, J.L., Lerner, M.P., and Nordquist, R.E. (1982): Elevated γ-glutamyl transpeptidase levels in malignant melanoma. *Cancer*, **49**, 1439–1443.

22. Sahm, D.F., Murray, J.L., Munson, P.L., Nordquist, R.E., and Lerner, M.P. (1983): γ-Glutamyl transpeptidase levels as an aid in the management of human cancer. *Cancer*, **52**, 1673–1678.

23. Saccheti, L., Castaldo, G., and Salvatore, F. (1988): The γ-glutamyl transpeptidase isoenzyme pattern in serum as a signal discriminating between hepatobiliary diseases, including neoplasia. *Clin. Chem.*, **34**, 352–355.

24. Moss, D.W. (1987): Diagnostic aspects of alkaline phosphatase and its isoenzymes. *Clin. Biochem.*, **20**, 225–230.

25. Ketterer, B. (1988): Protective role of glutathione and glutathione transferase in mutagenesis and carcinogenesis. *Mutat. Res.*, **202**, 343–361.

26. Ahluwalia, M., and Farber, E. (1984): Alteration in glutathione status in early hyperplastic nodules. *Proc. Am. Assoc. Cancer Res.*, **25**, 15–17.