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Primary research

## High dietary level of synthetic vitamin E on lipid peroxidation, membrane fatty acid composition and cytotoxicity in breast cancer xenograft and in mouse host tissue

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### Abstract

**Background:** d- $\alpha$ -tocopherol is a naturally occurring form of vitamin E not previously known to have antitumor activity. Synthetic vitamin E (sE) is a commonly used dietary supplement consisting of a mixture of d- $\alpha$ -tocopherol and 7 equimolar stereoisomers. To test for antilipid peroxidation and for antitumor activity of sE supplementation, two groups of nude mice bearing a MDA-MB 231 human breast cancer tumor were fed an AIN-76 diet, one with and one without an additional 2000 IU/kg dry food (equivalent to 900 mg of all-rac- $\alpha$ -tocopherol or sE). This provided an intake of about 200 mg/kg body weight per day. The mice were killed at either 2 or 6 weeks after the start of dietary intervention. During necropsy, tumor and host tissues were excised for histology and for biochemical analyses.

**Results:** Tumor growth was significantly reduced by 6 weeks of sE supplementation. Thiobarbituric acid reactive substances, an indicator of lipid peroxidation, were suppressed in tumor and in host tissues in sE supplemented mice. In the sE treated mice, the fatty acid composition of microsomal and mitochondrial membranes of tumor and host tissues had proportionately less linoleic acid (n-6 C 18-2), similar levels of arachidonic acid (n-6 C 20-4), but more docosahexanoic acid (n-3 C 22-6). The sE supplementation had no significant effect on blood counts or on intestinal histology but gave some evidence of cardiac toxicity as judged by myocyte vacuoles and by an indicator of oxidative stress (increased ratio of Mn SOD mRNA over GPX1 mRNA).

**Conclusions:** At least one of the stereoisomers in sE has antitumor activity. Synthetic vitamin E appears to preferentially stabilize membrane fatty acids with more double bonds in the acyl chain. Although sE suppressed tumor growth and lipid peroxidation, it may have side-effects in the heart.

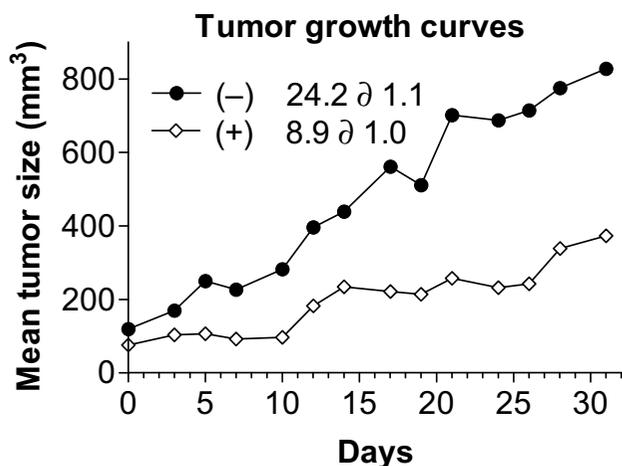
### Background

Vitamin E is an essential fat-soluble vitamin. The general

term vitamin E refers to eight naturally occurring and synthetic tocopherols and tocotrienols and their acetate and

succinate derivatives. Although the naturally occurring forms of vitamin E have lipid-soluble antioxidant properties that protect cell membranes against damage by free radicals, the acetate and succinate derivatives that are esterified at the C-6 position of the chromanol ring do not have antioxidant properties unless the esterification is hydrolyzed and free tocopherol is regenerated [1-3].

Synthetic vitamin E, a form of vitamin E commonly used as a dietary supplement, is the form used in the *in vivo* studies reported here. It is a mixture of eight stereoisomers in equal amounts designated "dl" or all-rac tocopherol and does have antioxidant properties [4]. Thus, different forms of vitamin E can have different antioxidant properties as well as differences in their absorption, distribution, and metabolism [4]. There are also reports that some forms of vitamin E have activity against cancer cells *in vitro* [3,5]. For example, tocotrienol and vitamin E succinate are reported to have antiproliferative activity in human cancer cells whereas some forms of vitamin E are reported to induce apoptosis in cancer cell types but not in normal cell types [3]. Specifically, these apoptogenic forms include RRR- $\alpha$ -tocopheryl succinate and RRR- $\delta$ -tocopherol while the  $\alpha$ ,  $\beta$ , and  $\gamma$  tocopherol and acetate derivatives of tocopherol were not apoptogenic [3]. There are few reports on the use of vitamin E for treating tumorous cancers *in vivo* but the results are inconsistent [4-10]. It is therefore essential that the form of vitamin E used in any research study as well as the dosage and the mode of administration be defined.



**Figure 1**  
Rate of human MDA-MB-231 breast cancer tumor growth in nude mice fed the AIN-76 food formula without (-) and with (+) synthetic vitamin E supplementation (2000 IU/kg dry food) for six weeks (mean  $\pm$  SE, n = 5 mice/group). Linear regression slopes of data indicated. Slopes are significantly ( $p < 0.01$ ) different due to vitamin E supplement.

The study reported on here was originally designed to test the ability of a high dietary dose of sE to prevent lipid peroxidation in nude mice bearing a human breast cancer xenograft MDA-MB-231 [11,12]. To assure suppression of lipid peroxidation, sE was added to the diet at a dose level. For example, the vitamin E requirement for mice when all-rac- $\alpha$ -tocopherol acetate is used as the dietary source is 32 mg/kg for diets in which lipids comprise less than 10 percent of the diet [1]. Thus, the 900 mg/kg diet used in this study is 28 fold higher than this estimated requirement. This is in contrast to human recommendations which are given in mg of  $\alpha$ -tocopherol equivalents with the adult RDA set at 15 mg/day and the upper limit set at 1000 mg/day. The observed antitumor activity of this sE dietary supplementation was an unexpected experimental result as sE was not previously reported to have antitumor activity although vitamin E may give modest protection against the risk of breast cancer [5]. Based on the observed antitumor activity of high dose sE in the diet, it became important to investigate if the sE supplement, when fed at such a high dose, was associated with the modification of the fatty acid composition of mitochondrial and microsomal membranes and if the sE was acting as an antioxidant or possibly a prooxidant in tumor and in host tissues. Given that such a high dose of sE has antitumor activity, it also became important to look for possible side-effects in host tissues prior to further preclinical trials.

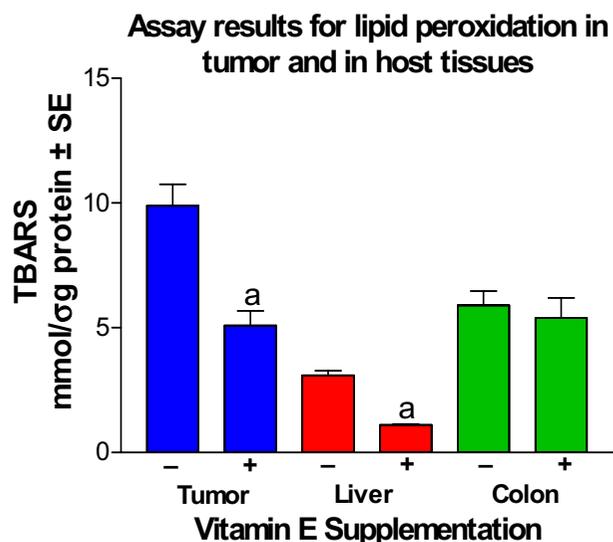
## Results

### Tumor growth

The mean tumor growth rate was calculated using linear regression analysis of mean tumor size over time. After two weeks of dietary intervention, the mean growth rate of the group of mice fed the sE supplement was half that of mice not fed the sE supplement however this difference was not quite significant at the  $p < 0.05$  level. Tumor growth rate in mice not sacrificed after 2 weeks of dietary intervention was followed for an additional four weeks. Analysis of mean tumor growth rate for the entire 42 days of intervention revealed that the mean tumor growth rate of mice fed the sE supplement was 63% slower than the mean tumor growth rate of mice not fed the sE supplement (significant at  $p < 0.01$  as illustrated in Fig. 1).

### Lipid peroxidation

Tumor and host tissue malondialdehyde and malondialdehyde by-products were analyzed by the thiobarbituric acid reactive substrates (TBARS) assay to estimate the extent of lipid peroxidation after six weeks of intervention. The TBARS findings are summarized in Fig. 2. The mean value of TBARS was significantly ( $p < 0.01$ ) lower in the tumor and in the liver of the mice fed sE but was not significantly different ( $p > 0.05$ ) in the colon.

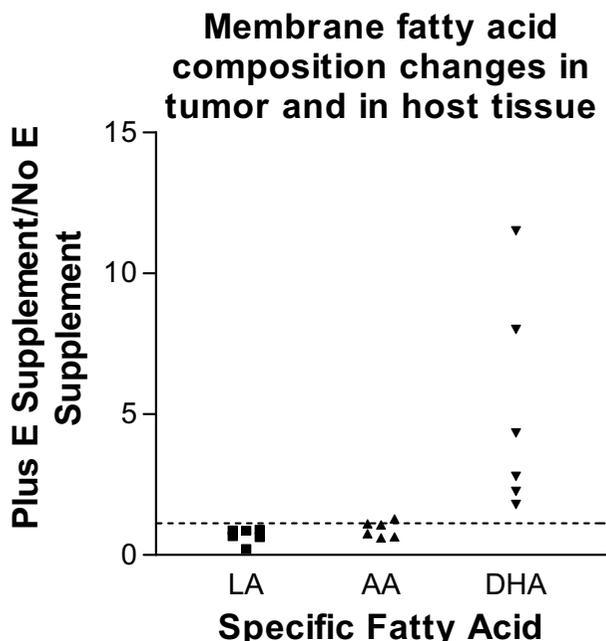


**Figure 2**  
Effect of six weeks dietary intervention with the AIN-76 food formula without (-) or with (+) synthetic vitamin E supplement (2000 IU/kg dry food) on lipid peroxidation as assessed by thiobarbituric acid reactive substrates (TBARS). Number of mice analyzed ranged from 3 to 5. <sup>a</sup> Significantly ( $p < 0.01$ ) lower than without vitamin E supplement

**Fatty acid composition of microsomal and of mitochondrial membranes**

Data on the percent of the total fatty acid composition for linoleic acid (LA), for arachidonic acid (AA), and for docosahexanoic acid (DHA) in the microsomal and in the mitochondrial fractions of tumor, of liver, and of colon after two weeks of dietary intervention both with and without sE supplementation are summarized in Table 1. The mean levels of LA were lower in both microsomal and mitochondrial fractions of the tumor, the liver, and the colon of mice fed sE than in mice without the sE. That 6 of 6 mean values of LA were lower with sE supplementation indicates a consistent pattern of membrane LA composition change in the tumor and in the host tissues. Conversely, the mean level of DHA was higher in the microsomal and mitochondrial fractions of the tumor, the liver, and the colon of mice fed sE than in mice without the sE supplement. That 6 of 6 mean values of DHA were higher with sE supplementation indicates a consistent pattern of membrane DHA change in the tumor and in the host tissues. The mean level of AA in the membrane fractions of the three tissues was not consistently changed.

To further explore the effect of sE supplementation on membrane fatty acid composition, the relative level of



**Figure 3**  
Mean ratio (% of total fatty acid composition in: + sE supplement/- sE supplement) of linoleic acid (LA), arachidonic acid (AA), and docosahexanoic acid (DHA) in microsomal and mitochondrial fractions of tumor, liver, and colon after two weeks consumption of the vitamin E supplement (analyses of all mean values in Table 1). Mean was lower for LA (0.70) and was higher for DHA (4.9).

each of the three fatty acids in each fraction of each tissue was expressed as a ratio of the level with sE supplement over the level without sE supplement. The results of this exercise are plotted in Fig. 3. The ratio is consistently lower than unity for LA (mean 0.70) and consistently higher than unity for DHA (mean 4.9). The mean ratio for AA was near one, indicating no significant difference.

**Body weight**

Data on change in body weights at two weeks and at six weeks from the body weight at the start of dietary intervention are summarized in Table 2. The mean values for change in body weights at two or at six weeks are not significantly different due to diet. The body weight loss observed at six weeks can be attributed to the presence of the growing tumor.

**Blood counts**

Results of blood counts, as summarized in Table 3, reveal no significant differences in white cell (WBC), in red

**Table 1: The percent of total fatty acid composition of linoleic acid (LA, n6 18-2), arachidonic acid (AA, n6 20-4), and docosahexaenoic acid (DHA, n3 22-6) in microsomal and in mitochondrial fractions of tumor, of liver and of colon following a two week dietary intervention of AIN-76 food formula without (-) and with (+) supplementation with synthetic vitamin E (2000 IU/kg dry food). Mean  $\pm$  SD and number of mice (n) are listed.**

Vitamin E supplement	Fraction	n	LA, 18-2 <sup>a</sup>	AA, 20-4	DHA, 22-6 <sup>b</sup>
<b>Tumor</b>					
-	Micro	4	8.7 $\pm$ 5.0	7.4 $\pm$ 1.2	0.3 $\pm$ 0.3
+	"	5	1.9 $\pm$ 2.1	7.9 $\pm$ 4.2	1.3 $\pm$ 1.3
-	Mito	4	7.0 $\pm$ 5.9	9.6 $\pm$ 2.8	0.2 $\pm$ 0.5
+	"	5	4.4 $\pm$ 5.9	5.8 $\pm$ 3.7	2.3 $\pm$ 4.9
<b>Liver</b>					
-	Micro	4	12.9 $\pm$ 5.1	4.9 $\pm$ 1.8	1.0 $\pm$ 0.9
+	"	5	11.3 $\pm$ 1.7	5.4 $\pm$ 0.9	1.8 $\pm$ 0.3
-	Mito	4	14.2 $\pm$ 1.5	8.0 $\pm$ 0.9	0.5 $\pm$ 0.1
+	"	5	12.9 $\pm$ 1.2	6.1 $\pm$ 1.8	1.4 $\pm$ 0.5
<b>Colon</b>					
-	Micro	3	5.3 $\pm$ 2.1	6.3 $\pm$ 2.2	0.3 $\pm$ 0.3
+	"	5	4.6 $\pm$ 1.2	4.1 $\pm$ 1.3	2.4 $\pm$ 1.1
-	Mito	3	9.4 $\pm$ 2.1	2.9 $\pm$ 0.2	0.8 $\pm$ 0.5
+	"	5	6.3 $\pm$ 1.3	3.7 $\pm$ 1.6	1.8 $\pm$ 1.0

<sup>a</sup>6 of 6 means lower with vitamin E supplement ( $p < 0.01$  by Fisher's exact test) <sup>b</sup>6 of 6 means higher with vitamin E supplement ( $p < 0.01$  by Fisher's exact test)

**Table 2: Change in body weight (g) at two weeks and at six weeks from the start of dietary intervention with AIN-76 formula  $\pm$  supplementation with 2000 IU/kg of dry food with synthetic vitamin E (mean  $\pm$  SE, number of mice in parenthesis).**

Vitamin E supplement	Two weeks	Six weeks
-	0.38 $\pm$ 0.30 (15)	-0.25 $\pm$ 0.53 (8)
+	0.22 $\pm$ 0.22 (17)	-0.16 $\pm$ 0.29 (5)

No significant difference due to supplement at two or six weeks  
Weight loss at six weeks attributed to growing tumor.

blood cell (RBC) or in platelet (PLT) numbers due to two weeks of sE supplementation.

#### Colon histology

Colon crypt height expressed in number of cells from the crypt base to the crypt mouth was scored as an indicator of damage attributable to sE supplementation. The crypt column heights, (number of cells, mean  $\pm$  SE) was 21.24  $\pm$  0.54 (15) for mice without the sE supplementation and 21.63  $\pm$  0.51 (11) for mice with the sE supplementation. These means are not significantly different. No histologi-

**Table 3: Hematological measurements in nude mice bearing MDA-MB-231 breast carcinoma xenografts after supplementation with (+) or without (-) synthetic Vitamin E (2000 IU/kg food) (mean  $\pm$  SE, numbers of mice in parentheses) for two weeks.**

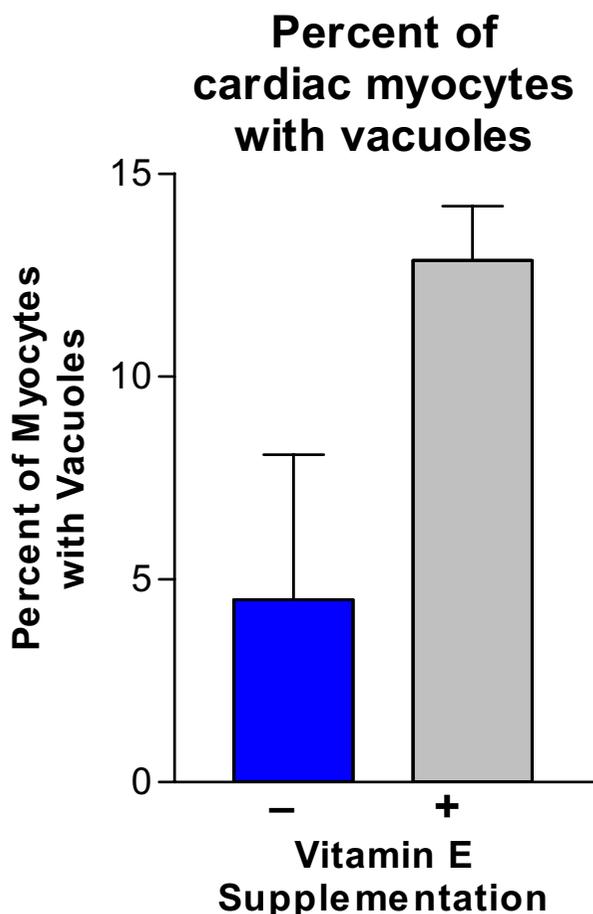
Vitamin E supplement	WBC m/ $\mu$ l	RBC m/ $\mu$ l	PLT k/ $\mu$ l
- (3)	0.62 $\pm$ 0.14	8.77 $\pm$ 0.04	471 $\pm$ 173
+	1.04 $\pm$ 0.43	8.50 $\pm$ 0.15	461 $\pm$ 130

WBC, RBC, and PLT counts were not significantly different due to diet.

cal differences attributable to the dietary intervention with sE were observed.

#### Heart histopathology and antioxidant enzyme gene expression

Histological cross-sections of the heart taken one-third of the distance from the apex were studied for indications of side-effects of high dietary levels of sE. Vacuole formation in the cardiac myocytes has been used as a marker of cardiotoxicity [13]. The mean percent of cardiac myocytes with one or more vacuoles in the subendocardial



**Figure 4**

The percent of cardiac myocytes with vacuoles in the subendocardial myocytes of the left ventricle scored in 4  $\mu\text{m}$  thick hematoxylin and eosin stained sections. At least 500 myocytes were scored per heart. The means ( $\pm$  SE) of 4 (-) and 7 (+) hearts are graphed.

myocytes of the left ventricle was significantly ( $p < 0.05$ ) higher in mice fed the sE supplement (Fig. 4). Based on this indication of cardiotoxicity, the remaining portion of the heart that had been snap frozen in liquid nitrogen at the time of sacrifice was used to look for differences in expression of antioxidant genes using quantitative RT PCR methodology.

The results of the RT PCR findings are summarized in Table 4. Of the antioxidant enzyme mRNAs measured, the only mRNA that differed significantly due to diet was the inducible form of heme oxygenase, which is identical to heat shock protein 32. A complete set of data was taken on each heart, which allowed calculation of the ratio of each

antioxidant gene transcript to each of the other gene transcripts. The results of this ratio analysis reveal that the feeding of sE supplement caused the ratio of MnSOD over GPX-1 to significantly increase (see Table 4).

#### **Assay for chromosomal breakage or loss**

The presence of one or more micronuclei in peripheral erythrocytes in mice is an accepted marker of chromosomal breakage or loss that occurred prior to the extrusion of the nucleus during erythrocyte differentiation. Results of the scoring of the numbers of peripheral erythrocytes with and without a micronucleus are summarized in Table 5. Included in Table 5 are data from mice treated with a series of injections of doxorubicin. Doxorubicin (DOX) is a cytotoxic drug with prooxidant properties known to cause genotoxic damage resulting in an increase in number of erythrocytes with a micronucleus. The results from the DOX treated mice served as a positive control. Results of statistical analyses demonstrate a significant increase in erythrocytes with micronuclei due to DOX treatment but no significant difference due to sE supplementation. Note that the highest mean incidence of micronuclei occurred in the group of mice fed the sE supplement and treated with DOX.

#### **Discussion**

Vitamin E was established as an essential micronutrient for proper fetal development [1] and the amount of vitamin E required to maintain proper fetal development has been defined in International Units. In addition to its role in reproduction, vitamin E is known to be a lipid-soluble antioxidant that blocks peroxidation of polyunsaturated fatty acids in cellular membranes and is known to stabilize biological membranes [14–17]. Because vitamin E has multiple biological activities, it is best to report dosage in weight units. The recommended dietary allowance and tolerable upper intake limit for sE, the commercially available form, has been calculated to be about 22 and 1470 mg per day respectively for 70 kg adult humans [1].

In animal species, oral intake of up to 200 mg/kg body weight per day was reported not to result in noticeable side effects [18]. Thus, we selected to use a dietary dose of sE at the level of 200 mg/kg body weight per day in tumor bearing mice in an attempt to maximize inhibition of lipid peroxidation in the cellular membranes of the tumor without an expectation of finding harmful side-effects. Specifically, this experiment was originally designed to test the hypothesis that DOX, a prooxidative cancer chemotherapeutic drug, inhibits tumor growth by increasing lipid peroxidation products in the tumor to cytostatic or cytotoxic levels and that suppression of lipid peroxidation by sE would suppress the antitumor effects of DOX [11,12]. The results demonstrated that sE supplementation did markedly suppress DOX-induced lipid peroxida-

**Table 4: Results of quantitative RT-PCR for antioxidant enzyme gene expression in the heart of mice fed the AIN-76 food formula with (+) or without (-) a synthetic vitamin E supplement (2000 IU/kg dry food) for six weeks.**

Vitamin E supplement	Enzyme <sup>a</sup>						
	GPX-I	CAT	CZ SOD	Mn SOD	EC SOD	HO-1 <sup>b</sup>	Mn SOD <sup>b</sup> GPX-I
-	.325 ± .019	.495 ± .048	.915 ± .038	2.106 ± .166	.064 ± .005	.020 ± .002	6.55 ± 0.56
+	.252 ± .028	.522 ± .029	.915 ± .054	2.538 ± .175	.059 ± .005	.013 ± .002	10.49 ± 1.16

<sup>a</sup> Abbreviations: GPX-I – glutathione peroxidase; CZ SOD – copper zinc superoxide dismutase; CAT – catalase; Mn SOD – manganese superoxide dismutase; EC SOD – extracellular superoxide dismutase; HO-1-inducible form of heme oxygenase <sup>b</sup> Means differ to significant extent using two-tailed t test. Data are presented as the mean ± SE number of transcripts for each mRNA after normalization to expression of beta actin. N = 5 mice per group.

tion, yet tumor growth was still suppressed in mice treated with DOX and sE. It was concluded that increased levels of lipid peroxidation products were not the sole cause of tumor growth inhibition by DOX. As reported here (Fig. 1), the dietary supplementation with this high dose of sE, by itself, retarded tumor growth to a significant extent.

It is not known how such a high intake of sE works to suppress tumor growth. What is known from the current study is that the sE supplement reduced lipid peroxidation (Fig. 1) and preferentially stabilized membrane polyunsaturated fatty acids with more double bonds (Fig. 3). It has been proposed that vitamin E may incorporate into cellular membranes by association of the tocopherol side chain with the polyenoic fatty acid residues in the membrane fatty acids [15,19]. This interaction may stabilize cell membranes by making highly unsaturated fatty acids less liable to peroxidation as well as by making highly unsaturated fatty acids less available for phospholipid hydrolysis by phospholipase [15,16]. The latter action may reduce mobilization of arachidonic acid (AA) from the membranes and in this way reduce the amount of AA available as a substrate to cyclooxygenase and/or lipoxygenase to produce eicosanoids with mitogenic and with inflammatory properties [17,20]. Other possible antitumor actions include: induction of apoptosis, interference with hormone production, modulation of cellular signaling and gene transcription, and induction of differentiation [4,21–24].

In comparison to the inhibitory effect of vitamin E on mammary tumor growth (this report), Cognault et al. report a stimulatory effect of vitamin E on mammary tumor growth. Specifically, Cognault et al. report that when the diet was high in omega-3 PUFAs, tumor growth was significantly increased by vitamin E supplementation. However, when the diet was relatively low in omega-3 PUFAs, the promotional effect of vitamin E was not observed [8]. It is noteworthy that the diet used in the presently reported study was very low in omega-3 PUFAs and that this di-

et, combined with the high dietary level of sE, resulted in suppression of tumor growth. Thus, the different results may be attributable to the type and the amount of omega-3 polyunsaturated fatty acids (PUFAs) in the diet.

Evidence for cardiotoxicity, as observed in this study, was not expected as supplementation with vitamin E at extremely high doses gave no significant indications of harmful side-effects in animals or in humans [18,25]. Perhaps vitamin E supplementation acts to inhibit lipid peroxidation but at the same time acts to down regulate other protective antioxidant systems as suggested by the antioxidant enzyme gene expression data in Table 4. If gene expression levels in Table 4 reflect antioxidant enzyme activity, then an increase in SOD and decrease in GPX without an increase in CAT would result in accumulation of inorganic and organic peroxides and hydroperoxides that can react with Fe<sup>2+</sup> to yield highly reactive free radical products that can damage cellular proteins and DNA as well as lipids. Likewise, the sE supplementation used caused low expression of the stress-induced isoform of heme oxygenase, (HO-1). HO-1 catalyzes the breakdown of prooxidant heme to biliverdin with release of carbon monoxide and iron ion. It should be realized that these observations are suggestive of cardiotoxicity but are not definitive.

Early studies suggested that vitamin E lessened the cardiotoxic effects of DOX chemotherapy [26] whereas other studies indicate potentiation of DOX induced cardiotoxicity [27,28]. Shinoyawa et al. [28] suggest that vitamin E, when combined with DOX, may increase levels of toxic products in the heart and in the tumor while Liu and Tan [27] suggest that vitamin E may have prooxidant properties at high dosage levels.

The micronuclei results in Table 5 suggest that while sE protects against lipid peroxidation, it did not protect the genome of erythroblasts against the prooxidant effects of DOX in this study. Thus, while the toxic effect of DOX was

**Table 5: Results of peripheral erythrocyte (RBC) assay for chromosomal breakage or loss. Frequency of micronuclei (MN) per 2000 erythrocytes counted in blood smear from each mouse (mean  $\pm$  SE, n = number of mice).**

Vitamin E supplement <sup>a</sup>	$\pm$ DOX <sup>b</sup>	n	MN/2000 RBCs <sup>c</sup>
-	-	5	7.6 $\pm$ 1.4 <sup>d</sup>
-	+	6	15.5 $\pm$ 2.5 <sup>e</sup>
+	-	5	5.6 $\pm$ 1.1 <sup>d</sup>
+	+	8	19.3 $\pm$ 1.9 <sup>e</sup>

<sup>a</sup>Added (+) synthetic vitamin E at 2000 IU/kg dry AIN-76 food <sup>b</sup>DOX = doxorubicin treatment: 5 mg/kg body weight i.v. in a lateral tail vein starting two weeks after start of dietary modification and given at 0, 4, 8, 12, 19, 26 and 33 days. Blood smears were made and stained with acridine orange one day after the last injection of DOX. <sup>c</sup>Results of 2-way ANOVA, significant main effect due to DOX. Results of SNK multiple comparison test, means in column which do not share a superscript letter are significantly different,  $p < 0.05$

not significantly enhanced by vitamin E supplementation, neither was the toxic effect of DOX lessened by vitamin E supplementation.

### Conclusion

The adage that more is better may not hold true for vitamin E as there may be beneficial effects at certain levels but not at high levels. Clearly, more research is needed on the antitumor effects of sE but caution is advised that sE may also have side-effects in the heart.

### Methods and Materials

Methods involving: preparation of cells, animals, tumor and body weight measurements, necropsy and tissue processing, products of lipid peroxidation, gas chromatography, and statistical analyses have been described in a prior issue of this journal [12]. The source of dl- $\alpha$ -tocopherol (synthetic vitamin E) was ICN Biochemical (Costa Mesa, CA). This experiment was conducted at the University of Texas Health Science Center at San Antonio. All animal use and handling was approved by UTHSCSA Institutional Animal Care and Use Committee.

#### Diet components, chemicals and composition

Purified high nitrogen casein, pure cornstarch, Alphacel (non-nutritive bulk cellulose), AIN-76 vitamin mixture, AIN-76 mineral mixture and choline bitartrate (99% pure) were obtained from ICN Nutritional Biochemicals, Cleveland, OH. Imperial brand (Sugarland, TX) extra fine pure cane sugar and 100% pure corn oil (Wesson) were purchased locally. D.L. methionine was purchased from Sigma, St. Louis, MO. Corn oil contains about 50% linoleic acid, 23% oleic acid, 10% C16 fatty acids and <1% n-3 PUFAs. The composition of the diet was as follows: 5% corn oil, 50% sugar, 20% casein, 15% cornstarch, 1% AIN-76 vitamin mix (the mix contains 20 g/kg of dl- $\alpha$ -to-

copherol acetate), 3.5% AIN-76 mineral mix, 0.2% choline bitartrate, 0.3% D.L. methionine, and 5% Alphacel fiber [12].

#### Histological analyses of heart and colon

At sacrifice, the heart and colon were removed. The heart was crosscut through the ventricles 1/3 of the distance between the apex and the atrium, and then the apex section was fixed in OmniFix II (Mt. Vernon, NY) and then embedded, cut surface down, in molten paraffin. This provided a consistent histological section for estimates of myocyte vacuolization and toxicity. Four  $\mu$ m thick sections were stained with hematoxylin and eosin. The number of myocytes with vacuoles in the subendocardial region of the left ventricle was counted. Cross sections of the descending colon were scored for number of cells in a crypt column from crypt base to mouth.

#### Blood counts

Blood was obtained by cardiac puncture at sacrifice and assayed within 3 hours of collection. A Coulter STKS hematology analyzer was used for counts of red blood cells, white blood cells, and platelets in EDTA anticoagulated blood of the mice.

#### Micronucleus assay

At the time of necropsy, blood was collected by heart puncture using a 1 ml syringe fitted with a 22G needle. Immediately, small drops of blood were placed on clean microscopic slides. The blood was pushed behind another slide held at a 45° angle to form a thin smear over an area of 3–4 cm. All smears were air-dried for 30–45 minutes. The cells on the slides were fixed in absolute methanol for 30 minutes. All slides were air-dried and stored in a box kept in a dark place at room temperature or at 4°C. The slides were stained with acridine orange before microscopic evaluation.

All slides were examined under 1000 $\times$  magnification using a fluorescent microscope fitted with appropriate filters for acridine orange stain. For each animal, the percentage of 1000 erythrocytes with micronuclei was scored.

#### RNA extraction and quantitative reverse transcription-polymerase chain reaction

All heart samples were immediately frozen in liquid nitrogen for RNA extraction. RNA was extracted by standard methods [29] and analyzed by reverse transcription followed by real-time quantitative polymerase chain reaction (PCR) for the transcripts of interest. The methodology of quantitative PCR has been described in detail previously [30]. Specific quantitative assays were designed from mouse sequences available in GenBank™. Primers and probes were designed from nonconserved sequences of the genes (allowing for isoform specificity),

spanning sites where two exons join (splice sites) when such sites are known (preventing recognition of the assay to any potential contaminating genomic DNA). Standard RNA was made for all assays by the T7 polymerase method (Ambion, Austin, TX), using total RNA isolated from the mouse heart. The correlation between the  $C_t$  (the number of polymerase chain reaction cycles required for the fluorescent signal to reach a detection threshold) and the amount of standard was linear over at least a 5-log range of RNA for all assays (data not shown). The level of transcripts for the constitutive gene product  $\beta$ -actin was quantitatively measured in each sample to control for sample-to-sample differences in RNA concentration. Polymerase chain reaction data are reported as the number of transcripts/number of  $\beta$ -actin molecules.

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