Dietary Fish Oil and Vitamin E Enhance Doxorubicin Effects in P388 Tumor-Bearing Mice

Qi-Yuan Liu and Benny K.H. Tan*

Department of Pharmacology, Faculty of Medicine, National University of Singapore, Singapore 117597, Republic of Singapore

ABSTRACT: In this study, four kinds of rodent diets, CO, FO, CVe, and FVe, were used by addition of canola oil, oil mixture (fish oil + canola oil), canola oil plus vitamin E, and oil mixture plus vitamin E, respectively, to a basic diet, AIN-93G, to investigate the influence of dietary fish oil and vitamin E on doxorubicin (DOX) treatment in P388 ascitic mice. Animal life span (LS) and heart damage were recorded in mice fed the four different diets and treated with different doses of DOX. The optimal doses of DOX for antitumor effect as manifested by increased LS were 6.0 and 9.0 mg/kg. Both fish oil and vitamin E significantly enhanced this effect. On the other hand, DOX at 12.0 mg/kg induced severe heart damage, which was also significantly aggravated by both fish oil and vitamin E, as shown by both decreased LS and increased serum creatine phosphokinase activity. Fish oil and vitamin E appeared to enhance the antitumor effect of optimal doses of DOX but to aggravate cardiotoxicity owing to DOX overdose.

Paper no. L8891 in *Lipids 37,* 549–556 (June 2002).

The effects of PUFA on tumor development and tumor growth have been intensively investigated in both animal models and humans in the past two decades. Although rigid scientific proof has yet to be obtained, it is generally believed that n-6 PUFA in terrestrial plant oils enhance tumor growth, but n-3 PUFA in fish oil have a protective effect (1). Furthermore, two long-chain n-3 PUFA that are found in significant amounts in fish oil, *viz.*, EPA (20:5n-3) and DHA (22:6n-3), may exert multiple activities to retard the growth of tumor cells and xenografts (2,3). On the other hand, it is known that certain physical and functional properties of cell membranes can be modified when PUFA content is changed, and lipid nutrition directed at producing this modification has been suggested in cancer therapy (4). By altering the properties of membrane lipids, FA-based diets may provide a new approach for enhancing the effectiveness of certain antineoplastic therapies.

Vitamin E (vit E) includes eight naturally occurring compounds of two classes, tocopherols and tocotrienols, with different biological activities. Tocopherols are the most important chain-breaking antioxidants within cellular membranes, mainly owing to their ability to donate phenolic hydrogens to lipid free radicals (5). They are present in oily seeds, leaves, and other green parts of higher plants. It is generally agreed that the relative antioxidant activity of the tocopherols *in vivo* is in the order of $\alpha > \beta > \gamma > \delta$ (6). In addition to their antioxidant property, tocopherols and their derivatives have been reported to have pro-oxidant properties in some biological systems (7,8).

Doxorubicin (DOX) has been widely used in the treatment of a variety of solid tumors and hematological malignancies. Several of its biological effects are supposedly derived from its quinone moiety acting as an alkylating/arylating electrophile or a pro-oxidant. The quinone structure permits DOX to act as an electron acceptor in reactions mediated by oxoreductases, such as cytochrome P450 reductase (9), NADH dehydrogenase (10), and xanthine oxidase (11), to convert the quinone to a semiquinone free radical. Under anaerobic conditions, the semiquinone undergoes further reduction accompanied by reductive cleavage of the sugar residue to generate a quinone methide, which binds covalently to nucleophiles and is known to form adducts with DNA (12). In the presence of O_2, however, this semiquinone radical is spontaneously and rapidly reoxidized in a process that generates a superoxide radical (13). The superoxide radical and its dismutation product, hydrogen peroxide, undergo Haber–Weiss and Fenton reactions, respectively, to form the hydroxyl radical, which is able to extract a doubly allylic hydrogen atom from PUFA and thus initiate lipid peroxidation (14). The nonenzymatic formation of reactive oxygen species is mediated by the formation of complexes between DOX and iron, which can either react with O_2 to yield superoxide radical and hydrogen peroxide or bind O_2 in a superoxo- or peroxo-like form (15). Despite its antitumor effect, clinical effectiveness of DOX is hindered by its unique dose-limiting cardiotoxicity (16), possibly because of the sensitivity of the heart to reactive free radicals, due to a low activity of antioxidant enzymes (17). Separates studies, however, have found both effectiveness (18,19) and ineffectiveness (20) of vit E in cardioprotection in DOX-treated animals.

In this study, we prepared four experimental diets by adding natural oils to the basic components of AIN-93G, a purified diet for laboratory rodent growth (21). These diets differed in their contents of n-3 PUFA and tocopherols. We tested their effects on the therapeutic efficacy of various doses of DOX in mice induced with P388 ascitic tumor [which is the standard transplantable murine tumor most sensitive to DOX (18)], recording life span (LS) of the tumor-bearing mice, and examining animal heart damage by measurement of serum creatine phosphokinase (CPK) level (a biochemical marker of animal myocardial damage).

*To whom correspondence should be addressed at Dept. of Pharmacology, Faculty of Medicine, National University of Singapore, MD2, 18 Medical Dr., Singapore 117597, Republic of Singapore.
E-mail: phctankh@nus.edu.sg

Abbreviations: CPK, creatine phosphokinase; DOX, doxorubicin; HNE, 4-hydroxynonenal; LS, life span; MDA, malondialdehyde, vit E, vitamin E.

EXPERIMENTAL PROCEDURES

Cell lines and chemicals. The P388 cell line was obtained from ATCC (American Type Culture Collection, Manassas, VA), and the cells were grown in DBA/2 mice for one generation before use in the following animal experiments. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

Diets. Four experimental diets with AIN-93G as a base food were prepared by Specialty Feed Service (Glen Forest, Australia) as follows: (i) CO: AIN-93G + 10% canola oil; (ii) FO: AIN-93G + 8.5% fish oil + 1.5% canola oil; (iii) CVe: AIN-93G + 500 mg/kg natural vit E + 10% canola oil; (iv) FVe: AIN-93G + 500 mg/kg natural vit E + 8.5% fish oil + 1.5% canola oil.

Canola oil, a favorable dietary oil, is an appropriate control to use to study the effect of long-chain n-3 PUFA in fish oil because it contains a relatively high proportion of linoleic acid (18:2n-6) and α-linolenic acid (18:3n-6), and a low proportion of EPA and DHA. Canola oil (1.5%) was added to fish oil (8.5%) in FO and FVe diets in order to increase linoleic acid content to above 1%, which is the requirement for optimal rodent growth. The FA composition of the four diets is shown in Table 1. Total n-6 PUFA content was similar in the four diets, whereas n-3 PUFA contents, particularly EPA and DHA, were greater in FO and FVe than in CO and CVe. On the other hand, AIN-93G basic diet contains 75 mg/kg *d*-α-tocopherol acetate (21). The natural vit E used in our experiments is a mixed tocopherol concentrate, Covi-ox® T-70 (Cognis Nutrition & Health, Broadmeadows, Victoria, Australia), with relative amounts of α- (14%), β- (1%), γ- (62%), and δ-tocopherols (23%). Five hundred milligrams of Covi-ox® T-70 contains 350 mg of total tocopherols. According to the manufacturer (Specialty Feed Service, Glen Forest, Australia), canola oil contained 330, 16, 846 and 56 mg/kg of α-, β-, γ-, and δ-tocopherols, respectively, whereas fish oil contained 340 mg/kg of α-tocopherol only. The calculated contents of individual vit E species in four diets are shown in Table 2, indicating that CVe and FVe contained a significantly higher content of all tocopherol species compared to CO and FO, respectively.

Comparisons between CO and FO and between CVe and FVe were made to evaluate the effect of fish oil, whereas comparisons between CO and CVe and between FO and FVe were made to examine the effect of vit E on tumor-bearing or normal mice with or without DOX treatment.

Measurements of LS. All experiments with animals were conducted according to guidelines in "International Guiding Principles for Animal Research," *WHO Chronicles,* Vol. 39, 1985. Male BDF1 mice purchased from Animal Resources Center (Canning Vale, Western Australia) were used at 6–8 wk of age when their body weights ranged between 19.0 and 21.0 g. The animals were kept in filter-covered plastic cages, housed in a temperature-controlled room with a diurnal 12 h light cycle, and provided with tap water and the assigned experimental diets *ad libitum* for 10 d prior to experimentation and continuously thereafter. This period of time was found to be sufficient to modify the PUFA composition of phospholipids in most organs to stable levels in mice (22). The animals were inoculated intraperitoneally on day 0 with P388 cells (10^6 cells/mouse), followed by injection of DOX or saline also intraperitoneally on days 1 and 6. Deaths among the experimental animals were recorded at least twice a day.

Determination of DOX cardiotoxicity. In the second experiment, the BDF1 mice were maintained, fed, and administered DOX or saline as described above without the inoculation of tumor cells. The animals were killed on day 7 by cervical dislocation, blood was collected by cardiac puncture, and serum was obtained after the clotting of blood at room temperature for 1 h. Serum was kept frozen (−76°C) until analysis. Cardiotoxicity was determined by measuring serum CPK activity, calculated according to the Sigma Diagnostics Procedure No. 47-UV. A

TABLE 1
FA Composition in Four Experimental Diets[a]

FA (%)	CO	FO	CVe	FVe
Individual				
14:0	ND	4.3	ND	4.5
16:0	5.6	19.8	4.6	19.2
18:0	1.3	2.2	2.0	5.0
16:1	ND	5.1	ND	4.1
18:1	60.1	19.3	60.3	18.4
20:1	1.5	ND	1.5	ND
18:2n-6	20.3	16.6	20.1	13.5
18:3n-3	10.2	ND	10.5	ND
18:4n-3	0.6	1.3	0.6	1.7
20:4n-6	ND	1.9	ND	1.9
20:5n-3	0.5	8.7	0.5	9.1
22:6n-3	ND	20.9	ND	22.5
Total	100	100	100	100
Class[b]				
∑SFA	6.9	26.3	6.6	28.7
∑MUFA	61.6	24.4	61.8	22.5
∑PUFA	31.6	49.4	31.7	48.7
∑n-3 PUFA	11.3	30.9	11.6	33.3
∑n-6 PUFA	20.3	19.8	20.7	17.1
∑n-3 PUFA/∑n-6 PUFA	0.56	1.56	0.56	1.95
Average double bond[c]	1.4	2.4	1.4	2.4

[a]Diet CO, AIN-93G + 10% canola oil; FO, AIN-93G + 8.5% fish oil + 1.5% canola oil; CVe, AIN-93G + 500 mg/kg natural vitamin E + 10% canola oil; FVe, AIN-93G + 500 mg/kg natural vitamin E + 8.5% fish oil + 1.5% canola oil; ND, not detected.
[b]SFA, saturated FA; MUFA, monounsaturated FA.
[c]"Average double bond" means average number of double bonds per molecule of FA.

TABLE 2
Vitamin E Content (mg/kg) in Four Experimental Diets[a]

Species of vitamin E	CO	FO	CVe	FVe
α-Tocopherol acetate	75	75	75	75
α-Tocopherol	33	33.9	82	82.9
β-Tocopherol	1.6	0.2	5.1	3.7
γ-Tocopherol	84.6	12.7	301.6	229.7
δ-Tocopherol	5.6	0.8	86.1	81.3
Total	200	122.2	549.8	472.6

[a]For description of diets see Table 1.

CPK Test kit (CK-20; Sigma Chemical Co.) based on the method described by Szasz *et al.* (23) was used in the study.

Lipid analysis of P388 ascitic tumor cells and cardiac tissue. In the third experiment, BDF1 mice were fed experimental diets 10 d prior to the experimentation and thereafter. P388 cells (10^7 cells/mouse) were intraperitoneally inoculated into animals on day 0. On day 7, the mice were killed by cervical dislocation. The hearts were collected and ascitic tumor cells obtained by laving the peritoneum with ice-cold saline. The tumor cells were pelleted at 700 × *g* for 5 min and washed twice with the same buffer solution. Total lipid was extracted using a method described by Folch *et al.* (24). The phospholipid fraction was separated by silicic acid chromatography (25), saponified in alkali (26), and methylated with BF_3/methanol (27). The fractions of FAME with different chain lengths and unsaturations were separated in a Hewlett-Packard 5890 gas chromatograph using a DB-225 column (J&W Scientific, Folsom, CA). Peaks were identified by comparison with the retention times of known standards and peak areas quantified.

Statistical analysis. Data were analyzed by Student's *t*-test or ANOVA as shown in the text and the legends for the tables and figures. Experimental data are shown as mean ± SD. $P < 0.05$ was set as the level of significance throughout the study.

TABLE 3
ANOVA on Life Span (LS) of P388 Ascitic Mice[a]

Two-way ANOVA

Source	*P0* (*n* = 28)	*P12* (*n* = 24)
Fish oil	0.052	0.018
Vit E	0.010	0.023
Fish oil × vit E	0.854	1.000

Multiway ANOVA

Source	*P6,9* (*n* = 48)
DOX	0.262
Fish oil	0.025
Vit E	<0.001
DOX × fish oil	0.567
DOX × vit E	0.844
Fish oil × vit E	0.601

[a]Canola oil served as the control for fish oil. Column *P0* considers data on the LS of animals without doxorubicin (DOX) treatment; *P12,* with 12.0 mg/kg DOX treatment; and *P6,9,* with 6.0 and 9.0 mg/kg DOX treatment; *n,* number of mice; vit E, vitamin E.

RESULTS

LS of P388 ascitic mice. The individual LS of P388 ascitic mice fed the four experimental diets and administered vari-

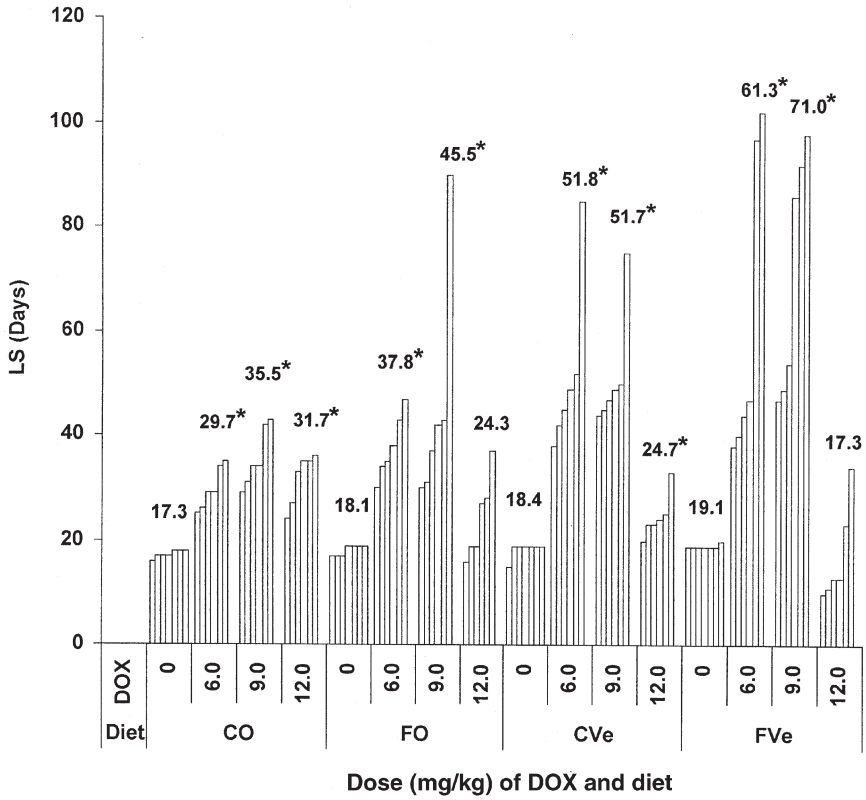

FIG. 1. Effects of different doses of doxorubicin (DOX) and experimental diets on life span (LS) of P388 ascitic mice. Each column represents the LS of one animal, whereas the value above the cluster of columns represents the mean LS in each group. *$P < 0.05$ represents the difference between the saline and DOX-treated groups on the same diet (Student's *t*-test). Diets: CO, AIN-93G + 10% canola oil; FO, AIN-93G + 8.5% fish oil + 1.5% canola oil; CVe, AIN-93G + 500 mg/kg natural vitamin E + 10% canola oil; FVe, AIN-93G + 500 mg/kg natural vitamin E + 8.5% fish oil + 1.5% canola oil.

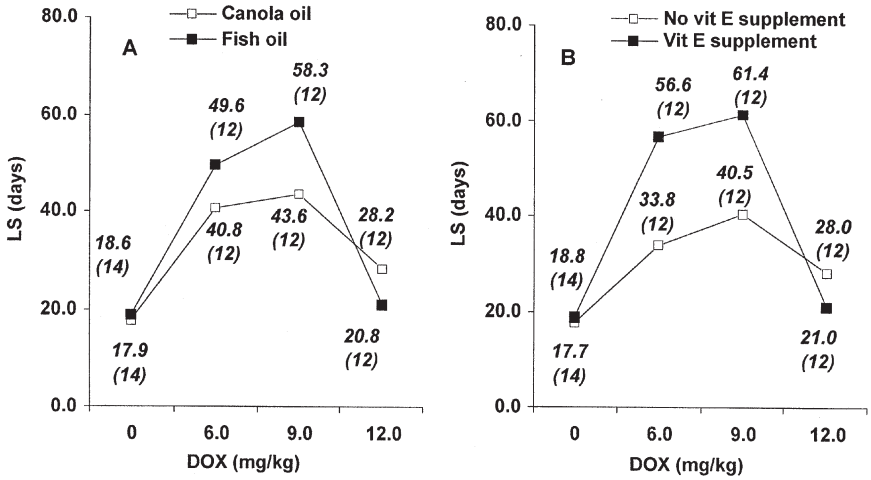

FIG. 2. Effects of fish oil and vitamin E (vit E) on LS of P388 ascitic mice treated with various doses of DOX. The values outside and inside the parentheses at each point represent the mean LS and number of animals, respectively, in the group. (A) Effect of fish oil; (B) effect of vit E. Canola oil served as the control for fish oil. For abbreviations see Figure 1.

ous doses of DOX are shown in Figure 1. The results of statistical analyses of the data are shown in Figures 1 and 2 and Table 3. In the absence of DOX treatment, vit E supplement increased the mean LS from 17.7 to 18.8 d ($P = 0.010$, two-way ANOVA) (Fig. 2B, Table 3). Both 6.0 and 9.0 mg/kg DOX significantly increased animal LS in all diet groups ($P < 0.05$, Student's *t*-test) (Fig. 1), but there was no significant difference ($P = 0.262$, multiway ANOVA) in therapeutic efficacy between these two doses (Table 3). Both fish oil and vit E, however, were found to significantly enhance the effect of DOX at these doses ($P = 0.025$ and <0.001, respectively, multiway ANOVA) (Table 3). Fish oil increased the mean LS from 40.8 to 49.6 d in mice treated with 6.0 mg/kg DOX and from 43.6 to 58.3 d in mice treated with 9.0 mg/kg DOX (Fig. 2A). Vit E increased the mean LS from 33.8 to 56.6 d in mice treated with 6.0 mg/kg DOX and from 40.5 to 61.4 d in mice treated with 9.0 mg/kg DOX (Fig. 2B). When DOX dose was increased to 12.0 from 9.0 mg/kg, animal LS was significantly decreased ($P < 0.001$, multiway ANOVA followed by Tukey's test for DOX only), suggesting the negative effect of DOX at this high dose. Both fish oil and vit E significantly aggravated this negative effect ($P = 0.018$ and 0.023, respectively, two-way ANOVA) (Table 3), with fish oil decreasing the mean LS from 28.2 to 20.8 d (Fig. 2A), and vit E decreasing the mean LS from 28.0 to 21.0 d (Fig. 2B).

DOX-induced CPK activity. The experimental data of plasma CPK levels in mice fed the four experimental diets and treated with various doses of DOX are shown in Table 4, part A. The results of statistical analysis on the data are shown in Table 4, part B, and Figure 3. DOX increased plasma CPK activity dose-dependently (Table 4). Two-way ANOVA (Table 4) showed that vit E inhibited plasma CPK activity in mice without DOX (decrease of 0.3 unit/mL serum; $P < 0.001$) or with 6.0 mg/kg DOX (decrease of 0.3 unit/mL serum; $P = 0.007$) but did not significantly modify CPK activity with 9.0 mg/kg DOX. In contrast, vit E enhanced CPK activity in the presence of 12.0 mg/kg DOX (increase of 0.7 unit/mL serum; $P = 0.002$, two-way ANOVA). On the other hand, fish oil increased DOX-induced CPK activity to an extent proportional to DOX dose (increases of 0, 0.3, 0.6, and 1.5 unit/mL serum in 0, 6.0, 9.0, and 12.0 mg/kg DOX-treated mice with $P = 0.935$, 0.005, <0.001, and <0.001, respectively, two-way ANOVA) (Fig. 3).

FA composition of ascitic tumors and animal hearts. Lipid analysis of the FA composition in phospholipids indicated that n-6 PUFA content was comparable in both P388 ascitic tumor and mouse heart of all four groups, whereas n-3 PUFA as well as total PUFA content was significantly larger in both tissues of FO- and FVe-fed mice than those in CO- and CVe-fed mice

TABLE 4
Effects of Fish Oil and Vit E on CPK Activity in Mice Treated with Various Doses of DOX[a]

A: CPK activity (unit/mL)[b]

	DOX (mg/kg)			
Diet	0	6.0	9.0	12.0
CO	0.6 ± 0.1	0.8 ± 0.1	1.1 ± 0.2	1.9 ± 0.2
FO	0.7 ± 0.1	1.1 ± 0.3	1.7 ± 0.3	3.3 ± 0.1
CVe	0.4 ± 0.1	0.6 ± 0.2	0.9 ± 0.2	2.5 ± 0.3
FVe	0.3 ± 0.1	0.8 ± 0.2	1.6 ± 0.2	4.1 ± 0.7

B: Results of two-way ANOVA[b]

Source	*P0* (*n* = 24)	*P6* (*n* = 24)	*P9* (*n* = 24)	*P12* (*n* = 24)
Fish oil	0.935	0.005	<0.001	<0.001
Vit E	<0.001	0.007	0.095	0.002
Fish oil × vit E	0.142	0.456	0.965	0.687

[a]The experimental data are shown as mean ± SD of six determinations on the samples obtained from four mice in each diet group. Canola oil served as the control of fish oil. Column *P0* considers the data on groups given saline treatment; *P6,* with 6.0 mg/kg DOX treatment; *P9,* with 9.0 mg/kg DOX treatment; and *P12,* with 12.0 mg/kg DOX treatment. *n,* number of determinations.

[b]Multiway ANOVA followed by Tukey's test for DOX only, $P < 0.001$ for 0 vs. 6.0 mg/kg, $P < 0.001$ for 6.0 vs. 9.0 mg/kg, and $P < 0.001$ for 9.0 vs. 12.0 mg/kg. For description of diets see Table 1. For abbreviations see Table 3.

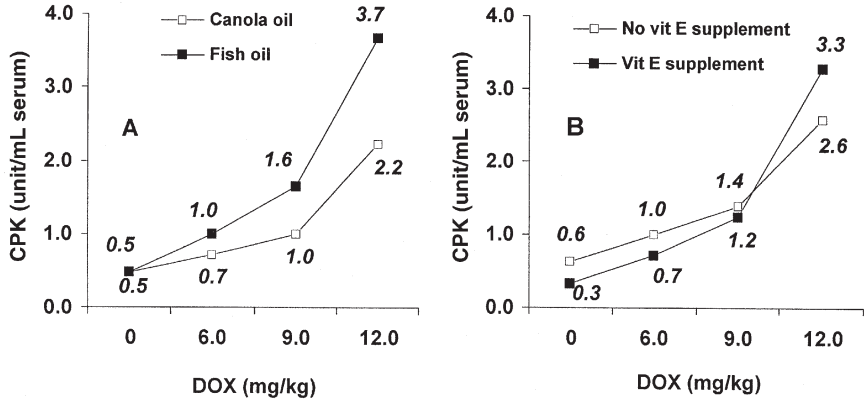

FIG. 3. Effects of fish oil/vit E interactions with DOX on CPK activity in normal mice. The value at each point represents the mean CPK activity from 12 determinations on the samples from eight mice in each group. (A) Effect of fish oil; (B) effect of vit E. Canola oil served as the control for fish oil. CPK, creatine phosphokinase; for other abbreviations see Figures 1 and 2.

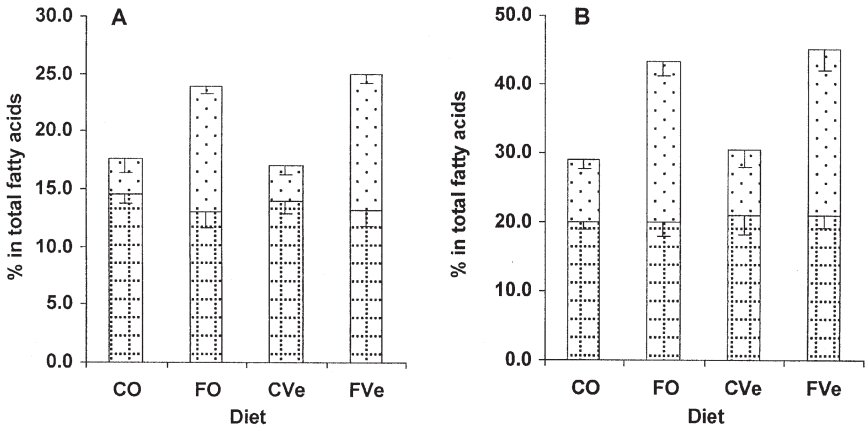

FIG. 4. Effects of experimental diets on PUFA in phospholipids of P388 ascitic tumor and mouse heart. (A) P388 ascitic tumor; (B) animal heart. The columns represent mean ± SD from five separate determinations on samples from three mice in each group. For diets see Figure 1. Key: n-3 PUFA, bars with stippling; n-6 PUFA, bars with vertical and horizontal lines.

(Fig. 4). The increased content of n-3 PUFA in FO and FVe groups can be attributed largely to EPA and DHA (data not shown), both of which were present in large amounts in FO and FVe diets.

DISCUSSION

Four AIN-93G-based rodent diets, CO, FO, CVe, and FVe, were designed for this study. CO and CVe contained 10% canola oil, whereas FO and FVe contained 8.5% fish oil plus 1.5% canola oil. CVe and FVe additionally included 500 mg/kg natural vit E. FO and FVe with CO and CVe as their respective controls were used to investigate the effect of fish oil, and CVe and FVe with CO and FO as their respective controls were applied to assess the effect of vit E.

This study demonstrated the therapeutic effect of vit E in P388 mice (6.2% increase in mean LS; $P = 0.010$, two-way ANOVA). This was consistent with the previous reports of tumor inhibitory and chemopreventive effects of tocopherols (28–30). Vit E may inhibit tumor growth *via* the cytotoxic effects of tocopherols and their derivatives (31–33), induction of apoptosis through activation of $P21^{WAF1/CIP1}$ (34), reduction of prostaglandin E_2 production (35), and abatement of muscle wasting in cancer cachexia (36).

In the present study, the optimal DOX antitumor effect was observed at 6.0 and 9.0 mg/kg doses. When DOX dose was increased to 12.0 mg/kg, the animal LS significantly decreased, indicating that DOX at this high dose produced a severe negative effect. The major negative effect of DOX might be its cardiotoxicity, as it was shown that serum CPK activity increased dose-dependently. Transmission electron microscopy also demonstrated that DOX at 12.0 mg/kg induced myofibrillar fragmentation and damaged mitochondria in the heart (data not shown).

The effects of DOX at various doses were modified by both fish oil and vit E. Fish oil not only significantly enhanced 6.0 and 9.0 mg/kg DOX therapeutic efficacy in P388 ascitic mice (21.6 and 33.7% increases in mean LS, respectively;

P = 0.025, multiway ANOVA) but also elevated mouse plasma CPK level to an extent proportional to the DOX dose administered, implying that fish oil might enhance DOX cytotoxicity in both tumor cells and heart. The chemistry of DOX lends itself to enzymatic and nonenzymatic generation of superoxide radical and the secondary reactive oxygen species derived from superoxide, including hydrogen peroxide and hydroxyl radical (10,14). The hydroxyl radical is able to extract a doubly allylic hydrogen atom from the unsaturated lipids to form lipid radicals (37), which may react quickly with O_2 to form lipid peroxyl radicals that in turn produce lipid endoperoxides (37), or else initiate the lipid peroxidation cycle, converting other unsaturated lipids into lipid hydroperoxides (38). A number of lipid endoperoxides and hydroperoxides and their aldehyde derivatives have been implicated as causative agents for cytotoxic processes in cells (39). Lipid peroxidation has been suggested to be the cause of DOX cytotoxicity in both tumor (40) and heart tissue (41). The increased membrane lipid unsaturation would consequently provide more targets for peroxidative events generated by the metabolism of DOX, thus increasing the cytotoxicity to tissues. On the other hand, the interaction between fish oil and DOX cytotoxicity might also occur through the enhancement of DOX accumulation owing to the increased membrane lipid unsaturation and thus fluidity (4,42). The lipid analysis in the present study showed that PUFA content (an index of cell membrane unsaturation) was increased in both ascitic tumor and animal heart in mice fed FO and FVe, suggesting the possibility that dietary fish oil could directly enhance DOX-induced lipid peroxidation as well as elevate intracellular DOX accumulation in both tumors and heart. Although fish oil markedly exacerbated 12.0 mg/kg DOX cardiotoxicity and abolished the DOX therapeutic effect, the increased LS was achieved when DOX was administered at optimal doses, i.e., tumor inhibition could be achieved with DOX at optimal doses while deleterious effects on the heart occurred only with DOX overdose. This suggests that heart and tumor may have differential responses to the interaction of fish oil and DOX and/or differential sensitivities to the cytotoxicity induced by DOX combined with fish oil.

Vit E seemed to have an inhibitory effect on 6.0 mg/kg DOX-induced CPK release. Many studies found that vit E injection protected against DOX cardiotoxicity (18,19). The cardioprotective effect was achieved in the present study by dietary vit E. The scavenging activity of vit E on lipid peroxidation has been proposed to account for the cardioprotective effect of DOX in DOX-treated animals (18,19), although mechanism(s) other than its antioxidant activity might also contribute to the cardioprotection (43). On the other hand, vit E has been reported to directly enhance the inhibitory effect of DOX on tumor growth (34), and this may partly explain the enhanced therapeutic effectiveness of 9.0 mg/kg DOX. It is thus possible that vit E might enhance the therapeutic efficacy of DOX at optimal doses *via* cardioprotection and/or enhancement of tumor growth inhibition.

Through a synergistic interaction between tocopherols and overdose DOX, vit E supplement aggravated 12.0 mg/kg DOX cardiotoxicity and thus decreased animal LS (25%; P = 0.023 in two-way ANOVA). Tocopherols transform to chromanoxyl radicals in the scavenging of lipid radicals. In the absence of adequate reductants, such as ascorbic acid, the chromanoxyl radicals will be irreversibly degraded to tocopheryl quinones (44), which are able to initiate and/or enhance lipid peroxidation (6). Owing to the potential pro-oxidant activity of vit E, it may interact synergistically with DOX to produce reactive free radicals and induce lipid peroxidation. Overdose DOX treatment produces oxidative stress, which may overwhelm and/or reduce the antioxidant capability in cells. In the absence of an efficient antioxidant apparatus, such as in the heart, the reactive oxygen metabolites of tocopherols, together with DOX, engender the production of vast amounts of oxygen free radicals, thereby causing extreme cytotoxicity to cells through drastically increased lipid peroxidation.

It thus seems that, at least in animals, vit E may express antioxidant or other activities to protect against heart damage when combined with DOX at optimal doses, but produce a pro-oxidant effect to enhance DOX-induced cardiotoxicity when combined with overdose DOX. This may explain why, in some studies, vit E was reported to be ineffective in cardioprotection (20) or even to be deleterious to the heart of DOX-treated animals (45). Another point to note is that, although α-tocopherol content was similar between fish oil and canola oil, the latter had a greater γ-tocopherol content than fish oil. However, fish oil but not canola oil enhanced the therapeutic efficacy of DOX, implying that α-tocopherol, but not γ-tocopherol, may be the dominant species of vit E with respect to its activity. This is consistent with the fact that α-tocopherol has both a higher (absolute value) tissue distribution (46) and a higher antioxidant activity (6) than γ-tocopherol.

In addition, the function of both PUFA and tocopherols as alkylating/arylating agents is known to account for some of their biological effects. The electrophiles generated from lipid peroxidation, such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE), may react with DNA bases. MDA modifies purine bases to generate a tricyclic adduct with guanine (47) and an acyclic adduct with adenine (48). HNE reacts directly with 2′-deoxyguanosine to produce a tricyclic substituted propane adduct and is readily oxidized to the epoxide derivative, which can covalently modify both 2′-deoxyguanosine and 2′-deoxyadenosine to form etheno adducts (49). Failure to repair these DNA lesions can lead to apoptosis (50). On the other hand, γ- and δ-tocopherol quinones generated from γ- and δ-tocopherols, respectively, are cytotoxic arylating electrophiles because they contain an α,β unsaturated carbonyl structure that forms Michael adducts with compounds containing a thiol nucleophilic group, such as glutathione and proteins (51). Whether treatment with PUFA, tocopherols, and DOX leads to the interactions through their alkylating/arylating activities and thereby enhances therapeutic efficacy remains to be studied.

Animal xenograft studies found that human lung cancer (52) and mammary carcinoma (53) in nude mice showed a greater sensitivity to DOX when the mice were fed fish oil.

Furthermore, the present work comprehensively investigated the influence of dietary fish oil and vit E, individually and in combination, on the therapeutic efficacy of DOX and has shown their additively enhancing effects on optimal doses of DOX in P388 ascitic mice. Increasing DOX dose, however, led to severe heart damage, which was exacerbated by fish oil and vit E. Thus overall, it appears that both fish oil and vit E modulate the effects of DOX in the laboratory mouse like a double-edged sword, on the one hand enhancing its antitumor effect and on the other, aggravating its cardiotoxicity.

ACKNOWLEDGMENTS

This work was supported by a Research Grant from the National University of Singapore (No. R-184-000-018-112). The authors thank Annie Hsu for technical assistance in animal experiments and Frances Lim for her help in the use of the gas chromatograph for the estimation of FAME.

REFERENCES

1. Fay, M.P., Freedman, L.S., Clifford, C.K., and Midthune, D.N. (1997) Effect of Different Types and Amounts of Fat on the Development of Mammary Tumors in Rodents: A Review, *Cancer Res. 57,* 3979–3988.
2. Gonzalez, M.J. (1992) Lipid Peroxidation and Tumor Growth: An Inverse Relationship, *Med. Hypotheses 38,* 106–110.
3. Calviello, G., Palozza, P., Nicuolo, F.E., Maggiano, N., and Bartoli, G.M. (2000) n-3 PUFA Dietary Supplementation Inhibits Proliferation and Store-Operated Calcium Influx in Thymoma Cells Growing in Balb/c Mice, *J. Lipid Res. 41,* 182–188.
4. Burns, C.P., and North, J.A. (1986) Adriamycin Transport and Sensitivity in Fatty Acid-Modified Leukemic Cells, *Biochim. Biophys. Acta 888,* 10–17.
5. Frei, B. (1994) Reactive Oxygen Species and Antioxidant Vitamins: Mechanisms of Action, *Am. J. Med. 97* (Supp. 13A), 5S–12S.
6. Kamal-Eldin, A., and Appelqvist, L. (1996) The Chemistry and Antioxidant Properties of Tocopherols and Tocotrienols, *Lipids 31,* 671–701.
7. Terao, J., and Matsushita, S. (1986) The Peroxidizing Effect of α-Tocopherol on Autoxidation of Methyl Linoleate in Bulk Phase, *Lipids 21,* 255–260.
8. Mukai, K. (1993) Synthesis and Kinetic Study of Antioxidant and Prooxidant Actions of Vitamin E Derivatives, in *Vitamin E in Health and Disease* (Packer, L., and Fuchs J., eds.), pp. 97–120, Marcel Dekker, New York.
9. Berlin, V., and Haseltine, W.A. (1981) Reduction of Adriamycin to a Semiquinone Free Radical by NADPH Cytochrome P450 Reductase Produces DNA Cleavage in a Reaction Mediated by Molecular Oxygen, *J. Biol. Chem. 256,* 4747–4756.
10. Doroshow, J.H. (1983) Anthracycline Antibiotic-Stimulated Superoxide, Hydrogen Peroxide and Hydroxyl Radical Production by NADH Dehydrogenase, *Cancer Res. 43,* 4543–4551.
11. Pan, S.S., and Bachur, N.R. (1980) Xanthine Oxidase Catalyzed Reductive Cleavage of Anthracycline Antibiotics and Free Radical Formation, *Mol. Pharmacol. 17,* 95–99.
12. Cullinane, C., and Phillips, D.R. (1990) Induction of Stable Transcriptional Blockage Sites by Adriamycin: GpC Specificity of Apparent Adriamycin-DNA Adducts and Dependence on Iron(III) Ions, *Biochemistry 29,* 5638–5646.
13. Lown, J.W., Chen, H.H., Plambeck, J.A., and Acton, E.M. (1982) Further Studies on the Generation of Reactive Oxygen Species from Activated Anthracyclines and the Relationship to Cytotoxic Action and Cardiotoxic Effects, *Biochem. Pharmacol. 31,* 575–581.
14. Gutteridge, J.M. (1984) Lipid Peroxidation and Possible Hydroxyl Radical Formation Stimulated by the Self-Reduction of a Doxorubicin-Iron(III) Complex, *Biochem. Pharmacol. 33,* 1725–1728.
15. Gianni, L., Zweier, J.L., Abraham, L., and Myers, C.E. (1985) Characterization of the Cycle of Iron-Mediated Electron Transfer from Adriamycin to Molecular Oxygen, *J. Biol. Chem. 260,* 6820–6826.
16. Lefrak, E.A., Pitha, J., Rosenheim, S., and Gottlieb, J.A. (1973) A Clinicopathologic Analysis of Adriamycin Cardiotoxicity, *Cancer 32,* 302–314.
17. Doroshow, J.H., Locker, G.Y., and Myers, C.E. (1980) Enzymatic Defenses of the Mouse Heart Against Reactive Oxygen Metabolites, *J. Clin. Invest. 65,* 128–135.
18. Myers, C.E., McGuire, W.P., Liss, R.H., Ifrim, I., Grotzinger, K., and Young, R.C. (1977) Adriamycin: The Role of Lipid Peroxidation in Cardiac Toxicity and Tumor Response, *Science 197,* 165–167.
19. Wang, Y.M., Madanat, F.F., Kimball, J.C., Gleiser, C.A., Ali, M.K., Kaufman, M.W., and van Eys, J. (1980) Effect of Vitamin E Against Adriamycin-Induced Toxicity in Rabbits, *Cancer Res. 40,* 1022–1027.
20. Breed, J.G., Zimmerman, A.N., Dormans, J.A., and Pinedo, H.M. (1980) Failure of the Antioxidant Vitamin E to Protect Against Adriamycin-Induced Cardiotoxicity in the Heart, *Cancer Res. 40,* 2033–2038.
21. Reeves, P.G., Nielsen, F.H., and Fahey, G.C.J., Jr. (1993) AIN-93 Purified Diets for Laboratory Rodents: Final Report of the American Institute of Nutrition *ad hoc* Writing Committee on the Reformulation of the AIN-76A Rodent Diet, *J. Nutr. 123,* 1939–1951.
22. Swanson, J.E., Black, J.M., and Kinsella, J.E. (1988) Dietary Menhaden Oil: Effects on the Rate and Magnitude of Modification of Phospholipid Fatty Acid Composition of Mouse Heart and Brain, *Br. J. Nutr. 59,* 535–545.
23. Szasz, G., Gruber, W., and Bernt, E. (1976) Creatine Kinase in Serum I: Determination of Optimum Reaction Conditions, *Clin. Chem. 22,* 650–656.
24. Folch, J., Lee, M., and Sloane Stanley, G.H. (1957) A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissues, *J. Biol. Chem. 226,* 497–509.
25. Wilder, P.J., Overman, D.K., Tenenholz, T.C., and Gutierrez, P.L. (1990) Differences in Myristic Acid Synthesis and in Metabolic Rate for P388 Cells Resistant to Doxorubicin, *J. Lipid Res. 31,* 1973–1982.
26. Browse, J., McCourt, P.J., and Somerville, C.R. (1986) Fatty Acid Composition of Leaf Lipids Determined After Combined Digestion and Fatty Acid Methyl Ester Formation from Fresh Tissue, *Anal. Biochem. 152,* 141–145.
27. Morrison, W.R., and Smith, L. (1964) Preparation of Fatty Acid Methyl Esters and Dimethylacetals from Lipids with Boron Fluoride-Methanol, *J. Lipid Res. 5,* 600–608.
28. Fleshner, N., Fair, W.R., Huryk, R., and Heston, W.D. (1999) Vitamin E Inhibits the High-Fat Diet Promoted Growth of Established Human Prostate LNCaP Tumors in Nude Mice, *J. Urol. 161,* 1651–1654.
29. Factor, V.M., Laskowska, D., Jensen, M.R., Woitach, J.T., Popescu, N.C., and Thorgeirsson, S.S. (2000) Vitamin E Reduces Chromosomal Damage and Inhibits Hepatic Tumor Formation in a Transgenic Mouse Model, *Proc. Natl. Acad. Sci. USA 97,* 2196–2201.
30. Decensi, A., and Costa, A. (2000) Recent Advances in Cancer Chemoprevention, with Emphasis on Breast and Colorectal Cancer, *Eur. J. Cancer 36,* 694–709.
31. Ramanathan, R., Das, N.P., and Tan, C.H. (1994) Effects of γ-Linolenic Acid, Flavonoids, and Vitamins on Cytotoxicity and Lipid Peroxidation, *Free Radic. Biol. Med. 16,* 43–48.

32. Thornton, D.E., Jones, K.H., Jiang, Z., Zhang, H., Liu, G., and Cornwell, D.G. (1995) Antioxidant and Cytotoxic Tocopheryl Quinones in Normal and Cancer Cells, *Free Radic. Biol. Med. 18,* 963–976.
33. Cornwell, D.G., Jones, K.H., Jiang, Z., Lantry, L.E., Southwell-Keely, P., Kohar, I., and Thornton, D.E. (1998) Cytotoxicity of Tocopherols and Their Quinones in Drug-Sensitive and Multidrug-Resistant Leukemia Cells, *Lipids 33,* 295–301.
34. Chinery, R., Brockman, J.A., Peeler, M.O., Shyr, Y., Beauchamp, R.D., and Coffey, R.J. (1997) Antioxidants Enhance the Cytotoxicity of Chemotherapeutic Agents in Colorectal Cancer: A p53-Independent Induction of $P21^{WAF1/CIP1}$ *via* C/EBPβ, *Nature Med. 3,* 1233–1241.
35. Jiang, Q., Elson-Schwab, I., Courtemanche, C., and Ames, B.N. (2000) γ-Tocopherol and Its Major Metabolite, in Contrast to α-Tocopherol, Inhibit Cyclooxygenase Activity in Macrophages and Epithelial Cells, *Proc. Natl. Acad. Sci. USA 97,* 11494–11499.
36. Buck, M., and Chojkier, M. (1996) Muscle Wasting and Dedifferentiation Induced by Oxidative Stress in a Murine Model of Cachexia Is Prevented by Inhibitors of Nitric Oxide Synthesis and Antioxidants, *EMBO J. 15,* 1753–1765.
37. Pryor, W.A., and Stanley, J.P. (1975) A Suggested Mechanism for the Production of Malonaldehyde During the Autoxidation of Polyunsaturated Fatty Acids. Nonenzymatic Production of Prostaglandin Endoperoxides During Autoxidation, *J. Org. Chem. 40,* 3615–3617.
38. Buege, J.A., and Aust, S.D. (1978) Microsomal Lipid Peroxidation, *Methods Enzymol. 52,* 302–310.
39. Esterbauer, H., and Cheeseman, K.H. (1990) Determination of Aldehydic Lipid Peroxidation Products: Malonaldehyde and 4-Hydroxynonenal, *Methods Enzymol. 186,* 407–421.
40. Benchekroun, M.N., Pourquier, P., Schott, B., and Robert, J. (1993) Doxorubicin-Induced Lipid Peroxidation and Glutathione Peroxidase Activity in Tumor Cell Lines Selected for Resistance to Doxorubicin, *Eur. J. Biochem. 211,* 141–146.
41. Powell, S.R., and McCay, P.B. (1995) Inhibition of Doxorubicin-Induced Membrane Damage by Thiol Compounds: Toxicologic Implications of a Glutathione-Dependent Microsomal Factor, *Free Radic. Biol. Med. 18,* 159–168.
42. Liu, Q.-Y., and Tan, B.K.H (2000) Effects of *cis*-Unsaturated Fatty Acids on Doxorubicin Sensitivity in P388/DOX Resistant and P388 Parental Cell Lines, *Life Sci. 67,* 1207–1218.
43. Carrasquedo, F., Glanc, M., and Fraga, C.G. (1999) Tissue Damage in Acute Myocardial Infarction: Selective Protective by Vitamin E, *Free Radic. Biol. Med. 26,* 1587–1590.
44. Liebler, D.C., and Burr, J.A. (1992) Oxidation of Vitamin E During Iron-Catalyzed Lipid Peroxidation: Evidence for Electron-Transfer Reactions of the Tocopheroxyl Radical, *Biochemistry 31,* 8278–8284.
45. Shinozawa, S., Gomita, Y., and Araki, Y. (1988) Effect of High Dose Alpha-Tocopherol and Alpha-Tocopherol Acetate Pretreatment on Adriamycin (Doxorubicin) Induced Toxicity and Tissue Distribution, *Physiol. Chem. Phys. Med. NMR 20,* 329–335.
46. Podda, M., Weber, C., Traber, M.G., and Packer, L. (1996) Simultaneous Determination of Tissue Tocopherols, Tocotrienols, Ubiquinols, and Ubiquinones, *J. Lipid Res. 37,* 893–901.
47. Marnett, L.J., Basu, A.K., O'Hara, S.M., Weller, P.E., Rahman, A.F.M.M., and Oliver, J.P. (1986) Reaction of Malondialdehyde with Guanine Nucleosides: Formation of Adducts Containing Oxadiazabicyclononene Residues in the Base-Pairing Region, *J. Am. Chem. Soc. 108,* 1348–1350.
48. Stone, K., Ksebati, M.B., and Marnett, L.J. (1990) Investigation of the Adducts Formed by Reaction of Malondialdehyde with Adenosine, *Chem. Res. Toxicol. 3,* 33–38.
49. Lee, S.H., Rindgen, D., Bible, R.H., Jr., Hajdu, E., and Blair, I.A. (2000) Characterization of 2′-Deoxyadenosine Adducts Derived from 4-Oxo-2-nonenal, a Novel Product of Lipid Peroxidation, *Chem. Res. Toxicol. 13,* 565–574.
50. Johnson, T.M., Yu, Z.X., Ferrans, V.J., Lowenstein, R.A., and Finkel, T. (1996) Reactive Oxygen Species Are Downstream Mediators of p53-Dependent Apoptosis, *Proc. Natl. Acad. Sci. USA 93,* 11848–11852.
51. Jones, K.H., Liu, J.J., Roehm, J.S., Eckel, J.J., Eckel, T.T., Stichrath, C.R., Triola, C.A., Jiang, Z., Bartoli, G.M., and Cornwell, D.G. (2002) γ-Tocopheryl Quinone Stimulates Apoptosis in Drug-Sensitive and Multidrug-Resistant Cancer Cells, *Lipids 37,* 173–184.
52. Hardman, W.E., Moyer, M.P., and Cameron, I.L. (2000) Dietary Fish Oil Sensitizes A549 Lung Xenografts to Doxorubicin Chemotherapy, *Cancer Lett. 151,* 145–151.
53. Hardman, W.E., Avula, C.P., Fernandes, G., and Cameron, I.L. (2001) Three Percent Dietary Fish Oil Concentrate Increased Efficacy of Doxorubicin Against MDA-MB 231 Breast Cancer Xenografts, *Clin. Cancer Res. 7,* 2041–2049.

[Received August 13, 2001, and in revised form May 15, 2002; revision accepted May 16, 2002]