Cancer Chemother Pharmacol (2001) 47: 34–40
DOI 10.1007/s002800000205

ORIGINAL ARTICLE

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# Suppression of tumor growth and metastasis by dietary fish oil combined with vitamins E and C and cisplatin

Received: 28 June 2000 / Accepted: 4 September 2000 / Published online: 16 November 2000


**Abstract** *Purpose*: The anticancer activity of Ω-3 polyunsaturated fatty acids (Ω-3 PUFA) has been shown in a large number of studies. This study was undertaken to analyze the combined effect of Ω-3 PUFA and antioxidative vitamins on the level of spontaneous metastatic dissemination. The supportive effect of this dietary combination on chemotherapy with cisplatin (CP) was determined in parallel. *Methods*: C57BL/6J mice bearing the Lewis lung carcinoma 3LL were fed ad libitum one of three isocaloric diets containing 5% soybean oil supplemented with 40 mg/kg α-tocopherol acetate (SO diet), or 4% fish oil plus 1% corn oil, and basal amounts of vitamin E (FO diet) or FO diet supplemented with vitamins E and C (FO + E + C diet). These diets were tested in combination with the conventional cytotoxic agent CP in a series of regimens. Tumor growth, feed consumption, body weight, lung metastasis and lung histology were followed. *Results*: Both the FO dietary groups showed significantly lower tumor development than the SO group in all examined parameters, indicating that Ω-3 PUFA have anticancer activity. However, the FO diet, in comparison with the FO + E + C diet induced a significantly slower rate of tumor growth, and lower metastatic load, as reflected in lung weight. The decrease in the anticancer activity of FO by the addition of vitamins E and C suggests that in situ oxidation of Ω-3 PUFA underlies their anticancer action. It is thus proposed that oxidized Ω-3 PUFA accumulates in the membranes and the cytosol of tumor cells, reducing their vitality and eventually leading to their death. No signs of anorexia or cachexia were observed in either FO group, in contrast to the SO group. CP treatment with the SO diet had no apparent therapeutic effect, while with the FO diets it reduced the metastatic load. The best regimen of this combined treatment was FO diet followed by CP treatment with FO diet supplemented with vitamins E and C after resection of the primary growth. This regimen could be translated to a combined therapy for human cancer. *Conclusions*: Diets enriched with Ω-3 PUFA may have beneficial anticancer effects in particular when containing only basal amounts of antioxidants such as vitamin E or C. Furthermore, the addition of drugs which promote oxidation of Ω-3 PUFA, such as ferrous salts (e.g. as prescribed for the treatment of anemia), may further increase these effects. However, the supportive effect of Ω-3 PUFA in chemotherapy (e.g. with CP) increases when vitamins E and C are also included.

**Key words** Ω-3 PUFA · Lipid peroxidation · Tumor suppression · Chemotherapy · Vitamins E + C

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## Introduction

There is ample evidence to support the notion that dietary components can induce or suppress tumor development. With respect to dietary fats, fish oil (FO) which is rich in Ω-3 polyunsaturated fatty acids (Ω-3 PUFA), has been shown in many studies to act as a tumor suppressor [20, 30, 32, 55]. Ω-3 and Ω-6 PUFA can selectively kill tumor cells in culture while causing little or no harm to normal cells [3, 4, 45]. This selective cytotoxic effect may be attributed to the formation of superoxide anions and lipid peroxidation since superoxide dismutase or vitamins A and E, when administered concomitantly with these fatty acids, are capable of blocking the anticancer activity of Ω-6 PUFA [3, 4, 15, 18]. Low contents of antioxidants and the associated deficiency of cellular antioxidation defenses in tumor cells [21] may be the origin of this effect.

In vivo experiments and epidemiological studies have indicated that only Ω-3 PUFA, in contrast to Ω-6 PUFA, preserves the anticancer activity [20, 27, 28, 30, 32, 42, 43, 55], although both have a similar effect on cell

membrane fluidity and permeability [31]. This basic difference may be related to metabolic processes of these fatty acids which are involved in tumorigenesis, such as synthesis of eicosanoids [1, 29, 37, 55], cytokine formation [8, 36], and insulin secretion [23, 53, 54]. However, the findings of epidemiological studies are inconsistent with regard to the effect of antioxidant vitamins on cancer development [7, 16, 26, 43].

The effect of dietary fats on chemotherapy is not yet fully elucidated. Peroxidation processes and negative side effects due to exhaustion of antioxidants have been found in studies in which chemotherapeutic agents were administered [44, 48]. On the other hand, the supplementation of antioxidants, in vitro and in vivo has been reported to enhance the cytotoxicity of chemotherapeutic agents in colorectal cancer [11].

In this study we tested a series of therapeutic regimens with FO diets in mice bearing the Lewis lung carcinoma, 3LL. We observed significant anticancer activity of dietary FO, a counter activity of the antioxidant vitamins E and C, when supplied together with FO and a marked potentiation of chemotherapy with cisplatin (CP) by FO + E + C diet, applied immediately after tumor resection.

## Materials and methods

### Mice

C57 BL/6J female mice at 8–9 weeks of age with an average weight of 23 g were purchased from Harlan Laboratories (Ein Karem, Jerusalem, Israel). The mice were kept in filter-covered plastic cages (ten mice per cage) and fed ad libitum with five distinct isocaloric diets which are outlined below. Animals were maintained and treated according to NIH guidelines.

### Diets

Five distinct diets were employed:

1. SO diet: standard purina diet (detailed in Table 1) supplemented with 5% soybean oil (SO).
2. FO diet: standard purina diet, essentially free of vitamins E and C, supplemented 4% FO, and containing 60% ethyl esters of Ω-3 PUFA eicosapentaenoic acid and docosahexaenoic acid and 0.5 mg/g vitamin E (Intermed Enterprises, Geneva, Switzerland), 1% corn oil and 2 mg/kg vitamin E (α-tocopherol; Rone Poulenc, Nutritional Animal, Antony Cedex, France).
3. FO + E diet: FO diet supplemented with an additional 20 mg/kg vitamin E.
4. FO + C diet: FO diet supplemented with 8 mg/kg vitamin C (Analar, BDH Laboratory Supplies, Poole, UK).
5. FO + E + C diet: FO diet supplemented with both vitamin E (as in FO + E diet) and vitamin C (as in FO + C diet).

Lipids extracted from these diets were determined by gas chromatography and their fatty acid and antioxidant contents are presented in Table 2. The FO diets were freshly prepared daily.

### Regimen

Three dietary groups of mice were tested simultaneously. All mice started their diet (ad libitum feeding) 7 days prior to tumor inoculation. After tumor resection, part of the FO dietary group was switched to the FO + E, FO + C and FO + E + C diets. Each of these groups included 20 mice as outlined below. Food intake and body weight were recorded every 2 days.

**Table 1** Composition of the basal diet without added oils

| Ingredient | g/kg |
|---|---|
| Maize | 587 |
| Defatted soybean meal (48% protein) | 320 |
| Wheat bran | 40 |
| DL-Methionine | 4 |
| L-Lysine | 5 |
| Limestone | 10 |
| Dicalcium phosphate | 9 |
| NaCl | 5 |
| Vitamin/microelement mix[a] | 20 |

[a] Per kg of diet: vitamin A 26000 IU, vitamin $D_3$ 4000 IU, vitamin K 90 mg, thiamine HCl 65 mg, riboflavin 30 mg, niacin 65 mg, pantothenic acid 245 mg, pyridoxine 20 mg, folic acid 10 mg, vitamin $B_{12}$ 0.004 mg, choline chloride 2 g, *p*-aminobenzoic acid 50 mg, manganese 65 mg, zinc 100 mg, iron 20 mg, copper 2 mg, iodine 1.30 mg, cobalt 0.8 mg, selenium 0.1 mg

**Table 2** Fatty acid content (percent weight in relation to total fatty acids) and added vitamins E and C and ethoxyquin in the diets (*ND* not detectable)

| Fatty acid | SO diet | FO diet | FO + E + C diet |
|---|---|---|---|
| Palmitic (16:0) | 13 | 19 | |
| Palmitoleic (16:1) | 0.5 | 10 | |
| Stearic (18:0) | 3.5 | 3 | |
| Oleic (18:1) | 27 | 18 | |
| Linoleic (Ω-6, 18:2) | 51.5 | 23 | |
| Linolenic (Ω-3, 18:3) | 4.5 | 0.5 | |
| Eicosenoic (20:1) | ND | 1 | |
| Arachidonic (Ω-6, 20:4) | ND | 1 | |
| Eicosapentaenoic (Ω-3, 20:5) | ND | 15 | |
| Docosapentaenoic (Ω-3, 20:5) | ND | 1.5 | |
| Docosahexaenoic (Ω-3, 20:6) | ND | 8 | |
| α-Tocopherol | 40 mg/kg | 22 mg/kg | 40 mg/kg |
| Vitamin C | | | 8.0 mg/kg |
| Ethoxyquin | 1.0 g/kg | 1.0 g/kg | 1.0 g/kg |

### Tumor and spontaneous metastasis

A highly metastatic clone (D122) of the 3LL Lewis lung carcinoma [17] was kindly provided by Prof. L. Eisenbach of our Institute. The cells were maintained in vitro in RPMI medium supplemented with 10% heat-inactivated calf serum, combined antibiotics, sodium pyruvate, and nonessential amino acids. Mice were inoculated in the right footpad with $5 \times 10^5$ D122 cells per mouse in 50 µl phosphate-buffered saline (PBS). Tumor size was monitored with a vernier caliper. In accordance with previous studies [17, 46], when the primary tumor reached a diameter of 8–9 mm, the tumor-bearing leg was amputated after ligation at the knee joint.

Experiments without cisplatin-treated mice were terminated when mortality started in the SO group. The surviving mice were then killed, and the lungs were weighed and assessed for metastatic spread by histological examination.

### Histological examination

The removed lungs were fixed in 10% buffered formalin, embedded in paraffin and stained with hematoxylin and eosin according to the

method of Lillie [34]. Sections were then examined by light microscopy.

### Cisplatin

CP (Abiplatin, lyophilized powder for injection; Abic, Netanya, Israel) was administered intravenously at a dose of 3 mg/kg body weight, similar to the dose used in human therapy, 1 day after resection of primary tumor growth and then 10 days later. Experiments were terminated 5 days after the second CP administration, and the lungs of the surviving mice were removed for assessment of metastatic load and histological examination (see above).

### Statistical analysis

The Mann-Whitney test was used. The significance of the difference between groups was analyzed by repeated measures of variance (ANOVA) using the computer program Statistica 5.0. The results are expressed as means ± SD in addition to *P*-values.

## Results

### Experimental design

This study was carried out with clone D122 [17] of the Lewis lung carcinoma line 3LL. This clone disseminates to the lung at a defined size of the primary growth in the footpad [17, 46] which provided the indication for the onset of lung metastasis. The effect of diet on tumor development was assessed in terms of two separate criteria: the size of the primary growth and the lung weight as a measure of metastatic load. Additional indirect criteria for tumor development were body weight and food consumption.

Two sets of experiments were carried out. In the first, the effect on tumor development of the three diets, without an additional therapeutic regimen, was tested. In the second, CP treatment was instituted after removal of the primary growth and combined with one of the three diets. In this set of experiments the diets before and after removal of the primary growth were either identical or changed after removal of the primary growth, as indicated.

### The effects of diets without CP treatment

The mice in the SO group developed local primary tumor in the footpad that reached 8 mm in diameter 25–28 days after tumor inoculation, while the tumor in mice in the FO + E + C group reached this size on days 33–35, and only on days 37–40 in those in the FO group. The average sizes of the primary tumor on day 25 after tumor inoculation in the three dietary groups are presented in Fig. 1.

Mice in the SO group also showed a progressive decrease in food intake and loss in body weight, which started 7 days after tumor inoculation and further decreased after removal of the primary tumor growth. These mice lost an average of 27% of their original weight and manifested clear signs of anorexia and cachexia toward the end of the experiment. In the FO and FO + E + C groups, the animals rapidly recuperated from the small loss in body weight and reduction in food intake after tumor inoculation. Body weight and food intake data are presented in Table 3.

### Lung pathology

Lung weights under the various dietary regimens, 24 days after removal of the primary growth, are summa-

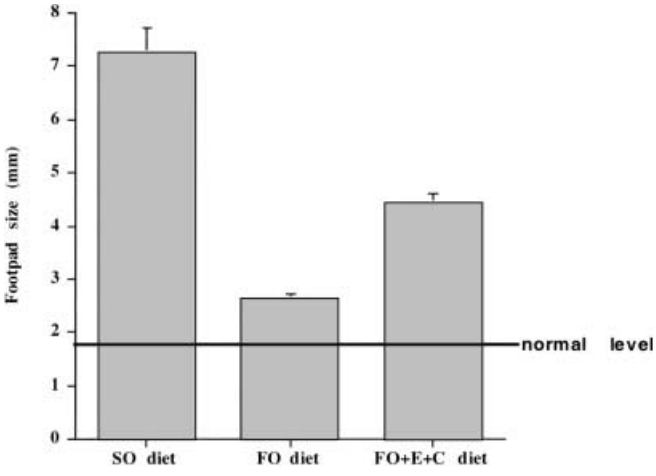


**Fig. 1** Footpad size (means ± SD) 25 days after inoculation of 3LL lung carcinoma into mice on SO, FO and FO + E + C diets. The differences between the FO group and SO group and between the FO group and FO + E + C group were highly significant ($P = 0.005$ and $P = 0.02$, respectively). The difference between the SO group and FO + E + C group was of moderate significance ($P = 0.05$)

**Table 3** Body weight and food intake (means ± SD) of tumor-free mice (control) or mice bearing 3LL carcinoma after resection of the primary tumor. Mice were either untreated or treated with CP under the various dietary regimens

| Dietary group | Final body weight (g) | Mean food intake (g/day) | |
|---|---|---|---|
| | | Beginning of study | End of study |
| SO | | | |
| Control | 26.2 ± 1.5 | 2.8 | 2.5 |
| 3LL, untreated | 18.1 ± 0.7 | | 1.6 |
| 3LL, CP treated | 17.1 ± 0.7 | | 1.1 |
| FO | | | |
| Control | 25.8 ± 1.3 | 2.5 | 2.3 |
| 3LL, untreated | 23.7 ± 0.9 | | 2.1 |
| 3LL, CP treated | 21.3 ± 0.8 | | 1.8 |
| Switched to FO + E | 23.3 ± 1.4 | | 2.1 |
| Switched to FO + C | 22.9 ± 1.2 | | 2.1 |
| Switched to FO + E + C | 23.9 ± 0.9 | | 2.2 |
| FO + E + C | | | |
| Control | 26.0 ± 1.1 | 2.7 | 2.3 |
| 3LL, untreated | 22.5 ± 0.9 | | 2.1 |
| 3LL, CP treated | 21.7 ± 0.6 | | 1.9 |

rized in Fig. 2. In healthy mice, the normal lung weight was $179 \pm 11$ mg, while in the tumor-bearing mice it was $754 \pm 113$ mg and $628 \pm 188$ mg in the SO and FO + E + C groups, respectively. The lung weight in the FO group was $317 \pm 80$ mg which is significantly different from the weights in both the SO group ($P = 0.0135$) and the FO + E + C group ($P = 0.025$; see Fig. 2). The difference between the lung weights in the SO group and the FO + E + C group was not significant ($P > 0.2$).

Pathological examination indicated that the pleural surfaces in the lungs of the SO group were occupied by numerous well-circumscribed white-gray nodules. Only a few small nodules were detected in the lungs of the FO group, while the FO + E + C group showed patterns which were intermediate in size and number between those seen in the FO and the SO groups. The tumors in the lungs of all three groups were composed of spindle or polygonal cells with numerous mitotic patterns. However, in the SO group, the lungs showed marked atypical giant cells, extensive necrosis and vessels invaded by cancer cells.

## Cisplatin plus diet

CP was administered intravenously at a dose of 3 mg/kg 24 h after resection of the primary growth, and then 10 days later. The state of tumor development was assessed 5 days after the second treatment. The effects of the various dietary regimens (see Materials and methods) on metastatic dissemination of 3LL under the conventional chemotherapy treatment with CP are summarized in Fig. 3. Lung weight after resection of primary growth was taken as an index of metastatic load, while the average lung weight in tumor-free mice (178 mg) was taken as the normal baseline. Mice in the SO group before and after CP treatment showed the highest lung weight ($863 \pm 110$ mg; Fig. 3, column 1), which is the upper border of lung weight at death. This finding indicates that under this regimen there was virtually no effect of CP treatment. In all other regimens in which FO diets were used (Fig. 3, columns 2–6) lung weights were considerably lower. In the groups on unchanged FO diets no significant difference ($P < 0.0892$) was detected between the FO (Fig. 3, column 2) and the FO + E + C (Fig. 3, column 3) groups. However, there was a significant difference in lung weights between the FO diet with CP (Fig. 3, column 2) and without CP (Fig. 2, column 2) ($571 \pm 111$ mg vs $317 \pm 81$ mg, respectively; $P < 0.0327$). However, the FO + E + C diet combined with CP (Fig. 3, column 3) was slightly, but not significantly, more beneficial than the diet without CP (Fig. 2, column 3) (average lung weights 509 ± 88 mg and $628.7 \pm 188$ mg, respectively).

Supplementation of vitamins E, C or their combination after resection of primary growth (Fig. 3, columns 4, 5 and 6), induced a significant reduction in metastatic load under CP treatment. As shown, switching from the FO diet (group 2) to the FO + E + C diet (group 6) after resection of the primary growth induced a marked reduction in metastatic load expressed as a lung weight close to normal ($261 \pm 46$ mg vs $178 \pm 14$ mg). The lung weight reached in this regimen was significantly lower than in all other treated groups, including the FO + E ($P = 0.0433$) and the FO + C ($P = 0.0147$) groups. This observation indicates that the anticancer potential of cancer chemotherapy with CP emerged only to a small extent with the continuous FO diet and became effective

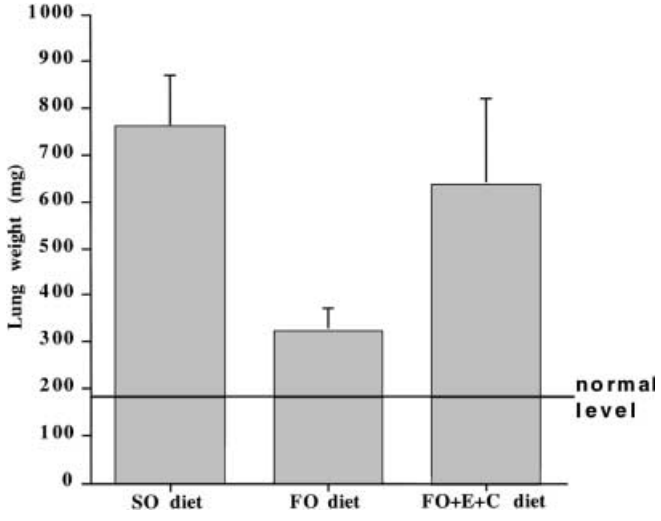


**Fig. 2** Metastatic load expressed in terms of lung weight. The lung weights (means ± SD) of mice bearing 3LL lung carcinoma on SO, FO and FO + E + C diets 24 days after resection of the primary tumor are shown. The differences in metastatic load between the FO group and the SO group or the FO + E + C group were significant ($P = 0.0135$ and $P = 0.025$, respectively). There was no significant difference between the SO and FO + E + C groups ($P > 0.2$)

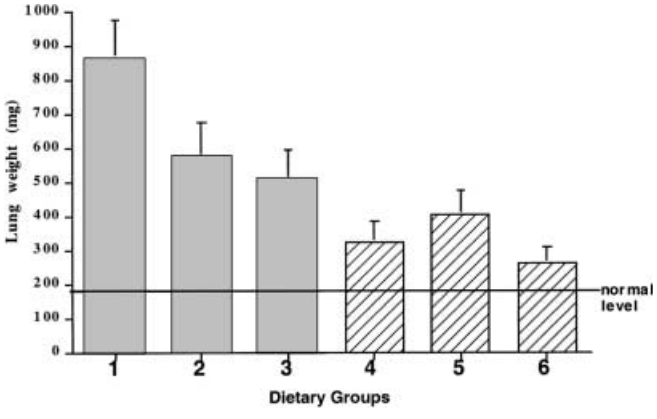


**Fig. 3** Level of metastatic load expressed in terms of lung weight of mice bearing 3LL carcinoma and treated twice with CP 1 day and 10 days after tumor resection. Six dietary groups are shown. *groups 1, 2 and 3* SO diet, FO diet and FO + E + C diet, respectively, given before and after resection of the primary growth; *groups 4, 5 and 6* FO diet switched after resection of the primary growth to FO + E, FO + C and FO + E + C diets, respectively. The lung weights shown were scored on day 16 after resection of the primary growth

if the FO diet was switched to the FO + E + C diet at the onset of metastatic dissemination.

CP administration caused a decrease in food consumption and body weight of animals on the SO diet (group 1) in which clear signs of cachexia were observed. In this respect animals of groups 2, 4, and 5, and more prominently of group 6, were significantly less affected in terms of body weight (see Table 3).

## Discussion

It has been long realized that Ω-3 PUFA exert a profound anticancer activity in animal tumor models [20, 30, 32, 55]. The experimental observations may be relevant to human cancer since a low incidence of certain tumors has been reported in populations known for their high intake of these fatty acids [2, 5, 6, 9, 28, 39, 52]. However, the mechanism by which Ω-3 PUFA, in contrast to Ω-6 PUFA, exerts its anticancer activity is still unclear.

In principle, the fatty acids provided in the diet can be metabolized via two major routes: incorporation into the membrane phospholipids of the dividing tumor cells and formation of prostanoids and leukotrienes (reviewed in reference 39). Eicosanoids originating from Ω-3 PUFA can act as partial antagonists to the tumorigenic action of those derived from Ω-6 PUFA [1, 29, 37] and thus suppress the proliferation of the tumor cells.

The Lewis lung carcinoma line 3LL provides a convenient model for human cancer. Upon local implantation, e.g. in the footpad, it disseminates at a specific stage to the lung, and more importantly, removal of the primary growth increases considerably the metastatic load [17, 46]. In our previous study [55], we demonstrated a clear antimetastatic effect of an FO diet in this tumor model. In this study we verified that the FO diet, unlike the SO diet, reduced considerably the metastatic load in mice bearing 3LL carcinoma (see Fig. 2). Unexpectedly, though, the supplementation of the FO diet with the antioxidant vitamins E and C reduced the tumor suppression. Since the antioxidative machinery of tumor cells is low [21], the observed tumor suppression by the FO diet free of these vitamins could be interpreted as related to the oxidized products of Ω-3 PUFA.

It could thus be assumed that in the cell membranes, phospholipids bearing oxidized Ω-3 PUFA can eventually lead to membrane perforation and cell death. We have recently detected such free radicals in 3LL cells after incorporation of Ω-3 PUFA (Gluzman et al., unpublished results) by paramagnetic resonance spin trapping [38]. In this respect it is interesting to note that the addition of the pro-oxidant ferrous citrate to Ω-3 PUFA diet has been found to further reduce tumor growth in vivo [24].

The counteracting effect of antioxidants on the anticancer activity of Ω-3 PUFA has been demonstrated by others both in vitro and in vivo. The rate of growth of human breast cancer cells has been shown to be inhibited by Ω-3 PUFA due to the production of lipid hydroperoxides. However, the addition of vitamin E practically restores cell growth [10]. Similarly, α-tocopherol acetate blocks the Ω-3 PUFA-induced sensitivity of astrocytoma cells to radiation [51]. In vivo studies have also demonstrated that the rate of tumor growth on diets supplemented with Ω-3 PUFA also depends on oxidative status. The addition of vitamin E to the diet diminishes considerably the anticancer effect of Ω-3 PUFA in mice bearing mammary tumor [13] or lung adenocarcinoma [35].

CP is an effective cytotoxic drug which selectively inhibits DNA synthesis and mutagenicity, in particular in solid tumors [14]. The anticancer activity of CP has also been observed in 3LL-bearing mice in which it has been found to reduce metastatic load [47] and prolong survival [49]. In our experiments with the D122 clone, treatment of the tumor-bearing mice on the SO diet with CP had no effect on metastatic load (Fig. 3, group 1). However, in mice on the FO and FO + E + C diets a moderate decrease in lung metastasis was observed (Fig. 3, groups 2 and 3). In addition, the results with the FO diet alone (Fig. 2) were significantly better than with the FO diet plus CP.

Many cytotoxic drugs, such as CP, generate hydroxyl radicals that reduce or even exhaust the antioxidant reservoir of the attacked tissue. This activity may lead to severe side effects such as cardiotoxicity, mutagenesis, pulmonary fibrosis, neutropenia, suppressed activity of natural killer cells, anorexia, cachexia and other noxious processes at the cellular level [12, 44, 48]. Ω-3 PUFA, which act as antioxidants under normal physiological conditions (low oxygen tension), may act as pro-oxidants and free-radical proliferators under high oxidizing conditions, e.g. under deficiency of vitamins E and C [39, 40]. Therefore, it seems reasonable to assume that the administration of CP to mice on the FO diet (Fig. 3, group 2), which is low in antioxidants, could lead to a high level of oxidants which react with the Ω-3 PUFA and thus reduce their incorporation into tumor cells and their anticancer action [22]. On the other hand, one may suppose that supplementation with vitamins E and C before inoculation of the tumor cells (Fig. 3, group 3) would reduce the CP peroxidation processes [11, 12, 19, 33, 41, 50] of Ω-3 PUFA in tumor cells, probably because of vitamin accumulation. In the other dietary groups (Fig. 3, groups 4–6), the concomitant supplementation of the FO diet with vitamin E (group 4), vitamin C (group 5), or their combination (group 6), increased the overall effect of the CP treatment.

FO diet followed by CP treatment and the FO + E + C diet showed the best anticancer activity of this study, and should be considered as the preferred choice for treatment. This important finding indicates that the anticancer activity of CP can be highly effective when properly supported by an FO diet under a conditioned balance of oxidation and antioxidation, which can be adjusted by vitamins E and C. In a recent study [25], mice implanted with human breast cancer cells were

treated with the conventional cytotoxic drug CPT-11 and dietary supplementation with Ω-3 PUFA clearly potentiated the anticancer activity of the drug and reduced the adverse side effects. Another important observation in our study was the elimination of anorexia and cachexia in the CP-treated mice by the FO diets. This finding may have an additional clinical implication for dietary FO support in cancer chemotherapy.

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