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Fish oil supplementation in F1 generation associated with naproxen, clenbuterol, and insulin administration reduce tumor growth and cachexia in Walker 256 tumor-bearing Rats

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Abstract

Weanling female Wistar rats were supplemented with fish oil (1 g/kg body weight) for one generation. The male offspring received the same supplementation until to adult age. Rats supplemented with coconut fat were used as reference. Some rats were inoculated subcutaneously with a suspension (2×10^7 cells/mL) of Walker 256 tumor. At day 3, when the tumor was palpable, rats were treated with naproxen (N) (0.1 mg/mL), clenbuterol (Cb) (0.15 mg/kg body weight), and insulin (I) (10 U/kg body weignt). At day 14 after tumor inoculation, the animals were killed. Tumor was removed and weighed. Blood, liver, and skeletal muscles were also collected for measurements of metabolites and insulin. In both tumor-bearing untreated rats and tumor-bearing rats supplemented with coconut fat, tumor growth, triacylglycerol, and blood lactate levels were higher, and glycogen content of the liver, blood glucose, cholesterol and HDL-cholesterol levels were lower as compared with the non–tumor-bearing and fish oil supplemented groups. Fish oil supplementation of tumor-bearing rats led to a partial recovery of the glycogen content in the liver and a full reversion of blood glucose, lactate, cholesterol, and HDL-cholesterol levels. The treatment with N plus Cb plus I attenuated cancer cachexia and decreased tumor growth in both coconut fat and fish oil supplemented rats. In conclusion, chronic fish oil supplementation decreased tumor growth and partially recovered cachexia. This beneficial effect of fish oil supplementation was potentiated by treatment with naproxen plus clenbuterol plus insulin. © 2004 Elsevier Inc. All rights reserved.

Keywords: Cancer cachexia; Tumor growth; Walker 256 tumor; Fish oil; Insulin; Clenbuterol; Naproxen

1. Introduction

Cachexia is characterized by intense peripheral catabolism with depletion of carbohydrate, lipid and protein stores, and weight loss. Most cancer patients (20–70%) die with cachexia as the primary cause, depending on tumor type [1−3]. However, as a result of the multifactorial etiology involving immune-metabolic pathways, the basic mechanisms that induce cancer cachexia remain still unknown [4−7]. Several proinflamatory cytokines, including tumor necrosis factor (TNF), interleukin-1 (IL-1), interleukin-6 (IL-6), interferon-γ [8], low plasma levels of insulin [9], high plasma levels of glucagon, cortisol, catecholamines [10], vasopressin, and prostaglandin E2 [11] have been postulated to play a key role in the establishment of cancer cachexia. Reversion of cachexia and inhibition of tumor growth are required for a successful cancer treatment. Attempts to reverse cachexia in cancer patients have used pharmacological approaches to increase food intake and to reduce tumor growth.

Over the past 150 years, there was a substantial increase in the consumption of saturated and n-6 polyunsaturated fatty acids and a reduction in that of n-3 polyunsaturated fatty acids (PUFA) [12]. This change in Western dietary habits has been correlated with an increase in the development and tumor growth [13−15]. Epidemiological and experimental studies have shown that n-3 PUFA have the

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ability to chemoprevent and to chemosuppress tumor growth [16–20]. Fish oil (rich in n-3 PUFA) administration reduces the production of cytokines such as IL-6 and TNF in healthy individuals [21,22] and in patients with pancreatic cancer [23]. Barber et al. reported normalization of metabolic response to feeding in pancreatic cancer-bearing patients supplemented with fish oil [24].

Insulin administration to Walker 256 tumor-bearing rats can partially reverse cachexia and reduce tumor growth [25,26]. Under conditions of low plasma insulin levels, as in alloxan-induced diabetic rats, tumor grows faster than in the control group [25]. Walker 256 tumor growth causes important changes in glucose, glutamine, and pyruvate metabolism in isolated lymphocytes [27] and macrophages [28]. Insulin may act on the metabolism of these cells to improve their function, thus causing deceleration or even regression of tumor growth [27].

Clenbuterol, a β_2 adrenergic agonist, has been shown to increase muscle mass [29] and to decrease total body fat in normal subjects and hypermetabolic rats [30,31] and to retard atrophy in denervated muscles [32]. In tumor-bearing rats, clenbuterol has shown a muscle-sparing effect when given in association with total parenteral nutrition [33]. Stalion et al. [34,35] reported that even in the presence of a large tumor burden, β_2 adrenergic agonist reverses muscle depletion and muscle breakdown.

Prostaglandins (PG) play an important role in cell growth either *in vitro* or *in vivo*. A large amount of PGE_2 has been found in tumor tissues and in plasma from cancer patients [36]. PGE_2 causes important metabolic alterations and has been implicated in the development of cachexia [11]. Inhibitors of PG biosynthesis has been found to induce a marked decrease of tumor growth [37], cancer cachexia [38], metastasis [39], and the survival [40] of animals bearing several types of tumors.

As mentioned above, insulin, clenbuterol, and PGE_2 have been shown to improve cachexia and to reduce tumor growth but there is no report on their association to treat cancer. This may suggest a good strategy for cancer treatment.

In most studies carried out to investigate the effect of dietary fatty acids on tumor growth, the animals were fed with a particular diet for a short period of time before or after tumor implantation. A more likely scenario, however, is a dietary regimen being given for lifelong starting from conception. We are not aware of any study that has investigated the effect of lifelong consumption of a particular fatty acid rich diet on tumor growth and cancer. In this study, coconut fat (saturated fat) and fish oil (n-3 PUFA) were given to female rats throughout pregnancy and lactation and to their male offspring after weaning. Walker 256 tumor growth and cancer-cachexia were evaluated in adulthood of male rats. The effect of fish oil supplementation associated with the treatment with naproxen (PG inhibitor), clenbuterol, and insulin was also examined. Body weight gain, tumor weight, blood levels of insulin, glucose, lactate, cholesterol, HDL-cholesterol, total lipids, triacylglycerol, and glycogen content of the liver, soleus muscle, and gastrocnemius muscle were determined. The thiobarbituric acid reactive substances (TBARS) content of tumor was determined to evaluate the possible involvement of lipid peroxidation in the effect of fish oil and coconut fat supplementation.

2. Methods and materials

2.1. Study design

The National Animal Ethics Committee approved this study. Weanling female Wistar rats (aged 21 days) were maintained under controlled temperature (23°C), humidity and 12-hour light, 12-hour dark cycle and were divided into three groups. One group received a normal chow diet *ad libitum* (control rats), and the other two groups were orally supplemented with either saturated coconut fat (S) or fish oil rich in n-3 PUFA (P). The fish oil used was a mixed marine triacylglycerol preparation containing 180 g eicosapentaenoic acid and 120 g docosahexaenoic acid/kg. The oil and fat were daily given as a single bolus at a dose of 1 g/kg body weight. At the 90 days of age, the female were mated with male Wistar rats that had been fed on a normal laboratory chow. Females continued to receive fat and oil supplementation throughout gestation and lactation. After weaning (21 days age), the male offspring received the same diet and supplementation as their mothers. At 90 days of age, half of each group was injected in the right flank with a sterile suspension of 2×10^7 Walker 256 tumor cells obtained from an ascitic tumor-bearing rat. At day 3, when the tumor was palpable, some animals were treated with naproxen (N) added to drinking water (0.1 mg/mL), clenbuterol (Cb) (0.15 mg/kg) daily injected subcutaneously, as well as insulin (I) NPH U-100 (10 U/Kg) also injected subcucaneously and given every 2 days to avoid death by hypoglycemia. The following 12 groups were set up: non–tumor-bearing (C, control), non–tumor-bearing supplemented with coconut fat (S) or fish oil (P), Walker 256 tumor-bearing rats receiving regular chow (W), tumor-bearing supplemented with S (WS) or P (WP), tumor-bearing supplemented with S or P and treated with naproxen (WSN or WPN), with naproxen plus clenbuterol (WSNCb or WPNCb), and with naproxen plus clenbuterol plus insulin (WSNCbI or WPNCbI). Body weight was regularly determined during the feeding period. At day 14 after tumor implantation, the animals were killed by decapitation without anesthesia. Tumor and heart were removed and their weight determined. Blood was collected for the measurement of glucose, lactate, cholesterol, HDL-cholesterol, triacylglycerol, and total lipids. The liver, soleus muscle and white portion of gastrocnemius muscle were removed for determination of the glycogen content.

2.2. Chemicals, oils, drugs, enzymes, and hormones

Chemicals and enzymes used were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Fish oil MaxEpa was obtained from Seven Seas (Hull, England, UK), naproxen from Syntex Corporation (Roche, Rio de Janeiro-RJ, Brazil), clenbuterol was kindly donated by Boehringer Ingelheim (São Paulo-SP, Brazil) and insulin was obtained from Biobrás (Montes Claros-MG, Brazil).

2.3. Blood metabolite determination

Serum glucose concentration was determined by using a glucose oxidase–based assay kit as described by Trinder [41] and quantified in spectrophotometer (Ultrospec 3000, Pharmacia Biotech, USA) by measuring the absorbance at 505 nm. Serum total cholesterol (Co) and HDL-cholesterol (HDL-C), and triacylglycerol (TAG) were measured using the methods described by Jung et al. [42] and Young [43], respectively, and quantified by measuring the absorbance at 500 nm (Co, HDL-C) and 540 nm (TAG). Total lipids were determined using the method described by Henry [44] and quantified in spectrophotometer at 530 nm. For lactate assay, serum (0.5 ml) was added to 0.1 mL of perchloric acid (25%) and left for 10 minutes at 4°C, followed by a centrifugation at $3000 \times g$ for 5 min. The supernatant was collected and neutralized with Tris/KOH (2 mol/L/0.5 mol/L) and the concentration of lactate was determined as described by Engle and Jones [45] at 340 nm.

2.4. Glycogen content of the liver and skeletal muscles

Accurately weighed pieces of liver, gastrocnemius muscle and soleus muscle (0.07 g) were put into 0.5 mL KOH aqueous solution (1 mol/L) and left for 20 min at 70°C for tissue digestion. After that, a digested sample (0.1 mL) was added to 0.5 mL triethanolamine buffer containing amyloglucosidase and incubated for 2 h at room temperature. After centrifugation ($800 \times g$ for 5 min), the supernatant (0.2 mL) was added to 1 mL of glucose assay buffer as described by Fernandes et al. [25]. Glycogen was quantified by measuring the absorbance at 340 nm.

2.5. Determination of products of lipid peroxidation

The products of lipid peroxidation were measured as thiobarbituric acid reactive substances (TBARS) following the method described by Winterbourn et al. [46]. Briefly, tumor tissue was extracted in phosphate buffer (0.1 mol/L) pH 7.4. The extract (0.5 mL) was then mixed with 0.5 mL thiobarbituric acid (1% in NaOH 50 mmol/L) and 0.5 mL HCl 25%. The samples were then heated in a boiling water bath for 10 min and after cooling were extracted with 1.5 mL butanol. The mixture was centrifuged at $12{,}000 \times g$ for 10 minutes and the absorbance of the supernatant was determined at 532 nm. Thiobarbituric acid reacts with products of lipid peroxidation, mainly malondialdeyde, producing a colored compound [47].

2.6. Protein determination

Protein content of the tissue homogenates was measured by the method of Bradford [48] using bovine serum albumin (BSA) as standard.

2.7. Statistical analysis

Data are presented as mean ± SEM. Statistical analysis was performed by one-way ANOVA followed by a *post hoc* Tukey test. The value of $P < 0.05$ was taken to indicate statistical significance.

3. Results

The body weight gain of the rats supplemented with coconut fat or fish oil was similar to that of the control group (Table 1). However, the presence of the tumor caused a significant decrease of body weight (by 20 g). In the coconut fat supplemented group (WS), tumor weight was 24.5 g, and the body weight remained low (by 26 g). Naproxen treatment of coconut fat supplemented rats (WSN) decreased tumor weight by 20 g (from 29.5 to 9.4 g) and increased body weight by 20 g. The inclusion of clenbuterol (WSNCb) and insulin (WSNCbI) to the treatment provoked a tumor weight decrease by 9.2 g and 8.3 g and an increase of body weight by 15 g and 33 g, respectively. These values were also statistically different from the W and WS groups (Table 1).

In rats supplemented with fish oil (WP), tumor weight was 8 g and the body weight increased by 12 g, both were statistically different from the W group (Table 1). The association of naproxen (WPN), naproxen plus clenbuterol (WPNCBI), and naproxen plus clenbuterol plus insulin (WPNCBI) did not induce a further reduction in the tumor weight and in the body weight gain as compared with WP ($P \leq 0.05$) but these results were different from W rats.

Rats supplemented with coconut fat (S) or fish oil (P) did not show any change in the liver glycogen content as compared with control (C) (Table 2). On the other hand, glycogen content of the liver was decreased by 60% in the tumor-bearing rats as compared with C ($P \leq 0.05$). The liver glycogen content was 13% lower in WP rats as compared with the C group but it was 38% higher as compared with the W group. The administration of naproxen to tumor-bearing rats supplemented with fish oil (WPN) induced a full recovery of the liver glycogen content. The inclusion of clenbuterol and insulin to the treatment (WPNCb) did not change this metabolite any further. It is noteworthy that the combination of the three agents markedly increased the liver glycogen content regardless the supplementation with either coconut fat or fish oil (WSNCbI and WPNCbI).

Table 1
Body weight, tumor, and carcass weight (g) of rats from the groups: control (C), supplemented with coconut fat (S) or fish oil (P), Walker 256 tumor-bearing rats (W), tumor-bearing suplemented with fat or oil and treated with naproxen (WSN or WPN), naproxen plus clenbuterol (WSNCb or WPNCb), or naproxen plus clenbuterol plus insulin (WSNCb1 or WPNCb1)

Group	Non–Tumor-Bearing			Tumor-Bearing								
	C	S	P	W	Coconut Fat				Fish Oil			
					WS	WSN	WSNCb	WSNCbI	WP	WPN	WPNCb	WPNCbI
Initial body weight (g)	325.8 ± 15.2	315.6 ± 10.3	334.3 ± 8.8	340.8 ± 11.2	322.9 ± 7.4	307.3 ± 14.6	308.2 ± 27.2	349.6 ± 7.1	290.2 ± 18.3	344.0 ± 16.3	334.3 ± 15.4	345.0 ± 16.9
Final body weight (g)	348.6 ± 15.9	344.3 ± 8.7	356.3 ± 9.4	345.8 ± 10.8	321.5 ± 9.5	336.1 ± 12.7	332.0 ± 24.2	390.4 ± 3.3	310.0 ± 18.4	372.1 ± 17.3	367.4 ± 16.6	386.7 ± 22.2
Tumor weight (g)				29.5 ± 2.3	24.5 ± 1.4	$9.4^{b,c}$ ± 1.4	$9.2^{b,c}$ ± 1.7	$8.3^{b,c}$ ± 1.2	8.0^{b} ± 1.9	6.5^{b} ± 1.4	4.3^{b} ± 0.7	6.7^{b} ± 1.6
Carcass weight (g)				320.9 ± 10.3	296.5 ± 10.4	326.8 ± 13.1	322.8 ± 23.1	382.1 ± 2.8	302.0 ± 19.5	366.2 ± 15.9	363.0 ± 16.5	380.0 ± 22.2
Weight change (g) (Carcass weight-initial body weight)	22.8 ± 2.9	287 ± 3.6	22.0 ± 3.6	-19.9^{a} ± 5.5	-26.4^{a} ± 5.7	$19.5^{b,c}$ ± 3.4	$14.6^{b,c}$ ± 4.5	$32.5^{b,c}$ ± 14.7	11.8^{b} ± 2.2	22.2^{b} ± 4.6	28.7^{b} ± 2.6	35.0^{b} ± 5.7

Results are presented as mean ± SEM.
[a] $P < 0.05$ as compared to non–tumor-bearing rats.
[b] $P < 0.05$ as compared to W.
[c] $P < 0.05$ as compared to WS.

Table 2
Glycogen content in the liver, soleus muscle, and white portion of gastrocnemius muscle (μmol per g tissue ww) in the groups: control (C), supplemented with coconut fat (S) or fish oil (P), Walker 256 tumor-bearing rats (W), tumor-bearing supplemented with fat or oil and treated with naproxen (WSN or WPN), naproxen plus clenbuterol (WSNCb or WPNCb), and naproxen plus clenbuterol plus insulin (WSNCb1 or WPNCb1)

Group	Non–tumor-bearing			Tumor-Bearing								
	C	S	P	W	Coconut Fat				Fish Oil			
					WS	WSN	WSNCb	WSNCb1	WP	WPN	WPNCb	WPNCb1
Liver	101.9 ± 12.61	107.6 ± 14.08	111.0 ± 12.55	64.89^{a} ± 5.20	65.08 ± 5.03	$144.6^{b,c}$ ± 23.67	$166.1^{b,c}$ ± 21.04	$190.5^{a,b,c}$ ± 29.44	89.41^{b} ± 2.57	$167.9^{b,d}$ ± 24.32	$155.8^{b,d}$ ± 24.10	$201.1^{a,b,d}$ ± 28.00
Soleus	27.39 ± 1.13	29.19 ± 1.29	34.17^{e} ± 1.56	21.60^{a} ± 1.38	22.55 ± 1.61	$37.10^{b,c}$ ± 1.55	$51.81^{b,c}$ ± 6.06	$49.61^{b,c}$ ± 5.00	31.49^{b} ± 2.23	$53.17^{a,b,d}$ ± 5.24	$42.26^{a,b,d}$ ± 4.43	$62.72^{a,b,d}$ ± 4.52
Gastro-cnemius	41.41 ± 4.20	42.22 ± 3.96	44.01 ± 3.74	30.40^{a} ± 2.13	31.51 ± 1.86	$39.29^{b,c}$ ± 2.92	$44.38^{b,c}$ ± 5.49	$43.00^{b,c}$ ± 3.46	40.17^{b} ± 2.39	42.25^{b} ± 2.89	41.45^{b} ± 3.64	47.94^{b} ± 3.58

Results are presented as mean ± SEM.
[a] $P < 0.05$ as compared to non–tumor-bearing rats.
[b] $P < 0.05$ as compared to W.
[c] $P < 0.05$ as compared to WS.
[d] $P < 0.05$ as compared to WP.
[e] $P < 0.05$ as compared to control.

As compared with C rats, the glycogen content of soleus muscle was increased by fish oil supplementation, whereas coconut fat had no effect. The W group had a significant decrease (by 22%) of the glycogen content of soleus muscle, as compared non–tumor-bearing animals, but it was not changed by coconut fat supplementation. Treatment with naproxen (WSN), naproxen plus clenbuterol (WSNCb), and naproxen plus clenbuterol plus insulin (WSNCbI) induced a remarkable increase in the glycogen content of soleus muscle. WP rats had the soleus muscle glycogen content restored to control values. The combination of fish oil supplementation with naproxen (WPN), naproxen plus clenbuterol (WPNCb), and naproxen plus clenbuterol plus insulin (WPNCbI) induced a significant further increase.

The supplementation with coconut fat or fish oil did not modify the glycogen content of gastrocnemius muscle in non–tumor-bearing rats as compared with controls. In Walker 256 tumor-bearing rats, however, it was significantly lowered. Saturated fat supplementation did not change the glycogen content of gastrocnemius muscle, whereas the administration of naproxen (WSN), naproxen plus clenbuterol (WSNCb), naproxen plus clenbuterol plus insulin (WSNCbI) restored it to control values. Fish oil supplementation (WP) by itself restored the glycogen content of gastrocnemius muscle to control values. Treatment with naproxen (WPN), naproxen plus clenbuterol (WPNCb), and naproxen plus clenbuterol plus insulin (WPNCbI) did not cause any further increase.

Supplementation of non–tumor-bearing with coconut fat or fish oil did not change cholesterol (Co), HDL-cholesterol (HDL-C), triacylglycerol (TAG), total lipids, lactate, glucose, and insulin as compared with control rats (Table 3). The presence of the tumor (W) significantly decrease all measurements, except for cholesterol and total lipids that remained similar to non–tumor-bearing animals. The concentration of the metabolites and insulin in plasma of tumor-bearing rats was not changed by coconut fat supplementation. The administration of naproxen (WSN), naproxen plus clenbuterol (WSNCb), naproxen plus clenbuterol plus insulin (WSNCbI) restored all measurements to control values. Fish oil supplementation (WP) fully abolished the changes induced by the tumor and the pharmacological treatment did not cause any additional effect.

The content of TBARS in the tumor tissue was not different among the groups studied. The values were of 8 μmol/mg protein of the tumor tissue for all groups (data not shown).

4. Discussion

Tumor-bearing rats (W) and tumor-bearing supplemented rats with coconut fat (WS) showed a significant decrease in body weight, glycogen content in the liver and skeletal muscles, blood glucose, HDL-cholesterol, and insulin levels and an increase in plasma lactate (Tables 1–3).

Table 3

Serum concentration of cholesterol (Co), HDL-cholesterol (HDL-C), triacylglycerol (TAG), total lipids, lactate, glucose, and insulin in the groups: control (C), supplemented with coconut fat (S) or fish oil (P), Walker 256 tumor-bearing rats (W), tumor-bearing supplemented with fat or oil and treated with naproxen (WSN or WPN), naproxen plus clenbuterol (WSNCb or WPNCb), and naproxen plus clenbuterol plus insulin (WSNCb1 or WPNCbI)

Group	Non–tumor-bearing			Tumor-Bearing								
	C	S	P	W	Coconut Fat				Fish Oil			
					WS	WSN	WSNCb	WSNCbI	WP	WPN	WPNCb	WPNCbI
Co (mg/dL)	73.5 ± 3.6	75.9 ± 4.5	73.7 ± 2.5	73.6 ± 3.7	68.0 ± 3.9	62.7 ± 4.9	66.8 ± 3.3	64.2 ± 3.9	62.9 ± 4.3	63.0 ± 3.7	63.9 ± 4.4	61.3 ± 2.3
HDL-C (mg/dL)	35.7 ± 1.9	37.6 ± 2.7	34.6 ± 2.8	14.8^{a} ± 1.3	14.9 ± 1.8	$27.5^{b,c}$ ± 1.5	$32.5^{b,c}$ ± 1.5	$33.6^{b,c}$ ± 2.0	29.1^{b} ± 3.5	26.8^{b} ± 1.1	30.8^{b} ± 2.4	30.8^{b} ± 1.7
TAG (mg/dL)	102.5 ± 17.2	125.1 ± 6.6	110.8 ± 3.4	167.4^{a} ± 14.0	162.2 ± 6.9	$95.3^{b,c}$ ± 6.7	$89.7^{b,c}$ ± 8.8	$89.2^{b,c}$ ± 8.7	97.5^{b} ± 13.4	80.0^{b} ± 7.8	101.7^{b} ± 6.4	90.3^{b} ± 6.5
Total Lipids (mg/dL)	315.7 ± 17.2	367.4 ± 26.3	321.6 ± 18.7	318.3 ± 39.1	314.0 ± 23.8	324.0 ± 35.1	277.4 ± 17.3	246.0 ± 34.2	342.7 ± 28.0	267.6 ± 19.4	334.0 ± 40.8	263.7 ± 22.1
Lactate (μmol/mL)	1.99 ± 0.08	2.26 ± 0.08	2.20 ± 0.11	2.74^{a} ± 0.09	2.82 ± 0.14	$2.10^{b,c}$ ± 0.11	$2.02^{b,c}$ ± 0.11	$2.00^{b,c}$ ± 0.09	2.17^{b} ± 0.07	2.33^{b} ± 0.10	2.10^{b} ± 0.14	2.04^{b} ± 0.13
Glucose (mg/dL)	142.4 ± 3.0	142.3 ± 3.5	134.1 ± 2.3	109.4^{a} ± 3.3	114.2 ± 3.8	$140.8^{b,c}$ ± 2.2	$144.9^{b,c}$ ± 5.5	$139.1^{b,c}$ ± 2.1	133.6^{b} ± 2.0	142.8^{b} ± 5.3	144.3^{b} ± 3.9	141.2^{b} ± 10.3
Insulin (μU/mL)	77.2 ± 2.8	72.1 ± 8.6	76.7 ± 3.2	25.8^{a} ± 5.9	26.0 ± 6.6	$58.6^{b,c}$ ± 9.9	$62.8^{b,c}$ ± 11.6	$62.3^{b,c}$ ± 16.8	53.4^{b} ± 6.7	57.3^{b} ± 12.7	65.1^{b} ± 15.0	58.3^{b} ± 13.1

Results are presented as mean ± SEM.

[a] $P < 0.05$ as compared to non–tumor-bearing groups.

[b] $P < 0.05$ as compared to W.

[c] $P < 0.05$ as compared to WS.

These results confirm the establishment of cachexia [11,25−27]. Epidemiological studies have shown a link between fat-rich diets and the incidence of cancer [49]. Reduced risk of cancer in colon, breast, and prostate has been associated with low fat intake and diets rich in n-3 fatty acids [50]. Diets rich in n-6 PUFA have been shown to promote an increase in cancer risk [51−53]. Supplementation with fish oil (WP) for one generation caused a marked decrease in tumor growth and an improvement of cachexia. The increase in the amount of fat in the diet by only 0.1% is quite different from other studies that used between 8% and 20% of n-3 PUFA [54]. Also, the effect of a chronic supplementation of fish oil for F1 generation was not previously performed. Interestingly, this small dose of n-3 PUFA given for one generation caused remarkable beneficial effects on tumor growth and cachexia. The mechanisms involved however are still unclear. Modification in prostaglandin biosynthesis, cyclooxygenase-2 activity, angiogenesis, modulation of immune cell function, and lipid peroxidation in the tumor has been suggested to play a role [19,55]. The lytic effects of n-3 PUFA on cultured tumor cells are correlated with the degree of lipid peroxidation [56−58]. However, the content of lipid peroxidation products (TBARs) of the tumor was not changed by the treatments imposed. Thus, the beneficial effects of the fish oil on Walker 256 tumor-bearing rats are not mediated by causing lipid peroxidation of the neoplastic tissue.

Prostaglandins favor tumorigenesis and are found at high amount in tumors as compared with normal tissues from which cancers arise [59,60]. Walker 256 tumor-bearing rats show high plasma levels of prostaglandin E_2 that have been associated with tumor growth [11]. PGE_2 mediates tumor survival by inhibiting tumor cell apoptosis and inducing tumor cell proliferation [61]. In addition, PGE_2 alters cell morphology and increases cell motility and migration resulting in tumor progression [62]. The immune system plays an important role in tumor response. PGE_2 stimulates the production of cytokines such as IL-4, IL-5, and IL-10 by T helper 2 cells (Th_2) and markedly inhibits the production of Th1 cytokines such as IFN-γ and IL-2 [63]. Thus PGE_2 promotes humoral and Th_2 immune responses, which do not participate in tumor destruction and inhibits Th_1 responses that promotes tumor destruction. The relationship between enhanced cyclooxigenase-2 expression and tumor growth has been established in breast, colon, and prostate cancers [64,65]. Several cyclooxygenase inhibitors have anticancer activities [66−69]. Our results show that fish oil supplementation decreases tumor growth and ameliorates cachexia (Tables 1–3). The treatment with naproxen did not cause any further decrease in the tumor growth. These results suggest that fish oil and COX inhibitors possibly share the same mechanism to reduce tumor.

Clenbuterol antagonizes the skeletal muscle depletion, acting possibly via ATP-ubiquitin proteolytic pathway promoting its hyperactivation [70]. The inclusion of clenbuterol to the treatment did not provoke any further decrease on tumor growth in coconut fat or fish oil supplemented rats (WSNCb or WPNCb), but it slightly increased the body weight gain. This may result from an increase in protein synthesis [70-73] and a reduction in proteolysis [29,74]. Glycogen content of the liver and soleus muscle was also increased by clenbuterol treatment. The serum measurements also returned to normal levels. Stalion et al. [35] reported that increased cardiac mass is associated with high doses of β_2 agonist. Thus, an important task for the therapeutic use of this drug is to limit the adverse metabolic effects while retaining the anabolic properties. It is interesting to mention that the dose used did not cause any increase in cardiac mass (data not shown).

Tumor growth led to hypoglycemia and hypoinsulinemia (Table 3). Insulin solely [25,26] or combined [75] with the other hormones such as GH and somatostatin, has been used to treat cancer. The rats supplemented with S or P were treated with naproxen plus clenbuterol plus insulin to counteract the catabolism induced by the tumor. There was no further decrease of tumor growth but the inclusion of insulin in the treatment contributed to restore body weight and the glycogen content of the liver and soleus muscle to control values. Possibly, insulin prevented the lipolytic effect of clenbuterol and acted to further increase protein synthesis and to inhibit protein degradation. There was no further contribution of this combination concerning the serum measurements. Also, the dose of insulin used was safe and did not induce hypoglycemia that could kill the rats.

These results led us to propose that fish oil supplementation for one generation decreases tumor growth and partially prevents cancer cachexia. The treatment with naproxen plus clenbuterol plus insulin fully prevented cachexia and this effect was similar for rats supplemented with either coconut fat or fish oil. Thus, the combination of naproxen, clenbuterol and insulin associated with fish oil supplementation may consist a good strategy to treat cancer patients.

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References

[1] Barber MD, Ross JA, Fearon KCH. Cancer cachexia. Surg Oncol 1999;8:133–41.
[2] Costelli P, Tessitore L, Batetta B, Mulas MF, Spano O, Pani P, Baccino FM, Dessi S. Alterations of lipid and cholesterol metabolism in cachetic tumor-bearing rats are prevented by insulin. J Nutr 1999; 129:700–6.
[3] Tisdale MJ. Cancer cachexia: metabolic alterations and clinical manifestations. Nutrition 1997;13:1–7.

[4] Giacosa A, Frascio F, Sukkar SG, Roncella S. Food intake and body composition in cancer cachexia. Nutrition 1996;12:S20–3.
[5] Plata-Salamán CR. Central nervous system mechanisms contributing to the cachexia-anorexia syndrome. Nutrition 2000;16:1009–12.
[6] Tisdale MJ. Metabolic abnormalities in cachexia and anorexia. Nutr 2000;16:1013–4.
[7] Tisdale MJ. Cancer anorexia and cachexia. Nutrition 2001;17:438–42.
[8] Cahlin C, Körner A, Axelsson H, Wang W, Lundholm K, Svanberg E. Experimental cancer cachexia: the role of host-derived cytokines interleukin (IL)-6, IL-12, interferon-γ, and tumor necrosis factor-α evaluated in gene knockout, tumor-bearing mice on C57 Bl background and eicosanoid-dependent cachexia. Cancer Res 2000;60:5488–93.
[9] Fernandes LC, Machado UF, Nogueira CR, Carpinelli AR, Curi R. Insulin scretion in Walker 256 tumor cachexia. Am J Physiol 1990;258:E1033–6.
[10] Tracey KJ, Lowry SF, Fahei TJ III, Albert JD, Fong Y, Hesse D, Beutler B, Manogue KR, Calvano S, Wei H, Cerami A, Shires GT. Cachectin/tumor necrosis factor induces lethal shock and stress hormone responses in the dog. Surg Gynecol Obstet 1987;164:415–22.
[11] Siddiqui RA, Williams JF. Interactions of vasopressin and prostaglandin E_2 in the development of cancer cachexia. Med Sci Res 1987;15:45–6.
[12] Simopoulos AP. Essential fatty acids in health and chronic disease. Am J Clin Nutr 1999;70:560S–9S.
[13] Rose DP, Connoly JM. Effects of fatty acids and eicosanoid synthesis inhibitors on the growth of two human prostate cancer cell lines. Prostate 1991;18:243–54.
[14] Pandalai PK, Pilat MJ, Yamazaki K, Naik H, Pienta KJ. The effects of omega-3 and omega-6 fatty acids on in vitro prostate cancer growth. Anticancer Res 1996;16:815–20.
[15] Caygill CPJ, Charlett A, Hill MJ. Fat, fish oil and cancer. Br J Cancer 1996;74:159–64.
[16] Karmali RA. Historical perspective and potential use of n-3 fatty acids in therapy of cancer cachexia. Nutr 1996;12:S2–4.
[17] Sauer LA, Dauchy RT, Blask DE. Mechanism for the antitumor and anticachetic effects of n-3 fatty acids. Cancer Res 2000;60:5289–95.
[18] Sauer LA, Dauchy RT, Blask DE. Polyunsaturated fatty acids, melatonin, and cancer prevention. Biochem Pharmacol 2001;61:1455–62.
[19] Rose DP, Connolly JM. Omega-3 fatty acids as cancer chemopreventive agents. Pharmacol Ther 1999;83:217–44.
[20] Helton WS, Espat NJ. Defining mechanisms of ω-3 fatty-acid activity. Nutrition 2001;17:674–87.
[21] Endres S, Ghorbani R, Kelley VE, Gerogilis K, Lonnemann G, van der Meer JWM, Cannon JG, Rogers TS, Klempner MS, Weber PC, Schaefer EJ, Wolff SM, Dinarello CA. The effect of dietary supplementation with n-3 polyunsaturated fatty acids on the synthesis of interleukin-1 and tumor necrosis factor by mononuclear cells. N Engl J Med 1989;320:265–71.
[22] Meydani SN, Lichtenstein AH, Cornwall S, Meydani M, Goldin BR, Rasmussen H, Dinarello CA, Schaefer EJ. Immunological effects of National Cholesterol Education Panel Step-2 diets with and without fish-derived n-3 fatty acid enrichment. J Clin Invest 1993;92:105–13.
[23] Wigmore SJ, Fearon KCH, Maingay JP, Ross JA. Down-regulation of the acute-phase response in patients with pancreatic cancer cachexia receiving oral eicosapentaenoic acid is mediated via suppression of interleukin-6. Clin Sci 1997;92:215–21.
[24] Barber MD, McMillan DC, Preston T, Ross JA, Fearon KCH. The metabolic response to feeding in weight-losing pancreatic cancer patients and its modulation by a fish oil-enriched nutritional supplement. Clin Sci 2000;98:389–99.
[25] Fernandes LC, Carpinelli AR, Hell NS, Curi R. Improvement of cancer cachexia and decrease of Walker 256 tumor growth by insulin administration in rats. Cancer Ther Contr 1991;1:259–68.
[26] Moley JF, Morisson SD, Gorschboth CM, Norton JA. Body composition changes in rats with experimental cancer cachexia: improvement with exogenous insulin. Cancer Res 1988;48:2784–7.
[27] Fernandes LC, Marques da Costa MM, Curi R. Metabolism of glucose, glutamine an pyruvate in lymphocytes from Walker 256 tumor-bearing rats. Braz J Med Biol Res 1994;27:2539–43.
[28] Costa Rosa LFBP, Guimarães ARP, Safi DA, Curi R, Williams JF. Effect of various dietary fatty acids on enzyme activities of carbohydrate and glutamine metabolism and the metabolic responses of lymphocytes and macrophages during Walker-256 ascites cell tumor growth in rats. Biochem Mol Biol Int 1993;29:33–45.
[29] Reeds PJ, Hay SM, Dorward PM, Palmer RM. Stimulation of muscle growth by clenbuterol: lack of effect on muscle protein biosynthesis. Br J Nutr 1986;56:249–56.
[30] Yang YT, McElligot MA. Multiple actions of β-adrenergic agonists on skeletal muscle and adipose tissue. Biochem J 1989;261:1–10.
[31] Chance WT, von Almen D, Benson D, Zhang F-S, Fisher JE. Clenbuterol decreases catabolism and increases hypermetabolism in burned rats. J Trauma 1991;31:365–70.
[32] Zeman RJ, Lundermann R, Etlinger JD. Clenbuterol, a β_2–agonist, retards atrophy in denervated muscles. Am J Physiol 1987;252:E152–5.
[33] Chance WT, Cao L, Zhang F-S, Foley-Nelson T, Fisher JE. Muscle-sparing effect of clenbuterol in tumor-bearing rats maitained on total parenteral nutrition. Surg Forum 1989;40:411–3.
[34] Stallion A, Zhang F-S, Chance WT, Foley-Nelson T, Fisher JE. Reversal of cancer cachexia in rats by cimaterol and supplementatal nutrition. Surgery 1991;110:678–84.
[35] Stallion A, Foley-Nelson T, Chance WT, Fisher JE. Effects of increased B2-agonist dose in tumor-bearing animals. Nutr Cancer 1993;20:251–60.
[36] Williams ED, Karim SMM, Sandler M. Prostaglandin secretion by medullary carcinoma of the thyroid. A possible cause of the associated diarrhoea. Lancet 1968;Jan 6;1(7532):22–3.
[37] Lynch NR, Salomon JC. Tumor growth inhibition and potentiation of immunotherapy by indomethacin in mice. J Natl Cancer Inst 1978;62:117–21.
[38] Homem-de-Bittencourt PI Jr, Pontieri V, Curi R, Lopes OU. Effects of aspirin-like drugs on Walker 256 tumor growth and cachexia in rats. Braz J Med Biol Res 1989;22:1039–42.
[39] Fulton AM. Inhibition of experimental metastasis with indomethacin: role of macrophages and natural killer cells. Prostaglandins 1988;35:413–25.
[40] Bennett B, Houghton J, Leaper DJ, Stamford IF. Cancer growth, response to treatment and survival time in mice: beneficial effect of the prostaglandin synthesis inhibition flurbiprofen. Prostaglandins 1979;17:179–91.
[41] Trinder P. Determination of blood glucose an oxidase-peroxidase system with a non-carcinogenic chromogen. J Clin Pathol 1969;22:158–61.
[42] Jung DH, Biggs HG, Moorehead WR. Colorimetry of serum cholesterol with use of ferric acetate/uranyl acetate and ferrous sulfate/sulfuric acid reagents. Clin Chem 1975;21:1526–30.
[43] Young DS. Effects of Preanalytical Variables on Clinical Laboratory Tests. 2nd ed: Washington, DC: AACC Press, 1997.
[44] Henry RJ. Clinical Chemistry: Principles and Technics. New York: Harper & Row, 1964, pp. 833–8.
[45] Engle PC, Jones JB. Causes an elimination of erratic blanks in enzymatic metabolite assay involving the use of NAD+ in alkaline hydrazine buffers: improved conditions for the assay of L-glutamate, L-lactate, and other metabolites. Anal Biochem 1978;88:475–84.
[46] Winterbourn C, Gutteridge JMC, Halliwell B. Doxorubicin-dependent lipid peroxidation at low partial pressure of oxygen. J Free Rad Biol Med 1985;1(1):43–9.
[47] Miyasaka CK, Mendonça JR, Nishiyama A, Alves de Souza JA, Pires de Melo M, Pithon-Curi TC, Curi R. Comparative effects of fish oil

given by gavage and fish oil-enriched diet on leukocytes. Life Sci 2001;69:1739–51.
[48] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
[49] Boyd NF, Martin LJ, Noffel M, Lockwood GA, Tritchler DL. A meta analysis of studies of dietary fat and breast cancer risk. Br J Cancer 1993;68:627–36.
[50] Caygill CPJ, Charlett A, Hill MJ. Fat, fish oil and cancer. Br J Cancer 1996;74:159–64.
[51] Aronson WJ, Glaspy JA, Reddy ST, Reese D, Heber D, Bagga D. Modulation of omega-3/omega-6 polyunsaturated ratios with dietary fish oils in men with prostate cancer. Urology 2001;58:283–8.
[52] Collett ED, Davidson LA, Fan Y-Y, Lupton JR, Chapkin RS. n-6 and n-3 Polyunsaturated fatty acids differentially modulate oncogenic Ras activation in colonocytes. Am J Physiol 2001;280:C1066–75.
[53] Cowing BE, Saker KE. Polyunsaturated fatty acids and epidermal growth factor receptor/mitogen-activated protein kinase signaling in mammary cancer. J Nutr 2001;131:1125–8.
[54] Dommels YEM, Alink GM, van Bladeren PJ, van Ommen B. Dietary n-6 and n-3 polyunsaturated fatty acids and colorectal carcinogenesis: results from cultured colon cells, animal models and human studies. Environ Toxicol Pharmacol 2002;12:233–44.
[55] Jiang WG, Bryce RP, Horrobin DF. Essential fatty acids: molecular and cellular basis of their anti-cancer action and clinical implications. Crit Rev Oncol Hematol 1998;3:179–209.
[56] Begin ME, Ells G, Das UN, Horrobin DF. Diferential killing of human carcinoma cells supplemented with n-3 and n-6 polyunsaturated fatty acids. J Natl Cancer Inst 1986;77:1053–62.
[57] Begin ME, Ells G, Horrobin DF. Polyunsaturated fatty acid-induced cytotoxicity against tumor cells and its relationship to lipid peroxidation. J Natl Cancer Inst 1988;80:188–94.
[58] Begin ME, Ells G. Effects of C18 fatty acids on breast carcinoma cells cultured in vitro. Anticancer Res 1987;7:215–8.
[59] Subbaramaiah K, Zakin D, Weksler BB, Dannenberg AJ. Inhibition of cyclooxygenase: a novel approach in cancer prevention. Proc Soc Exp Biol Med 1997;216:201–10.
[60] Chulada PC, Thompson MB, Mahler JF, Doyle CM, Gaul BW, Lee C, Tiano HF, Morham SG, Amithies O, Langenbach R. Genetic disruption of Ptgs-1 as well as Ptgs-2, reduces intestinal tumorigenesis in Mim mice. Cancer Res 2000;60:4705–8.
[61] Sumitani K, Kamijo R, Toyoshima T, Nakanishi Y, Takizawa K, Hatori M, Naguno M. Specific inhibition of cyclooxygenase-2 results in inhibition of proliferation of oral cancer cell lines via suppression of prostaglandin E_2 production. J Oral Pathol Med 2001;30:41–7.
[62] Gately S. The contribution of cyclooxygenase-2 to tumor angiogenesis. Cancer Metastasis Rev 2000;19:19–27.
[63] Hilkens CM, Snijderset A, Snijdewint FG, Wierenga EA, Kapsenberg ML. Modulation of T-cell cytokine secretion by accessory-cell-derived products. Eur Respir J 1996;22:90S–4S.
[64] Dempke W, Rie C, Grothey A, Schmoll HJ. Cyclooxygenase-2: a novel target for cancer chemotherapy. J Cancer Res Clin Oncol 2001;127:411–7.
[65] Kirschenbaum A, Liu XH, Yao S, Levine AC. The role of cyclooxygenase-2 in prostate cancer. Urology 2001;58:127–31.
[66] Gelin J, Anderson C, Lundholm K. Effects of indomethacin, cytokines, and cyclosporin A on tumor growth and the subsequent development of cancer cachexia. Cancer Res 1991;51:880–5.
[67] Prescott SM, Fitzpatrick FA. Cyclooxygenase-2 and carcinogenesis. Biochim Biophys Acta 2000;1470:M69–78.
[68] Masferrer JL, Leahy KM, Koki AT, Zweifel BS, Settle SL, Woerner BM, Edwards AD, Flickinger AG, Moore RJ, Seibert K. Antiangiogenic and antitumor activities of cyclooxygenase-2 inhibitors. Cancer Res 2000;60:1306–11.
[69] Cassano G, Gasparre G, Susca F, Lippe C, Guanti G. Effect of prostaglandin E_2 on the proliferation, Ca^{2+} mobilization and cAMP in HT-29 human colon adenocarcinoma cells. Cancer Lett 2000;152: 217–22.
[70] Carbó N, López-Soriano J, Tarragó T, González O, Llovera M, López-Soriano FJ, Argilés JM. Comparative effects of β_2-adrenergic agonists on muscle waste associated with tumour growth. Cancer Lett 1997;115:113–8.
[71] Anderson PT, Hefferich WG, Parkhall LC, Meakel RA, Bergen WG. Ractopamine increases total and myofibrilar protein synthesis in cultured rat myotubes. J Nutr 1990;120:1677–83.
[72] Emery PW, Rothwell NJ, Stock MJ, Winter PD. Chronic effects of β_2-adrenergic agonists on body composition and protein synthesis in the rat. Biosci Rep 1984;4:83–91.
[73] Babit P, Booth FW. Clenbuterol prevents or inhibits loss of specific mRNAs in atrophying rat skeletal muscle. Am J Physiol 1988;254: C657–60.
[74] Benson DW, Foley-Nelson T, Chance WT, Zhang F-S, James JH, Fisher JE. Decreased myofibrilar protein breakdown following treatment with clenbuterol. J Surg Res 1991;50:1–5.
[75] Bartlett DL, Charland S, Torosian MH. Growth hormone, insulin, and somatostatin therapy of cancer cachexia. Cancer 1994;73:1499–1504.