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Exercise and Shark Liver Oil Supplementation Reduce Tumor Growth and Cancer Cachexia in Walker 256 Tumor Bearing Rats

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Abstract

This study investigated whether exercise associated to shark liver oil supplementation (1 g/kg b.w./day) affects tumor growth, cachexia, lipid peroxidation and proteins expression involved in cell death in Walker 256 tumor-bearing rats. Animals were divided into 4 groups: sedentary tumor-bearing (W), sedentary tumor-bearing shark liver oil supplemented (WSL), exercised tumor-bearing (EW) and exercised tumor-bearing shark liver oil supplemented (EWSL). Training sessions consisted of 6 bouts, 30 seconds each with 50% body-weight load attached to the trunk followed by 1 minute of resting (jump training). Five minutes after the finish jump training the exercise groups were subjected to 30 minutes of continuous swimming with a load equivalent to 6% of body weight, 4 times a week during 8 weeks. Tumor cells were injected at the 6th training week and all groups were killed 15 days post inoculation. Tumor weight (g) in W group was of 26.50 ± 1.79 and in the WSL, EW and EWSL was of 14.08 ± 0.91, 15.60 ± 0.55 and 12.60 ± 1.07, respectively. The group W showed hypoglycemia (68.67 ± 2.12 mg/dl), hyperlactacidemia (1.49 ± 0.06 mmol/L), hypertriacylglycerolemia (161.4 ± 9.4 mg/dl) and body weight reduction (13.21 ± 2.25 g) characterizing cachexia state. The groups WSL, EW and EWSL presented reduction of tumor cells proliferation *ex vivo*, and the production of hydroperoxide and apoptosis was increased. Bax/Bcl-2 expression ratio was increased only in the exercised groups. Shark liver oil supplementation and exercise alone were able in to avoid the installation of cachexia state and also reduced tumor growth, but the association of both cause further effect only in the tumor growth.

Keywords: Exercise; Cancer cachexia; Shark liver oil; Lipid peroxidation; Apoptosis

Introduction

Several studies have examined the role of exercise as an adjunct therapy in cancer treatment. It has been reported that exercise changes tumor progression [1,2] as well as improves wellbeing and quality of life of cancer patients [3,4]. These changes in tumor progression appear to be related to a process called lipid peroxidation (LP). Gago-Dominguez et al. [5] proposed that LP represents a protective mechanism in breast cancer, and reviewed the evidence of the role of this process on established reproductive, hormonal, and non-hormonal factors for breast cancer. Exercise increases oxygen consumption levels considerably by as much as more than 10-fold regarding to resting level. This will lead to an increase of reactive oxygen species (ROS) production [6].

LP is one of the most investigated features of ROS actions on membrane structure and function. It has been shown that lipid hydroperoxides and oxygenated products of LP degradation as well as lipid peroxidation initiators (e.g.: ROS) participate in many process such as signal transduction cascades [7], cell proliferation control, induction of differentiation, maturation, and apoptosis [8]. It has been shown that LP and ROS are triggers and essential mediators of apoptosis, which eliminates precancerous and cancerous, virus-infected and damaged cells that threaten our health [9].

Shark liver oil (SLO) is an ancient remedy among the fishermen who live along the west coast of Norway and Sweden. It was not until the early part of the last century that biochemists discovered substances in this oil that may account for its traditional use namely, alkylglycerols [10]. Alkylglycerols are glyceryl ether lipids that are structurally characterized by an ether linkage of a fatty acid attached to the chain length and by the number of double bonds; several derivatives of the ether lipid have been identified [11]. Alkylglycerols inhibit the growth of primary tumors and the metastasis of Lewis lung carcinoma growing in C57Bl/6 mice [12]. It has been reported a time-and concentration-dependent development of oxidative stress when sensitive HL-60 cells were exposed to membrane-targeted ether lipids [13]. Oxidative stress has been suggested as an inducer of apoptosis in human leukemic cells [14]. Induction of oxidative stress followed by apoptosis is also assumed to explain the inhibition of L-1 sarcoma-induced angiogenesis by alkylglycerols [15].

SLO supplementation as well as exercise has been used for cancer chemoprevention and also treatment. So far we are not aware of any study about the association of both exercise and SLO supplementation on cancer growth, cachexia, proliferation and death of tumor cells. Therefore, this study investigated whether exercise associated to shark liver oil supplementation (1 g/kg b.w./day) affects tumor growth, cachexia, lipid peroxidation and proteins expression involved in cell death in Walker 256 tumor-bearing rats. We hypothesize that both interventions might be beneficial for tumor-bearing individuals.

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Materials and Methods

Chemical and enzymes

All chemicals and enzymes were purchased from Sigma Chemical (St. Louis, MO), unless otherwise indicated.

Study design

All proceeds involving animals were approved by the Animal Ethics Committee of the Biological Science Building, Federal University of Paraná under number 293. Wistar rats (70 days old) were obtained from the animal house and maintained under controlled temperature 23°C and 12 h light/12 h dark cycle, with free access to water and a standard commercial chow food (Nutrilab - CR1, Nuvital Nutrients Ltda, Curitiba-PR, Brazil).

Rats were randomized to one of the four experimental groups: sedentary tumor-bearing (W), sedentary tumor-bearing SLO supplemented (WSL), exercised tumor-bearing (EW) and exercised tumor-bearing SLO supplemented (EWSL). Training sessions was carried out in a swimming pool apparatus, in which water temperature was maintained between 29°C and 32°C. The apparatus is composed of 12 individual swimming pools in which each rat swims individually. The depth of the water column is maintained at 50 cm. The exercise training protocol consisted of six 30-s sets, each followed by 1 min of rest, with a load equivalent to 50% of body weight load attached to the back, 4 times a week for 8 weeks. Rats were acclimated to the water environment for 2 days before the exercise protocol was initiated. So that they could further adapt to the water environment, rats underwent progressive training, beginning with a load corresponding to 30% of their body weight and reaching 50% at the eighth exercise session. Because the load is too great for the rats to stay above the water surface by swimming, they must perform serial jumps to keep breathing. In 30 s, the duration of each set, a rat performs 10 jumps, on average. This training protocol is characterized by power and anaerobic exercise [16]. Five minutes after the finish jump training the exercise groups were subjected to 30 minute of continuous swimming with a load equivalent to 6% of body weight, 4 times a week during 8 weeks. This training protocol is characterized aerobic exercise. Training sessions were performed on Mondays, Tuesdays, Thursdays, and Fridays; all trained rats rested on the other days.

Supplemented rats received SLO at a level of 1g per kg body weight per day, during the whole 8 weeks experiment period, always after exercise session as a single daily bolus by using a micropipette. The SLO (Ecomer®) was kindly donated by Naturalis Nutrição e Farma Ltda. At the 6th week of supplementation tumor cells were subcutaneously inoculated in the tumor groups in the right flank with a sterile suspension of 3 x 10^7 Walker 256 tumor cells, obtained from a rat where cells grew in the peritoneal cavity. Fifteen days after tumor inoculation the animals were killed by decapitation using a guillotine. Body weight was regularly measured during experimental period and tumor weight was determined at sacrifice. Blood was collected and serum prepared to measure the concentrations of glucose, lactate and triacylglycerol. Tumor tissue also was removed for studies. All groups were killed 48 h after the last session of exercise in order to eliminate any acute effects of the exercise on metabolic parameters.

Blood metabolite measurements

Serum glucose was determined by using a glucose oxidase-based assay kit [17] and quantified in a spectrophotometer (Ultrospec 2000, Pharmacia Biotech) by measuring the absorbance at 505 nm. Triacylglycerol (TAG) concentration was measured using an assay kit as described elsewhere [18]. Lactate was assayed enzimatically. For this procedure, 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 x *g* for 5 min. The supernatant was collected and neutralized with Tris/KOH (2 M/0.5 M), incubated with the assay buffer for 40min and the lactate concentration was determined by measuring the absorbance at 340 nm [19].

Walker 256 tumor cells proliferation *ex vivo*

This was performed according to Nunes et al. [20]. At sacrifice whole tumor was removed and chopped with a scalpel into a flask containing saline 0.9%. The cells suspension was obtained by filtration through a funnel with gauze. Red blood cells were discharged by means of a solution containing NH_4Cl (15.5 mM) and EDTA (5.5 mM) in a proportion 9:1, respectively. Tumor cells were prepared by centrifugation at 1000g for 5 min and were resuspended in RPMI 1640 medium. Then, tumor cells were cultured in RPMI 1640 medium enriched with 10% fetal calf serum containing 100 U/ml penicillin, 100 mg/ml streptomycin and 0.05 µCi of [2-C^{14}]-thymidine. Cells (10^5 cells/ml) were seeded in 96 well microplates and after 48 h of culture at 37°C in 5% CO_2; they were harvested onto glass fiber disks and washed using a Skatron cell harvester. Radioactive thymidine incorporation into DNA was determined in a Beckman LS 6000IC scintillation counter. Each cell preparation was cultured in sixtuplicate and the data are express in counts per minute (cpm). At the final preparation of cells they were 97% viable.

Lipid peroxidation products

Lipid peroxidation was measured as described by Nouroozzadeh et al. [21]. This method is based on the oxidation of hydroperoxide iron to ferric iron which is bound to xylenol orange. Walker 256 tumor cells (200g) were homogenized in 1 ml of methanol and centrifuged to 5000 x g for 5 minutes to 4°C. For each sample, of 90 µl of supernatant were put on 6 centrifuge tubes. Three tubes were added with 10 µl of TPP (triphenylphosphine) solution 10 mM, diluted in methanol, which is a selective for reduction by hydroperoxides and in others only 10 µl methanol. Tubes were incubated at room temperature for 30 minutes. At the end of incubation, 900 µl of the reagent FOX2 (100 µM xylenol orange, butylated hydroxytoluene 400 mM, 25 mM sulfuric acid and ammonium ferrous sulphate 250 mM in methanol 90%) was added to each tube and incubated again at room temperature for 30 minutes. The reading was held in spectrophotometer at 560 nm.

The concentration of hydroperoxide in the samples was determinate by subtracting the absorbance of the samples without TPP with the absorbance of samples treated with TPP. The value obtained was then interpolated in the standard curve of hydrogen peroxide, which was obtained with standard solutions of hydrogen peroxide in different concentrations (100.0, 50.0, 25.0, 12.5, 6.2, 3.1 and 1.6 µM). The concentration of hydroperoxide (nmol/ml) was corrected by protein concentration (mg/ml) which was measured by Bradford's method [22].

Apoptosis

The percentage of tumor cells undergoing apoptosis was evaluated using annexin V-FITC (Pharmingen, SanvDiego, CA, USA). Cells were stained with annexin V-FITC 30 min 4°C in the dark. The fluorescence was analyzed using flow cytometry; the positive cells were considered to be in an early stage of apoptosis.

Western blot analysis of Bax, Bcl-2 and NF-κB (p65, p52 and IκB)

Protein content in tumor extract was determined by Bradford's method [22], to normalize the amount of protein in different samples (60 µg/lane). Pieces of accurately weighed tumor tissue (0.1 g) were homogenized in lysis buffer (0.7 ml) containing 100 mM Trizma Base, pH 7.5, 10 mM EDTA, 10% sodium dodecyl sulfate (SDS), 10 mM sodium pyrophosphate, 10 mM sodium fluoride 10 mM sodium orthovanadate and 10 µg/ml aprotinin. Then, the samples were boiled for 5 min and subjected to SDS polyacrylamide gel electrophoresis (15% polyacrylamide) followed by electrophoretic transfer onto nitrocellulose membranes (Hybond-ECL; Amershan Bioscience, São Paulo, Brazil) using a semi-dry blotter apparatus (HOEFER-Mini VE; Amersham Biosciences). The membranes were incubated overnight with goat polyclonal IgG anti-Bax antibody, goat polyclonal IgG anti-Bcl-2, goat polyclonal IgG anti-p65 antibody, goat polyclonal IgG anti-p52 antibody and goat polyclonal IgG anti-IκB, at dilution of 1:500 they were then incubated with a secondary antibody-conjugated rabbit horseradish peroxidase diluted to 1:2000. Protein load control was done with anti-total β-Actin (1:500), in the same membranes after antibody stripping. Finally, the membranes were subjected to enhanced chemiluminescence (Super Signal Systems Pierce). Blots were scanned using Image J software to detect relative band intensities.

Determination of fatty acids content by HPLC and alkylglycerol by GC-MS

The lipids were extracted as previously described [23] from SLO and standard commercial chow. The lipids were saponified using 2 ml of an alkaline methanol solution (1 mol NaOH/l in 90% methanol) at 37°C, for 2 h, in a shaking water bath. Then, the alkaline solution was acidified to pH 3 with HCl solution (1 mol/l). Fatty acids were then extracted three times with 2 ml hexane. After the extraction procedure and saponification [24-26] the fatty acids were derivatized with 4-bromomethyl-7 methoxycoumarim [27], and the analysis performed on a Varian model LC-10A liquid chromatography. The samples were placed on a C8 column (25 cm × 4.6 mm i.d., 5 µm of particles), with a flow rate of 1 ml/min of acetonitrile/water (77:23, vol/vol), and a fluorescence detector (325 nm excitation and 395 nm emission). The standard mixture of fatty acids was obtained from Sigma Chemical Co. (St. Louis, MO). The elution sequence and limit of detection were determined. The minimum limit of quantification of the fatty acids ranged from 1 to 10 ng. One curve of calibration for each standard, determining coefficients of correlation and regression, was obtained. Alkylglycerol profile was determined by GC-MS.

Total lipids were extracted from SLO capsules (Ecomer®) using chloroform-methanol (2:1 vol/vol) according to Folch et al. [23] and Vitorino et al. [28]. Then the content was hydrolyzed and kept under 100°C for 2h. the samples were dried out under gas nitrogen and added 0.2 ml of ethanoyl ethanoate and 0.2 ml of pyridine followed by 30 min incubation at 100°C [29]. Alkyglycerols were separated on a GC-MS Saturn using a CP-Sil-% CB Chrom-pack® column (30 m x 0.25 mm) (Table 1).

Statistical analysis

Data are presented as mean ± standard error mean (SEM). Statistical analysis was performed by two-way analysis of variance (two-way ANOVA) by using supplementation and exercise as factors. To test for differences between groups the post hoc Bonferroni's test was applied. The value of $p<0.05$ was taken to indicate statistical significance.

Results

Fatty acids profile of regular chow and SLO are showed in Table 2A. Regular chow is rich in linoleic acid (omega-6), which constitutes 50% of total fatty acids, oleic (18%) and palmitic acid (25%). Very low quantities of α-linolenic acid (omega-3) were detected, and long chain polyunsaturated fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were not detected. In contrast, SLO presented about 18% of omega-3 fatty acids family. Palmitic and oleic acids were the most abundant components in this oil, 32% and 28% respectively. Alkylglycerol profile is shown in Table 2B. Regular chow has no presence of alkylglycerol whatsoever. In the capsule however the alkylglycerol in higher proportion was octadecenylglycerol (52.4%) followed by hexadecylglycerol (17%) and octadecylglycerol (8.4%) all three giving around 78%.

The tumor-bearing rats supplemented with SLO (WSL) showed 53% reduction in tumor growth ($p<0.05$ *vs.* W). Exercise caused a reduction of 59% in the exercised tumor-bearing rats ($p<0.05$ *vs.* W). When supplementation was associated with training (EWSL), tumor growth was 47.5% lower when compared to W group ($p<0.05$ *vs.* W) (Table 1). Carcass weight (Body weight after 15 days of tumor-bearing minus tumor weight) of sedentary rats fed regular chow after 15 days tumor-bearing was 13.21 g lower when compared to the day of tumor inoculation. Supplementation alone (WSL), exercise (EW) and both association (EWSL) were able to avoid carcass weight lost when compared with the day of tumor inoculation ($p<0.05$).

The W group showed hypoglycemia, hyperlactacidemia and hypertriacylglycerolemia featuring a cachetic state (Table 3). SLO supplementation (WSL) and exercise (EW) increased glycemia and decreased lactacidemia and triacylglycerolemia when compared to W group ($p<0.05$). SLO supplementation plus exercise (EWSL) induced additive effect on glycemia, lactacidemia but not in the triacylglycerolemia when compared to WSL and EW ($p<0.05$).

The proliferation of Walker 256 tumor cells (Figure 1) obtained from

	Tumor-bearing (W)	Tumor-bearing Supplemented (WSL)	Tumor-bearing Exercised (EW)	Tumor-bearing Exercised-Supplemented (EWSL)
Body weight before tumor inoculation (g)	340.9 ± 6.40	380.6 ± 8,05[a]	338.3 ± 6.55	300.8 ± 20.43[ab]
Body weight after 15 days of tumor-bearing (g)	354.1 ± 7.17	393.5 ± 8.92[a]	352.3 ± 7.12	310.0 ± 20.51[ab]
Tumor weight (g)	26.50 ± 1.79	14.08 ± 0.91[a]	15.60 ± 0.55[a]	12.60 ± 1.07[ab]
Carcass weight	327.8 ± 7.01	379.4 ± 9.05[a]	336.7 ± 6.80	297.4 ± 20.76[ab]
Weight change (g)	-13.21 ± 2.25	-2.15 ± 1.82[a]	-1.60 ± 1.47[a]	-3.35 ± 0.73[a]

Values are means ± SEM of 10 animals per group. [a]$p<0.05$ *vs.* W; [b]$p<0.05$ *vs.* WSL e EW

Table 1: Body weight, Tumor weight, and Carcass weight of Walker 256 tumor-bearing rats fed a regular diet (W), supplemented with shark liver oil (WSL), fed regular chow and exercised (EW) and, exercised and shark liver oil supplemented (EWSL).

Fatty acids (g/100 g of total fatty acids)	**Shark liver oil Ecomer®**	**Regular chow Nuvital®** (AR-1)
Lauric acid (12:0)	1.0 ± 0.05	-
Myristic acid (14:0)	4.8 ± 0.2	-
Palmitic acid (16:0)	32.6 ± 2.0	20.7 ± 3.8
Palmitoleic acid(16:1n-7)	6.5 ± 0.5	-
Estearic acid (18:0)	1.6 ± 0.07	2.3 ± 0.04
Oleic acid (18:1n-9)	28.2 ± 1.6	19.5 ± 1.5
Linoleic acid (18:2n-6)	1.9 ± 0.4	51.3 ± 4.2
α-Linolenic acid (18:3n-3)	0.6 ± 0.08	5.0 ± 0.2
Araquidonic acid (20:4n-6)	0.9 ± 0.1	0.1 ± 0.07
EPA (20:5n-3)	4.5 ± 0.3	-
DHA (22:6n-3)	12.6 ± 1.0	-

Data are presented as mean ± SEM of 3 independent measurements

Table 2A: Fatty acids profile obtained by HPLC analysis of regular chow provide to all groups and of shark liver oil capsule.

Alkylglycerol	**% of alkylglycerol**
Octadecenylglycerol (C18:1)	50.9
Hexadecylglycerol (C16:0)	18.0
Octadecylglycerol (C18:0)	8.1
Heptadecenylglycerol (C17:1)	1.5
Heptadecylglycerol (C17:0)	1.2
Others	20.3

Data are presented as mean ± SEM of 3 independent measurements

Table 2B: Percentage of alkylglycerol measured in the shark liver oil capsule.

sedentary SLO supplemented (WSL) have a significant reduction of 26% ($p<0.05$ vs. W). Tumor cells from exercised group (EW) presented similar reduction of 30% ($p<0.05$ vs. W), but was not different from WSL ($p>0.05$). SLO supplementation plus exercise (EWSL) caused an additive effect upon proliferation reduction *ex vivo* when compared to WSL and EW ($p<0.05$).

LP in Walker 256 tumor tissue is shown in the Figure 2. Supplementation with SLO liver oil induced an increase in the lipid peroxidation of 51% ($p<0.05$ vs. W). Exercise also caused an increase of 60% ($p<0.05$ vs. W). The association of supplementation with SLO liver oil and exercise (EWSL) did not cause any further effect ($p>0.05$ vs. WSL and EW).

Percentage of apoptotic cells detected as annexin-V positive is represented in the Figure 3. The supplementation with SLO liver oil (WSL) caused an increase by almost 2-fold in the apoptosis in the tumor cells obtained from supplemented group ($p<0.001$ *vs.* W). Exercise also caused an increase in the apoptosis of tumor cells from EW by 2.2-fold ($p<0.001$ *vs.* W). The association of exercise and SLO liver oil supplementation (EWSL) caused a further increase in the apoptosis of Walker 256 tumor cells by 2.6-fold which was significantly different when compared to W and WSL ($p<0.05$).

Bax and Bcl-2 proteins expression

Bax expression was higher in exercised groups EW and EWSL (Figure 4A). Bcl-2 expression in the WSL, EW and EWSL groups was reduced of 11, 25 and 23%, respectively compared to W ($p<0.05$) (Figure 4B). Bax/Bcl-2 ratio (Figure 4C) increased only in exercised groups (EW or EWSL) ($p<0.05$)

The p65 and p52 expression were reduced in exercised groups (EW and EWSL) ($p<0.05$) (Figures 5A and 5B, respectively). The IκB-α expression was higher in groups WSL, EW and EWSL, when compared to W ($p<0.05$) (Figure 5C).

Discussion

Cachexia is among the most debilitating and life-threatening feature of cancer. Walker 256 tumor-bearing rat offers an opportunity to investigate cancer cachexia and tumor growth due to its characteristics of inducing a higher level of cachexia in only 14 days

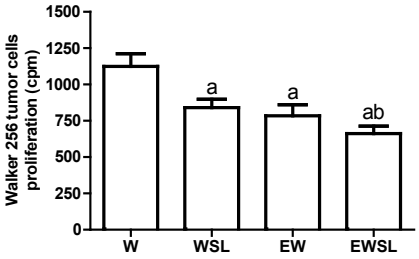

Figure 1: Walker 256 tumor cells proliferation *ex vivo* in counts per minute (cpm), cells were obtained from sedentary tumor-bearing rats fed a regular chow (W), supplemented orally with shark liver oil (WSL), exercised rats fed regular chow (EW) exercise and supplemented with shark liver oil (EWSL).Data are presented as mean ± SEM of 20, 21, 16 and 19 tumors per group to W, WSL, EW and EWSL, respectively. [a]$p<0.05$ vs. W; [b]$p<0.05$ vs. WSL e EW.

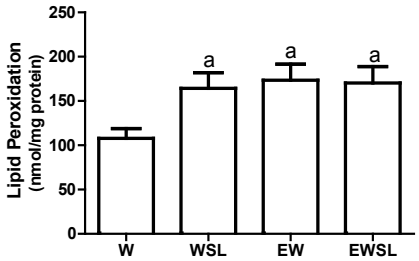

Figure 2: Lipid peroxidation (nmol/mg sample protein content) in tumor samples from tumor-bearing rats fed a control diet (W), supplemented orally with shark liver oil (WSL) and exercised rats fed regular chow (EW), exercise and supplemented with shark liver oil (EWSL). Data are presented as mean ± SEM of 12 animals per group to W, EW and 15 animals per group to WSL, EWSL. [a]$p<0.05$ vs. W.

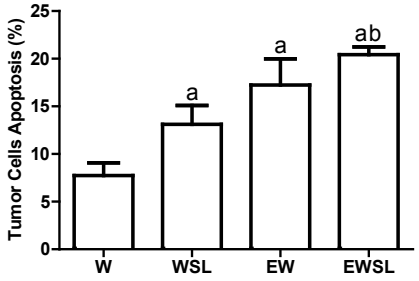

Figure 3: Apoptosis (%) of Walker 256 tumor cells from tumor-bearing rats fed a regular diet (W), supplemented orally with shark liver oil (WSL), exercised rats fed regular chow (EW), exercise and supplemented with shark liver oil (EWSL). Data are presented as mean ± SEM of 20, 21, 16 and 19 tumors per group to W, WSL, EW and EWSL, respectively.[a]$p<0.05$ *vs.* W; [b]$p<0.05$ *vs.* WSL.

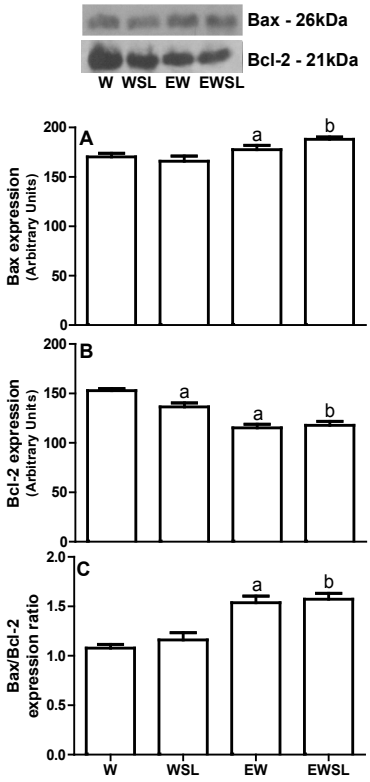

Figure 4: Expression of Bax (A) and Bcl-2 (B) proteins in Walker 256 tumor cells and in tumor-bearing rats fed a control diet (W), supplemented orally with shark liver oil (WSL) exercised rats fed regular chow (EW), exercise and supplemented with shark liver oil (EWSL). Bax/Bcl-2 expression ratio (C). [a]p<0.05 vs. W; [b]p<0.05 vs. WSL.

[1]. A lot of diseases have been linked to lifestyle, diet, smoking and sedentary lifestyle. The practice of regular physical activity and healthy eating habits are significant factors in the prevention and control of some types of diseases even cancer [30]. Here we show for the first time that physical exercise and SLO supplementation combined were able to reduce tumor growth, cachexia development, tumor cells proliferation and increased apoptosis of Walker 256 tumor cells and some of the proteins involved in the mechanism of action.

At the beginning of SLO supplementation until sacrifice rats from WSL group had a significant body weight gain when compared to the other groups (Table 1), around 40 g higher. Maybe this was caused by lipid intake because exercise balanced such effect. Exercise alone, SLO supplementation and both associated were able to prevent body weight lost (as measured by carcass weight) indicating an anti-cachectic effect. Furthermore, the blood biochemical parameters of cancer cachexia in Walker 256 such as hypoglycemia, triacylglycerolemia and lactacidemia were all restored to normal values [31]. These data show that alone or associated both factors (diet and exercise) have anti-cachectic properties. The cachectic state aggressiveness might be linked to tumor size and/or tumor cells proliferation ratio. Proliferation of tumor cells was reduced in the WSL, EW and EWSL groups. Which might partially explain the reduction in the proliferation capacity of these cells, as showed before for other tumor cells [32].

Another mechanism by which physical exercise and SLO supplementation can cause reduction of tumor growth seems to involve induction of apoptotic process by increasing lipid peroxidation in tumor tissue and modifying the pattern of expression of proteins linked to induction of cell death [33]. Polyunsaturated fatty acids are more prone to peroxidation than saturated or monounsaturated ones and it is known that peroxidation products may alter cellular membrane structure leading to cell death [34]. The ECOMER® fatty acid profile, quantified by HPLC (Table 2A), showed that 35% and 21% of the fatty acids are monounsaturated and polyunsaturated, respectively. This content is in agreement with that obtained by others [35]. Mund et al. [33] have demonstrated that tumor tissue obtained from rats supplemented with fish oil, a rich source of n-3 PUFAs, had a 3-fold higher degree of lipid peroxidation when compared to those fed regular chow. Increased fatty acid peroxidation is known to produce some deleterious effects, as induction of apoptosis [33]. Our data are in agreement with that, as demonstrated by the increased percentage of apoptotic tumor cells and the reduced expression of the anti-apoptotic protein Bcl-2 in samples harvested from supplemented groups, where lipid peroxidation was increased as well. The liver lipids composition in SLO may change among species, gender, season and other biological factors. Some studies have found small amounts of n-3 fatty acids in SLO of different species, with values around 0.4% - 5.3% to EPA and 6.5% - 17.5% to DHA [33,35,36]. Besides n-3 fatty acids, SLO is also rich in alkylglycerols, which can be another source for lipid peroxidation and induce tumor cells apoptosis. In fact we found by GC-MS a high content of alkylglycerol in the capsules offered to the supplemented animals (Table 2B). A study with leukemia patients supplemented with alkylglycerols reported an increased oxidative

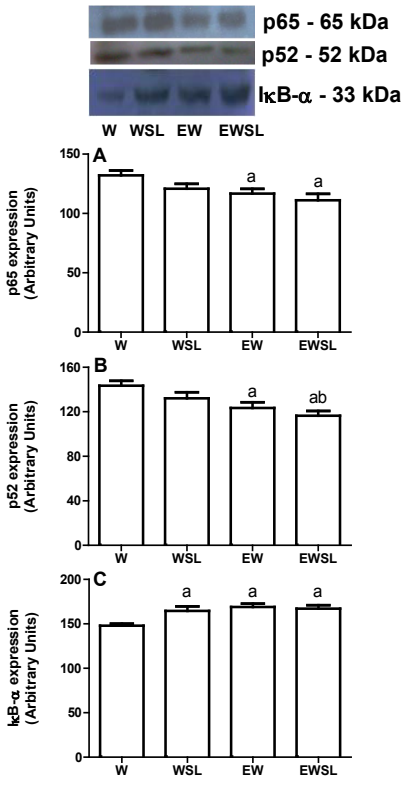

Figure 5: Expression of p65 (A), p52 (B) and IκB-α (C) proteins in Walker 256 tumor cells and in tumor-bearing rats fed a control diet (W), supplemented orally with shark liver oil (WSL) exercised rats fed regular chow (EW), exercise and supplemented with shark liver oil (EWSL).

	Tumor-bearing (W)	Tumor-bearing Supplemented (WSL)	Tumor-bearing Exercised (EW)	Tumor-bearing Exercised-Supplemented (EWSL)
Serum Glucose (mg/dl)	68.67 ± 2.12	76.73 ± 2.82[a]	82.27 ± 2.64[a]	85.42 ± 2.92[ab]
Serum Lactate (mmol/l)	1.49 ± 0.06	1.41 ± 0.05[a]	1.30 ± 0.04[a]	1.25 ± 0.06[abc]
Triacylglycerol (mg/dl)	161.40 ± 9.43	136.60 ± 11.78[a]	130.40 ± 13.20[a]	136.10 ± 13.00[a]

Abbreviations: W = tumor-bearing rats fed a regular diet; WSL = supplemented orally with shark liver oil; EW = exercised rats fed regular chow; EWSL = supplemented with shark liver oil. Data are presented as mean ± SEM of 14, 15, 12 and 14 animals per group to W, WSL, EW and EWSL, respectively, for glucose (mg/dl); 10 animals per group, for lactate (mmol/l) and 13, 16, 11 and 14 animals per group to W, WSL, EW and EWSL, respectively, for triacyglycerol (mg/dl). [a]p<0.05 vs W; [b]p<0.05 vs. WSL; [c]p<0,05 vs. EW

Table 3: Serum glucose (mg/dl), lactate (mmoL/l) and triacylglycerol (mg/dl) concentrations in tumor-bearing rats W or WSL and EW or EWSL.

stress after the nutritional intervention, which the authors pointed as the mechanism behind the increased number of apoptotic neoplastic cells in such patients [14].

Physical activity is also able to induce lipid peroxidation in animal and human models [37,38]. Our data corroborate such reports, because we found in the tumor tissues from exercised groups increased lipid peroxidation. The mechanisms by which physical activity-induced lipid peroxidation are not completely understood, but it is well known that during oxidative metabolism much of the oxygen consumed is bound to hydrogen following oxidative phosphorylation, forming water. However, it has been estimated that 4-5% of the oxygen consumed during respiration is not completely reduced to water, forming free radicals instead. Thus, as oxygen consumption increases during exercise, a concomitant increase occurs in free radical production and lipid peroxidation [38]. Physical exercise has been consistently shown to reduce the risk of some cancers [37], and lipid peroxidation might be one of the possible mechanisms involved in such effect.

Reduced activity of apoptotic pathways is a hallmark of tumor cells [39]. Here we show that exercised groups (EW and EWSL) have an increase in the percentage of apoptotic cells and a pro-apoptotic protein expression environment in the tumor tissue, characterized by increased Bax and decreased Bcl-2 expression. In addition, it is well known that NF-κB system proteins are involved in tumor cells survival and tumor progression [39]. Decreased expressions of p65 and p52 proteins in tumor cells have been linked to malignancy [40]. Moreover, the increased expression of IκB-α in tumor cells is usually related to decrements in NF-κB pathway activation [41]. Our results supported the influences of such protein expression pattern over tumor growth and tumor cells apoptosis. The percentage of apoptotic cells when exercise and supplementation were associated was higher than using both strategies alone. This result suggests that SLO supplementation and physical exercise might be inducing tumor cell death by not completely similar ways, which deserves further investigation.

Conclusions

In summary, our results suggest that shark liver oil supplementation and exercise alone were able in to avoid the installation of cachexia state and also reduced tumor growth, but the association of both only cause further effect in the tumor growth.

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