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Maternal nutritional supplementation with fish oil and/or leucine improves hepatic function and antioxidant defenses, and minimizes cachexia indexes in Walker-256 tumor-bearing rats offspring

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ABSTRACT

In this study, we hypothesized that throughout the pregnancy/weaning period, nutritional supplementation with leucine (which improves protein synthesis) and/or fish oil (rich in omega-3, which modulates oxidative stress) can minimize/improve cachexia-induced damage in rat offspring. Thus, we investigated the maternal supplementation with these nutrients over the modulation of cachexia index and liver function in tumor-bearing rats offspring. Pregnant rats were fed control, leucine, omega-3, and leucine/omega-3 diets, which were given throughout the gestational and weaning periods. The male offspring were subjected to a control diet until adulthood (120 days) and then distributed into 5 groups (n=4-6 per group): C, Control; W, tumor-bearing; WL, tumor-bearing group with a maternal leucine-rich diet; WO, tumor-bearing group with a maternal omega-3 diet; and WLO, tumor-bearing group with a maternal leucine-rich and omega-3 diet. The W group had a higher cachexia index (31.83 ± 2.9%), but this parameter decreased in the WO (P=0.0380) and WLO groups (P=0.0187). In addition, the W group had a lower survival rate, and the WLO group exhibited a trend toward increased survival (P=0.0505). The hepatic function in maternal supplemented groups was preserved, while the W group exhibited an increased aspartate-aminotransferase/alanine-aminotransferase ratios (P=0.0152) and also enhanced liver oxidative stress, with higher alkaline phosphatase (P=0.0190) and superoxide dismutase (P=0.0190) activities, and trended toward to higher malondialdehyde content (P=0.0556). In contrast, the maternal-supplemented groups had similar liver enzymes and malondialdehyde contents. Thus, we concluded that supplementing the maternal diet modulated/improved liver antioxidant responses and ameliorated the cachexia state in tumor-bearing rat offspring.

Abbreviations: AIN-93, American Institute of Nutrition- year 93; ALT, alanine amino transferase; AP, alkaline phosphatase; AST, aspartate aminotransferase; BCAA, branched-chain amino acid; GGT, gamma glutamyl transferase enzyme; GSH, glutathione reductase; GST, glutathione S-transferase; MDA, malondialdehyde; MST, median survival time; PUFA, polyunsaturated fatty acid; ROS, reactive oxygen species; SEM, standard error of the mean; SOD, superoxide dismutase.

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1. Introduction

Cancer is the second leading cause of death worldwide. Approximately 8.8 million people die from cancer every year, and new cases are expected to increase by 70% over the next two decades [1]. Moreover, 20% of cancer deaths are caused by cachexia [2], which is a multifactorial syndrome characterized by reduced anabolism and intense catabolism. This state of impaired metabolism, with the depletion of carbohydrates, lipids and primarily proteins, leads to involuntary weight loss and structural tissue damage, causing progressive functional impairment [3,4]. In addition, as an essential organ of metabolic homeostasis, the liver is related to this syndrome due to metabolic reprogramming, with suppressed ketogenic potential, adaptation to the metabolic state during starvation, immune suppression of the host, and increased oxidative stress [5-7]. Together, these factors overload organs and reduce patient survival [8,9].

Nutritional supplementation is recommended as a treatment for cachexia [10,11], and diet composition modulation is an interesting strategy because this condition is irreversible [3]. The prevention of many diseases and probably the cachectic state can begin during gestation, as has been proposed by several studies that investigated the influence of maternal diet on offspring during adulthood [12,13]. Nutritional composition is the most important environmental factor for modulating the expression of metabolic pathway genes, and during pregnancy, diet exerts a significant influence on organ development and fetus plasticity [14].

One widely used supplement is the branched-chain amino acid (BCAA) leucine, which has cellular signaling properties, increasing protein synthesis and reducing muscle proteolysis [15-17]. Likewise, the polyunsaturated fatty acid (PUFA) omega-3 improves the host immune response [18,19] and attenuates skeletal muscle protein catabolism [20]. Moreover, several studies have demonstrated that omega-3 is efficient in diminishing tumor growth [21-23], and this PUFA and leucine attenuate the cachexia state [24-26] in tumor-bearing rats when administered during tumor development.

One component of cancer, oxidative stress, is characterized by an imbalance between the minor antioxidant defense systems and by the augmented generation of reactive species [27], which is important for maintaining a high proliferation rate in cancer cells, through high reactive oxygen species (ROS) concentrations [28]. On the other hand, studies have confirmed the benefits of leucine or omega-3 treatment in patients with cancer [29], which also modulate oxidative stress during tumor development [30,31]. Thus, considering the lack of studies investigating cachexia modulation, it would be interesting to study the preventive roles of these components after nutritional supplementation throughout pregnancy and the weaning period against cancer-cachexia and the associated tissue damage caused by oxidative responses in both the liver and body reserves.

We hypothesized that nutritional supplementation with leucine and/or fish oil (rich in omega-3) throughout pregnancy and the weaning period could prevent or modulate the damaging effects of cancer. To test this hypothesis, we evaluated the effects of Walker-256 carcinosarcoma – an experimental cachexia model – in adult male rat offspring from mothers subjected to nutritional supplementation with leucine and/or fish oil, evaluating whether the maternal supplementation could improve the cachexia index and also the liver function, analyzing the main hepatic enzyme contents and the oxidative stress responses in those tumor-bearing animals.

2. Methods and Materials

2.1. *Animals*

Adult male and female Wistar rats, which were 90 days old, were obtained from the Animal Facility at the University of Campinas, UNICAMP, Brazil, and housed in collective cages in the experimental room located in the Laboratory of Nutrition and Cancer. The environmental conditions were controlled (light and dark 12/12 h; temperature 22 ± 2°C; and humidity 50-60%), and the animals were monitored daily, weighed three times/week and given access to food and water ad libitum.

2.2. *Experimental Procedure*

The experiments were approved by the Ethics Committee on Animal Experimentation of the Institute of Biology at the University of Campinas (protocol numbers: #2463-1; #3424-1) and conducted according to the current ethical standards of the United Kingdom Coordinating Committee on Cancer Research [32].

The animals were mated (2 females:1 male), following the harem method [33]. After pregnancy detection, females were separated from males. The pregnant rats (n= 4 per group) were distributed into four groups with the following diets: Control; Leucine; Omega-3; Leucine and Omega-3. Throughout pregnancy (21 days) and weaning (21 days), the dams received the indicated diets, and the offspring were reduced to 8 pups/litter. To verify the influence of maternal diet, after the weaning period, the male offspring (n=6) started to receive the control diet, as outlined in the following groups: C, fed a control diet throughout the intrauterine, lactation and adulthood periods, without tumor; W, fed a control diet throughout the intrauterine, lactation and adulthood periods, and tumor-bearing; WL, subjected to a leucine-rich diet throughout the intrauterine and lactation periods, fed a control diet in adulthood, and tumor-bearing; WO, subjected to a omega-3 diet throughout the intrauterine and lactation periods, fed a control diet in adulthood, and tumor-bearing; and WLO, subjected to a leucine-rich and omega-3 diet throughout the intrauterine and lactation periods, fed a control diet in adulthood, and tumor-bearing. The experimental design is presented in Fig. 1.

At 120 days of age, the adult rats received tumor implants via the subcutaneous injection of approximately 3×10^6 viable Walker-256 tumor cells into the right flank [34,35]. For this work, two experiments were performed to analyze hepatic biochemical parameters and to assess survival time. Experiment A was performed after 21 days of tumor evolution, and the number of animals was 4-6 per group. The animals were

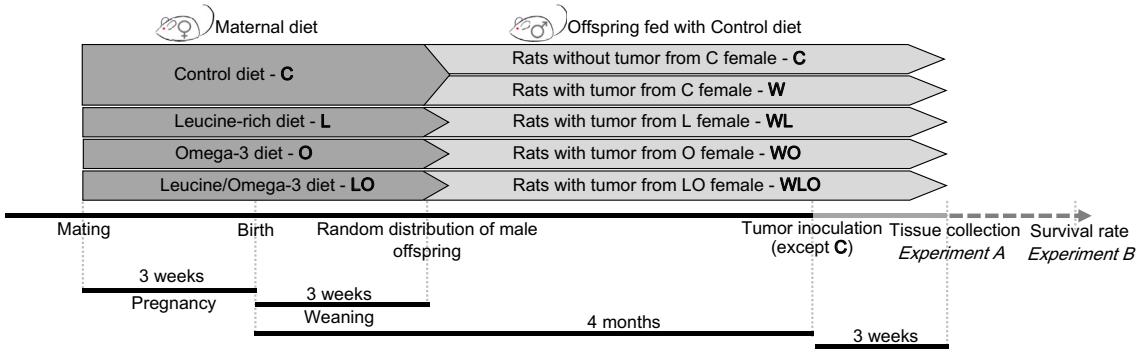

Fig. 1 – Schematic of the experimental procedure. Legend: C, Control; W, Tumor-bearing animals; WL, Tumor-bearing group with a maternal leucine-rich diet; WO, Tumor-bearing group with a maternal fish oil (rich in omega-3) diet; and WLO, Tumor-bearing group with a maternal leucine-rich and fish oil (rich in omega-3) diet. For details, see the Materials and Methods section. The minimum number of adult rats was 4-6 per group.

euthanized by decapitation (including the C group), and blood was collected. Tumors and livers were resected and weighed. Then, the carcasses were weighed. All of these parameters were used to calculate the cachexia index and biochemical parameters. Experiment B (Survival Rate) was conducted as described for Experiment A, but the control and tumor-bearing animals were monitored after tumor inoculation until the pre-agonic/agonic and death stage, which was used as the experimental endpoint; for this experiment, the number of animals was 5-6 per group. The schematic timeline is represented in Fig. 1.

2.3. *Diets*

Semi-purified diets were formulated in accordance with the American Institute of Nutrition (AIN-93; [36]). The control diet (C) was the AIN-93 semi-purified diet, containing 18% protein and 7% lipids (soybean oil source); all ingredients are presented in Table 1. The omega-3 diet (O) was formulated by replacing the soybean oil with 7% fish oil (cod liver oil -containing high quantities of omega-3 fatty acids in comparison to soybean oil) (Table 2). The leucine-rich diet (L) had the same composition as the control diet and was enriched with 3% L-leucine. The leucine-rich/omega-3 diet (LO) corresponded to the combination of the L and O diets, which contained 3% L-leucine and 7% fish oil and the same amount of the other ingredients, as shown in Table 1. The amounts of oil and L-leucine used in this study are consistent with our previous works and with other studies [35,37]. Individual cage-sized portions (25-30 g) of each diet were stored in sealed containers at -20°C to prevent fat oxidation. All diets were normoproteic, isocaloric and normolipidic (Table 1) and were prepared by our own Laboratory of Nutrition and Cancer at the University of Campinas.

2.4. *Cachexia Parameters*

The cachexia index was determined using the following formula: Cachexia Index = [(initial body mass – final body mass + tumor weight + body weight gain of Control group)/ (initial body mass + body weight gain of Control Group)] x 100% [38]. Total serum proteins and albumin were quantified spectrophotometrically using commercial kits [39] (Laborlab, Brazil), and the difference between the total serum protein

Table 1 – Ingredient composition of the diets (g/kg diet)

Ingredient	Control	Leucine	Omega-3	Leucine and Omega-3
Cornstarch [1]	397.5	387	397.5	387
Casein	200	200	200	200
Dextrin	132	122	132	122
Sugar	100	90	100	90
Fiber (cellulose microfiber)	50	50	50	50
Mineral mix [2]	35	35	35	35
Vitamin Mix [2]	10	10	10	10
L-Cystine	3	3	3	3
Choline bitartrate	2.5	2.5	2.5	2.5
Soy Oil	70	70	0	0
Cod Liver Oil [3]	0	0	70	70
L-Leucine [4]	0	30	0	30

[1] Cornstarch - Provided by Ingredion Products Brazil.
[2] Mineral and Vitamin Mix - Based on the AIN-93G vitamin and mineral mixes [36].
[3] Cod Liver Oil - Imported from Berg LipidTech (Norway) by Henrifarma Produtos Químicos e Farmacêuticos Ltda.
[4] L-Leucine- Provided by Ajinomoto Interamericana Ind. & Com. Ltda.

Table 2 – Oils and Diets Fatty Acid Composition

Name	Shorthand	Oils (%)		Diets (%)			
		Soy oil	Fish oil	Control	Leucine	Omega-3	Leucine and Omega-3
Saturated fatty acids							
Myristic acid	14:0	0.09±0.00	4.71±0.05*	0.01±0.00	0.01±0.00	0.39±0.01*	0.39±0.02*
Palmitic acid	16:0	10.73±0.01	10.11±0.14*	0.89±0.05	0.87±0.07	0.83±0.02*	0.84±0.04*
Stearic acid	18:0	3.84±0.00	1.96±0.04*	0.32±0.02	0.31±0.03	0.16±0.00*	0.16±0.01*
Monounsaturated fatty acids							
Palmitoleic acid	16:1 n-7	0.09±0.00	9.56±0.01*	0.01±0.00	0.01±0.00	0.78±0.02*	0.80±0.03*
Oleic acid	18:1 n-9	0.06±0.00	15.53±0.11*	0.01±0.00	0.01±0.00	1.27±0.03*	1.29±0.05*
Gondoic acid	20:1 n-9	ND	6.88±0.03*	ND	ND	0.56±0.01*	0.57±0.02*
Gadoleic acid	20:1 n-11	0.22±0.00	7.73±0.02*	0.02±0.00	0.02±0.00	0.63±0.01*	0.64±0.03*
Cetoleic acid	22:1 n-11	ND	9.67±0.04*	ND	ND	0.79±0.02*	0.80±0.03*
Polyunsaturated fatty acids							
Linoleic acid	18:2 n-6	50.70±0.02	1.71±0.04*	4.22±0.24	4.13±0.34	0.14±0.00*	0.14±0.01*
Linolenic acid	18:3 n-3	5.76±0.01	0.66±0.00*	0.48±0.03	0.47±0.04	0.05±0.00*	0.06±0.00*
Stearidonic acid	18:4 n-3	ND	1.99±0.00*	ND	ND	0.16±0.00*	0.17±0.01*
Eicosapentaenoic acid	20:5 n-3	ND	9.69±0.05*	ND	ND	0.79±0.02*	0.81±0.03*
Docosapentaenoic acid	22:5 n-3	ND	1.09±0.00*	ND	ND	0.09±0.00*	0.09±0.00*
Docosahexaenoic acid	22:6 n-3	ND	9.42±0.15*	ND	ND	0.77±0.02*	0.78±0.03*
PUFA n-6		50.70±0.02	1.71±0.04*				
PUFA n-3		5.76±0.01	22.84±0.20*				
Ratio n-6/n-3		8.80±0.02	0.08±0.00*				

The analyses of fatty acid composition were performed in duplicate in a capillary gas chromatograph. Values are means ± SEM. The fatty acid composition is presented as the percentage share of each individual fatty acid in the total pool of all fatty acids (%). *Compared with the C group, P<0.05: significant differences among oils in a T-test; ND: not detected.

and albumin contents was used to calculate the serum globulin content. The cachexia index and serum parameters were used to determine the general wasting status of the tumor-bearing groups.

2.5. *Hepatic Enzyme Activities and Oxidative Stress*

Hepatic tissue samples were weighed and homogenized in homogenizing buffer (1:4) (20 mM Tris, 1 mM DTT, 2 mM ATP and 5 mM $MgCl_2$; purchased from Sigma, USA), and the homogenate was centrifuged at 13,000g for 15 min at 4°C. The obtained supernatant was used to assay the activity and content of enzymes related to hepatic function and oxidative stress. The protein content of the tissue homogenates was measured using the Bradford method [40] and used to normalize the amounts of protein or the enzyme activities in the different samples.

Gamma glutamyl transferase enzyme (GGT) was analyzed in the hepatic tissue homogenate based on the reaction with L-gamma-carboxyl-4-nitroanilida, glycylglycine Z gamma-glutamylglycine and 5-amino-2nitrobenzoato, and the reaction was measured for the rate of formation of 5-amino-2-nitrobenzoato at 405 nm, using a UV spectrophometry assay (InVitro Company, Brazil); the enzyme content was normalized according to the total protein content in the sample [41]. The result was expressed as arbitrary units/μg protein. The enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were analyzed using the spectrophotometer method, reading at 340 nm, according to the manufacturer's guidelines (InVitro Company, Brazil). For AST analysis, the assay is based on the reaction with 2-oxoglutarate, L-aspartate, L-glutamate and oxaloacetate, reading the formation of L-malate from the reaction of oxaloacetate with $NADH^+ H^+$. For ALT, the assay is based on the reaction of 2-oxoglutarate, L-alanine, L-glutamate and pyruvate, reading the formation of L-lactate from the reaction of pyruvate with $NADH^+ H^+$ [42]. For each sample, the enzyme content was expressed as arbitrary units/μg protein, using the total liver protein content to normalize the reactions.

Aliquots of liver homogenate supernatant were analyzed to measure alkaline phosphatase (AP) activity, using the reaction of thymolphthalein monophosphate with AP, according to the kit manufacturer's instructions (Laborlab, Brazil). The AP results are expressed in nmol/μg protein/min [43,44].

Glutathione S-transferase (GST) activity was assessed in the homogenized liver samples based on the conjugation of 1 mM 1-chloro-2,4-dinitrobenzene (CDNB; Sigma) with glutathione, following the methodology described by Habig *et al.* [45]. The GST activity was expressed as nmol/μg protein/min and calculated using an extinction coefficient of 9.6, as described previously [44]. Liver glutathione reductase (GSH) was measured following the method described in our previous work [44]. The catalase activity was measured using the method described by Cohen [46], and the results are expressed as nmol/min/mg protein.

The superoxide dismutase (SOD) activity was measured as described by Winterbourn and colleagues [47]. The assay is based on the ability of superoxide dismutase to inhibit the reduction of nitro-blue tetrazolium by superoxide, and the results are expressed as units of SOD/mg of protein. The lipid peroxidation product malondialdehyde (MDA) was quantified by incubating the samples with n-methyl-2-phenylindole (MPO; Sigma) and then reading the absorbance at 590 nm, as described previously [48]. The results were normalized based on the sample protein content and expressed as nmol/μg protein.

2.6. *Statistical Analyses*

Median survival was determined using the Kaplan–Meier method and followed analysis with the Log-rank test (Mantel-Cox text),

comparing each survival curve, considering differences to be significant when P≤0.05 [49]. Other results are expressed as the mean and standard error of the mean (SEM). For comparisons among multiple groups, the data were evaluated using one-way ANOVA followed by the post-hoc Bonferroni test (Graph Pad Prism software, version 5.0, San Diego, CA) [49]. For direct comparisons between two groups (e.g., C vs. W), the data were analyzed using the Student's t-test [49]. Results were considered to be significant when P ≤0.05.

3. Results

3.1. *Body Weight Evolution of Rat Offspring (21 to 120 days of life)*

To confirm that the maternal diet (throughout pregnancy and the weaning period) did not affect offspring weight or health, we evaluated the body weight after the weaning period (21 days of life) and observed similar weight gain among the groups until the time of tumor inoculation (120 days). When analyzing the area under curve, all groups exhibited the same pattern (Fig. 2A). Before tumor implantation, biochemical analysis was performed to certify that all animals had the same health condition (total serum protein and albumin had similar values for all groups; data not shown).

3.2. *Morphometric Parameters After Tumor Implantation and Cachexia Indicators*

All animals had similar body weights at the time of tumor implantation (Fig. 2B – initial body weight). After tumor evolution (21 days), an analysis of the carcass weight, which represents the body weight after euthanasia, without the tumor weight and the gastrointestinal tract weight, revealed that the W group exhibited a significant reduction of carcass weight in comparison to the C group (area under curve: C group = 930±15 (mean ± standard error); W group = 819±20; P=0.0087). The WL group also had a lower carcass weight, but the value did not differ from that of the C group (area under curve: L group = 888±48); the same pattern was observed for the WO and WLO groups, which had the same carcass weight as the C group (area under curve: WO = 992±34 and WLO = 931±43), but the WO group had a greater carcass weight than the W group (P=0.0159; Fig. 2B).

As tumor evolution causes damaging effects that induce host protein wasting, we analyzed the total serum protein and albumin. The tumor-bearing animals exhibited a decrease in total protein and albumin content values, especially the W

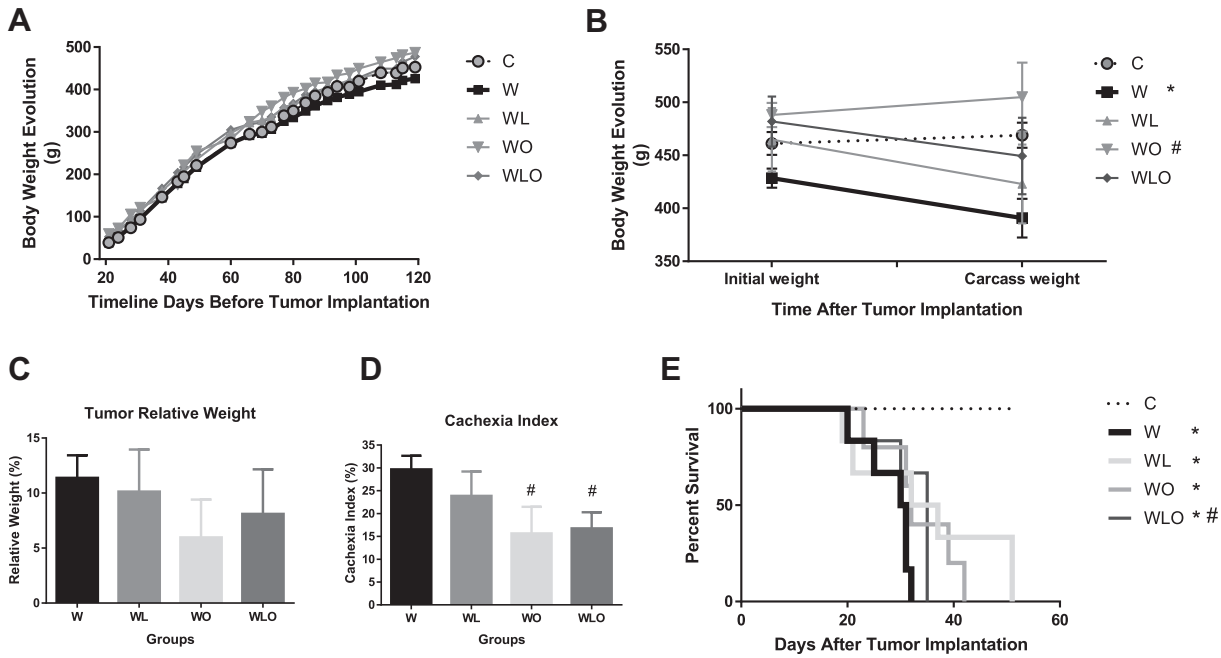

Fig. 2 – Morphological parameters of all experimental groups in response to maternal diet influence. A, Body weight gain of rat offspring from the end of the weaning period until tumor inoculation (g). B, Initial body and carcass weight after 21 days of tumor evolution (g). C, Relative tumor-to-carcass weight, expressed as a percentage (%). D, Cachexia index of tumor-bearing rats after 21 days of tumor development, expressed as a percentage (%); the cachexia index is determined using the following formula: cachexia index = [(initial body mass – carcass mass + tumor weight + body mass gain of control)/(initial body mass + body mass gain of control)] × 100 % [69]. E, Survival rate of the groups in days after tumor inoculation (N=5-6 animals per tumor-bearing group). Statistical analysis was based on the Kaplan-Meir method and used the log-rank test. Legend: C, Control; W, Tumor-bearing animals; WL, Tumor-bearing group with a maternal leucine-rich diet; WO, Tumor-bearing group with a maternal omega-3 diet; and WLO, Tumor-bearing group with a maternal leucine-rich and omega-3 diet. The minimum number of adult rats was 4-6 per group. For details, see the Materials and Methods section. Values are means ± SEM. *Compared with the C group, P≤0.05, #Compared with the W group, P≤0.05, based on one-way ANOVA followed by the Bonferroni test.

group in relation to the C group. The W group showed the greatest decrease, with a value 28% lower than the C group. The supplemented groups exhibited reductions of 23% (WL), 18% (WO), and 16% (WLO) in comparison to the control group (Table 3).

3.3. *Cachexia Index*

The relative tumor weight did not differ among the groups (Fig. 2C). The values were approximately 10% of body weight, which is considered to be a positive point for defining cachexia; however, the WO group trended toward a lower relative tumor-to-carcass weight than the other groups. The W group had a higher cachexia index (31.83 ± 2.9%). The WL group showed values similar to the W group. There was a decrease in the group treated with fish oil WO (P=0.0380), with a value 47.3% smaller than that of the W group. The WLO group also had a lower cachexia index (P=0.0187), with a decrease of 44.18% when compared to the W group (Fig. 2D).

3.4. *Survival Rate*

The obtained survival curves were significantly different (P= 0.0016; Fig. 2E). The median survival time (MST) was lower in the W group (30.5 d) than the groups supplemented during maternal life (WL=34.5 d; WO=32 d; WLO=35 d). The survival curve comparison (Log-rank test) showed that the W group had a significant survival decrease compared only to the WLO group (W < WL, P=0.1195; W < WO, P=0.1086; W < WLO, P= 0.0505.).

3.5. *Hepatic Physiological Parameters*

One damaging effect of tumor evolution is hepatomegaly, and a tendency of increase was observed in the W group in comparison to the C group (P=0.0544). The relative liver weights of all experimental tumor-bearing groups failed to increase significantly during cancer progression (Fig. 3A). The hepatic protein content was similar among the groups, except in the WO group, which presented an increase compared to the C (P=0.033) and W (P=0.014) groups (Fig. 3B).

Analyzing hepatic tissue function, we assessed the key enzymes (GGT, ALT and AST) that could be affected by tumor evolution. The measurement of GGT in the liver tissue indicated a slight decline in all tumor-bearing animals, with a significant decrease only in the WO group compared to the C (P=0.025) and W groups (P=0.009). The hepatic tissue ALT was diminished only in the WO group in relation to the C group (P= 0.0333), but the other groups showed similar values in relation to the C group. The liver AST trended toward an increase only in the W group compared to the C group (P= 0.0556), as well as the WL (P=0.0333), WO (P=0.0143) and WLO groups (P=0.0079). The liver AST/ALT ratio increased significantly in the W group compared to the C group (P=0.0152), whereas the WL (P= 0.0260) group's value decreased in relation to the W group.

3.6. *Hepatic Oxidative Stress*

Analyzing the damaging effects of tumor evolution on hepatic tissue, we observed in Fig. 4 that the W group presented higher values of AP (P=0.0190) and SOD (P=0.0309) than the C group (Fig.4A and B). The other antioxidant enzymes assessed in the W group (GST, catalase) exhibited the same patterns as the control group (Fig. 4C, D and E). The MDA liver content in the W group trended to increase in comparison to the C group (P=0.0556) (Fig. 4F), and the antioxidant response, as illustrated by the GST/MDA ratio, was decreased in the W group (P=0.0151) (Fig. 4G).

The influence of maternal diet on the liver tissue could be seen in all supplemented groups. In this way, we observed that alkaline phosphatase (Fig. 4A) values were similar in the WL, WO, WLO and C groups, although in these groups, AP was significantly decreased in comparison to the W group (WL, P= 0.0317; WO, P=0.0286; and WLO, showing a trend toward significance, P=0.0571). Analyzing SOD enzyme activities, we observed that the WL and WO groups had the same value as the C group, and only the WLO group had lower activity than the C group (P=0.0276) and the W group (P=0.0035) (Fig. 4B). In all experimental groups (WL, WO, WLO), no differences in GST activity were observed in relation to the C group (Fig. 4C). The GSH content was similar among the WL, WO and C groups, but there was a decrease in GSH in the WLO group in comparison to the C group (P=0.0067) (Fig. 4D). With respect to catalase activity, all groups showed values similar to the C group, although we verified a tendency toward a reduction in the WLO group (P=0.0541) (Fig. 4E). The MDA content had similar values in the WL, WO, WLO and C groups (Fig. 4F) but was significantly decreased in comparison to the W group (WL, P=0.0095; WO, P=0.0140; and WLO, P=0.0224) (Fig. 4F). The antioxidant response, as indicated by the GST/MDA ratio, showed a positive effect in the WL, WO and WLO groups, as

Table 3 – Serum parameters of cachexia in tumor-bearing rats, supplemented or not with leucine-rich and/or omega-3 diets during the maternal period.

	C	W	WL	WO	WLO
Total protein (g/dL)	5.837±0.195	4.070±0.169*	4.516±0.381*	4.436±0.325*	4.602±0.343*
Albumin (mg/dL)	3.849±0.103	2.815±0.322*	2.97±0.263*	3.151±0.283*	3.228±0.171*
Albumin/Globulin ratio (mg/dL)	1.969±0.107	2.645±0.620	2.167±0.383	2.609±0.497	2.595±0.359

C, Control (n=6); W, Tumor-bearing animals (n=5); WL, Tumor-bearing group with a maternal leucine-rich diet (n=6); WO, Tumor-bearing group with a maternal omega-3 diet (n=4); and WLO, Tumor-bearing group with a maternal leucine-rich and omega-3 diet (n=5). For details, see the Material and Methods section. Values are presented as means ± SEM. The data were analyzed using one-way ANOVA followed by the Bonferroni test to evaluate comparisons among groups.

* P<0.05, for comparison with the C group.

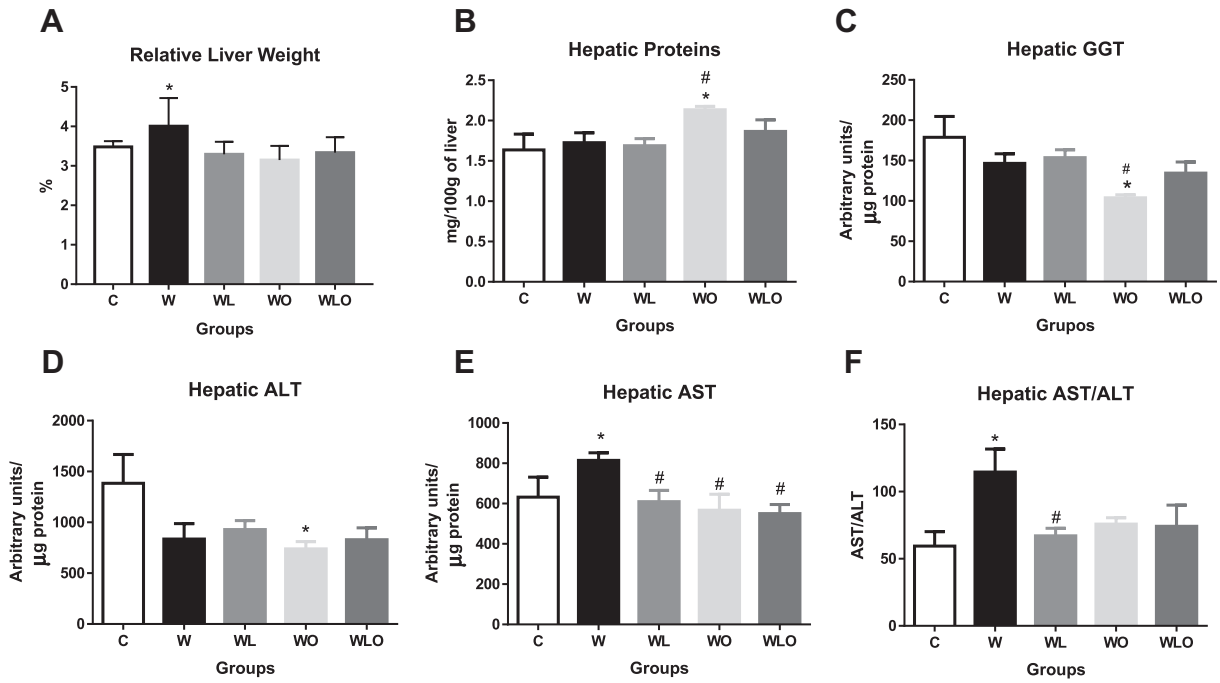

Fig. 3 – Hepatic parameters and enzyme activities and contents in all experimental groups after maternal diet changes. A, Relative liver weight. B Total hepatic proteins, expressed as mg/100 mg of liver, after 21 days of tumor development. C, Hepatic GGT content, expressed as arbitrary units/µg protein. D, Hepatic ALT content, expressed as arbitrary units/µg protein. E, Hepatic AST content, expressed as arbitrary units/µg protein. F, Hepatic AST/ALT ratio. The total tissue protein was used to normalize the enzyme activities [40]. Legend: C, Control; W, Tumor-bearing animals; WL, Tumor-bearing group with a maternal leucine-rich diet; WO, Tumor-bearing group with a maternal omega-3 diet; and WLO, Tumor-bearing group with a maternal leucine-rich and omega-3 diet. The minimum number of adult rats was 4-6 per group. For details, see the Materials and Methods section. Values are means ± SEM. *Compared with the C group, $P \leq 0.05$, #Compared with the W group, $P \leq 0.05$, based on one-way ANOVA followed by the Bonferroni test.

these groups presented significantly increased values in comparison to the W group (WL, P=0.0025; WO, P=0.0109; WLO, P=0.0334) (Fig 4G).

4. Discussion

Cancer-cachexia syndrome is responsible for poor prognosis and reduced patient survival, causing one in four cancer-related deaths [50]. Several studies showed that nutritional supplementation can be an easy alternative as an adjuvant treatment for cancer and the cachexia state, as well as for prevention [51,52]. Additionally, omega-3 only (or fish oil rich in omega-3) and leucine are already used for treatment in cancer, showing positive results in response to cachexia state [9,53,54]. Thus, in the present work, we chose to study the preventive effects of leucine alone or in combination with fish oil throughout pregnancy and weaning, which are two important periods of life that can be modulated by nutrition. This approach was chosen based on recent studies in the field of nutri-epigenomics, which investigates how maternal nutrition remodels the offspring epigenome, leading to stable changes that can predispose to or protect against adulthood diseases, including cancer [14,55,56]. Supporting these studies, we demonstrated that damaging tumor effects were modulated as a consequence of maternal influence, since we found preserved hepatic activity and antioxidant responses in all supplemented adult offspring. Most importantly, we found that maternal influence reduced the cachexia index (as seen in the WO and WLO groups) and also minimized the low survival rates (as seen in the maternal nutritional supplementation groups, especially in the WLO group), despite having similar tumor-to-body weight ratios.

Few studies have investigated improvements related to survival rate with combined leucine and omega-3 supplementation, which was investigated in this work. Indeed, we found that the W group exhibited shorter survival. Contrary, all supplemented groups had a median survival that was at least two days longer, suggesting that survival was proportionately longer. Additionally, most of the WLO animals died on the 35th day of tumor evolution, which likely showed that the influence of the maternal nutritional supplementation postponed the tumor effects and improved the host responses. Mabasa and colleagues observed that canola oil, which contains a lower omega-6/omega-3 fatty acid ratio, improved the survival rate in tumor-bearing rats [37]. Consistent with this study, our data revealed an improvement in survival rate in the groups subjected to maternal supplementation with fish-oil and/or combined leucine supplementation.

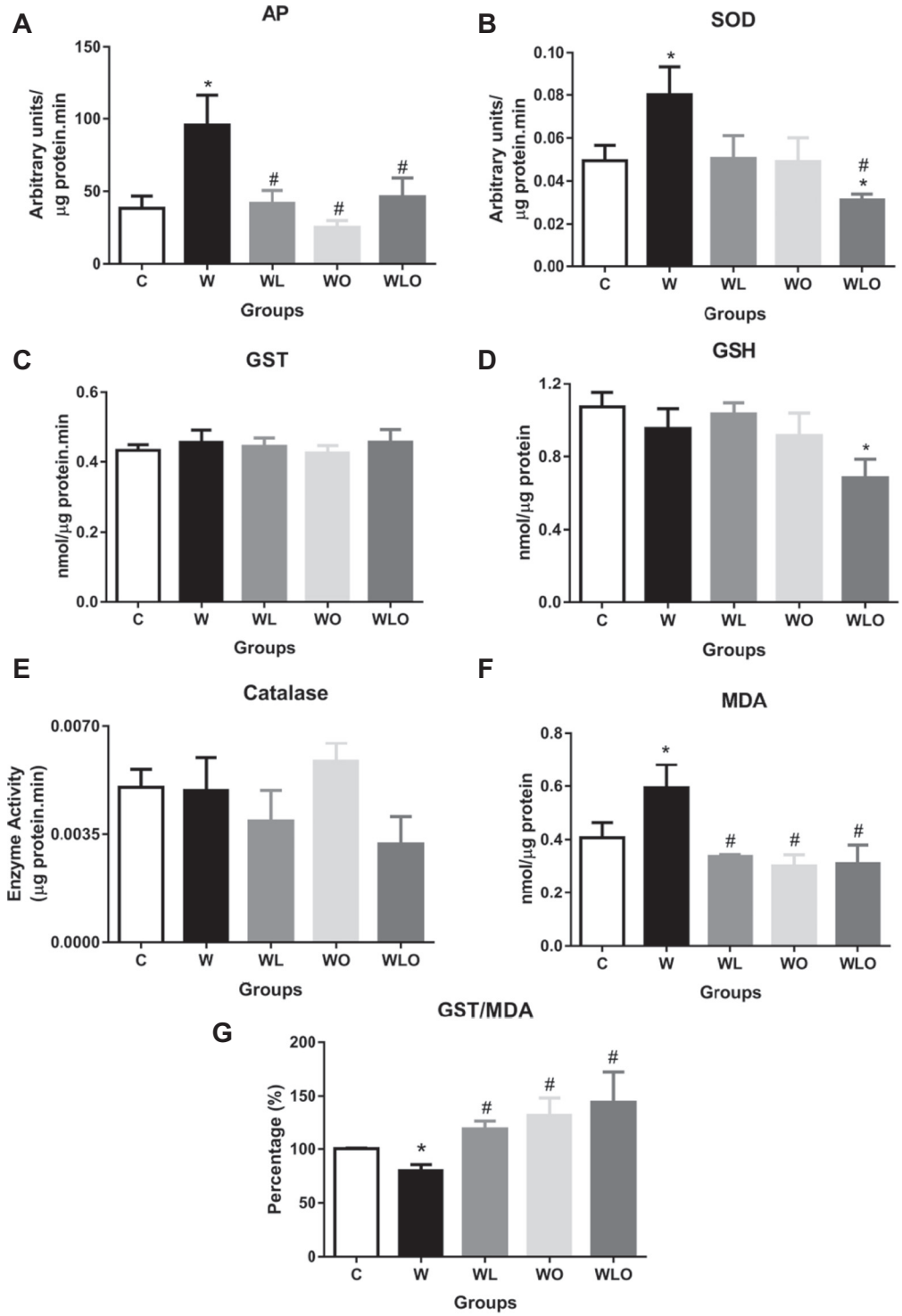

Fig. 4 – Hepatic antioxidant defenses and oxidative marker content in all experimental groups bearing Walker-256 tumors. A, **Alkaline phosphatase activity (AP), expressed as arbitrary units/µg protein/min.** B, **Superoxide dismutase activity (SOD), expressed as arbitrary units/µg protein/min.** C, **Glutathione-S-transferase activity (GST), in nmol/µg protein/min.** D, **Content of reduced glutathione (GSH), in nmol/µg protein.** E, **Catalase activity, expressed as arbitrary units/µg protein/min.** F, **Content of malondialdehyde (MDA), in nmol/µg protein.** G, **GST/MDA ratio, which indicates the relation between antioxidant defense and oxidative stress products, expressed as a percentage (%). Legend: C, Control; W, Tumor-bearing animals; WL, Tumor-bearing group with a maternal leucine-rich diet; WO, Tumor-bearing group with a maternal omega-3 diet; and WLO, Tumor-bearing group with a maternal leucine-rich and omega-3diet. The minimum number of adult rats was 4-6 per group. For details, see the Materials and Methods section. Values are means ± SEM. *Compared with the C group, $P \leq 0.05$, #Compared with the W group, $P \leq 0.05$, based on one-way ANOVA followed by the Bonferroni test.**

In this work, the different nutritional schemes led to similar growth in all animals from the time of separation from their mothers to adulthood; thus, we observed that these diets promoted similar health conditions before tumor implantation. The similar conditions of all animals were modified by Walker-256 tumor evolution, as the carcass weights were smaller than the initial body weights. This result demonstrated the expected severe tissue degradation caused by the tumor's development, which is observed in the cachexia state [3]. Therefore, we suggest that the maternal influence modulated the tumor's damaging effects, because the lower cachexia index was found especially in the WO and WLO groups, which reflected the less carcass-wasting in adult offspring. Recent studies have shown that both supplements – leucine and fish oil – efficiently modulate parameters of cachexia [24,26]. In our previous studies, a lower cachexia index was observed in animals treated with leucine during the tumor period [24,35]. Consistent with the literature, our data demonstrated that a lower cachexia index could be an effect of maternal nutrition influence, since the groups that were supplemented with fish oil alone or in association with leucine had the capacity to manage some physiological parameters, which led to a reduced cachexia index.

Hepatomegaly is common during tumor development and is likely caused by intense hepatic metabolism, resulting in increased C-reactive protein production (in response to inflammation) and/or edema [57]. Corroborating this fact, we observed an increased relative liver weight only in tumor-bearing rats of the W group, preserving this parameter in all supplemented adult offspring. In addition, liver enzymes, such as GGT, ALT and AST, also indicate liver activity in relation to detoxification processes and amino acid metabolism [58-60]. In this view, the association with increased AST activity and decreased ALT activity, which corresponded to an enhanced AST/ALT ratio, which was verified only in the W group, likely suggested liver dysfunction caused by tissue overload during cancer-cachexia progression [24,61,62]. Moreover, despite having tumors, all adult rat offspring that were subjected to maternal influence exhibited some damaging tumor effects but also exhibited amelioration of hepatic function and carcass weight, which was preserved in those animals, reflecting a lower cachexia index and modifying the survival rate. Another point related to liver function is the global cellular activity, as indicated by the alkaline phosphatase (AP) enzyme [63], whose levels increase in pathologies like cancer [64]. Our results confirmed this effect only in the W group. Again, despite having tumors, the groups that received maternal nutritional supplementation (WL, WO and WLO) had levels of AP similar to those of the controls, which indicated an improvement of tumor-induced liver dysfunction.

In the cachexia state, antioxidant defenses are impaired, and reactive oxygen species (ROS) levels are increased. This imbalance can promote tumor development and progression [28], which likely affect many tissues and organs in the host, especially the liver. Therefore, our results corroborated these findings, as some liver antioxidant enzyme activities and a product of lipid peroxidation and oxidative stress marker (MDA) [65,66] were significantly increased only in the W group, indicating a jeopardized response to oxidant damage. Having a higher SOD activity, the W group exhibited a primary defense, as SOD acts against the radical superoxide anion (O_2^-) when the cell is under oxidative stress by catalyzing the oxidation of the superoxide anion into oxygen (O_2) and peroxide (H_2O_2) [67]. However, the subsequent processes, such as the decomposition of H_2O_2 into water by the catalase enzyme and the detoxifying effect of GST for the maintenance of cellular integrity [68], probably had a minimal effect, as the increased MDA content contributed to lower liver function in the W group. On the other hand, the groups subjected to maternal diet supplementation had hepatic responses similar to those of the control group, especially related to liver enzyme and antioxidant activities, suggesting that maternal nutritional supplementation was able to reduce the damage caused by the tumor. This effect reduced the overload on the liver tissue, which is an organ that is required for metabolism during tumor progression. Thus, we found here, for the first time, that some important parameters are influenced by maternal nutritional supplementation with fish oil and/or leucine. More points must be investigated since the limitations of this study were most related with the length and size of the experiment. New experiments are now underway in our laboratory that involve assays related to cell signaling and epigenetic features to better understand how maternal diet can counteract the damaging effects of tumor evolution.

In summary, maternal nutritional supplementation with fish oil and/or leucine modulated the altered antioxidant enzyme activities in the liver, efficiently reducing oxidative stress (lower MDA levels). Additionally, and first demonstrated here, the use of leucine-rich and/or fish oil diets throughout the gestational and weaning periods ameliorated cachexia-impaired parameters, modifying the survival rate in tumor-bearing rat offspring. Thus, we suggest that maternal nutritional supplementation could be effective in preventing the harmful effects of cachexia.

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