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Basic nutritional investigation

Synergistic anti-tumor effects of melatonin and PUFAs from walnuts in a murine mammary adenocarcinoma model

Carolina P. Garcia M.S. [a], Alicia L. Lamarque Ph.D. [b], Andrea Comba Ph.D. [c], María A. Berra Ph.D. [a], Renata A. Silva M.S. [a], Diana O. Labuckas M.D. [b], Undurti N. Das M.D. [d,e,f], Aldo R. Eynard Ph.D. [c], Maria E. Pasqualini Ph.D. [c,*]

[a] Instituto de Biología Celular-Facultad de Ciencias Médicas, Universidad Nacional de Córdoba, Argentina
[b] Instituto Multidisciplinario de Biología Vegetal (IMBIV-CONICET), Cátedra de Química Orgánica. FCEF y N. UNC, Argentina
[c] Instituto de Investigaciones en Ciencias de la Salud de Córdoba (INICSA-CONICET) and Cátedra de Biología Celular, Histología y Embriología, Facultad de Ciencias Médicas, Universidad Nacional de Córdoba (FCM-UNC), Ciudad Universitaria, Córdoba, Argentina
[d] UND Life Sciences, Shaker Heights, Ohio, United States
[e] Jawaharlal Nehru Technological University, Kakinada, India
[f] Bio-Science Research Centre, Gayatri Vidya Parishad College of Engineering, Visakhapatnam, India

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ABSTRACT

Objective: The aim of this study was to determine the effects of some polyunsaturated fatty acids plus phytomelatonin from walnuts in the development of mammary gland adenocarcinoma.
Methods: BALB/c mice were fed a semisynthetic diet supplemented with either 6% walnut oil and 8% walnut flour containing phytomelatonin (walnut diet: WD); or 6% corn oil plus commercial melatonin (melatonin diet: MD), or the control group (CD), which received only 6% of corn oil. Membrane fatty acids of tumor cells (TCs) were analyzed by gas liquid chromatography, cyclo-oxygenase (COX) and lipoxygenase (LOX) derivatives, and plasma melatonin by high-performance liquid chromatography; apoptosis and tumor-infiltrating lymphocytes by flow cytometry.
Results: TCs from the MD and WD mice showed significant decreases in linoleic acid compared with the CD group ($P < 0.05$). Significantly lower levels of LOX-[13(S)-HODE] were found in TCs from the MD and WD group than in CD ($P < 0.0001$). COX-[12(S)-HHT] was lower and 12 LOX-[12(S)-HETE] was higher in TCs from the MD group than form the WD and CD arms ($P < 0.05$). Plasma melatonin, apoptosis, tumor infiltration, and survival time were significantly lower in CD mice than in MD and WD mice ($P < 0.05$).
Conclusions: This study shows that melatonin, along with polyunsaturated fatty acids, exerts a selective inhibition of some COX and LOX activities and has a synergistic anti-tumor effect on a mammary gland adenocarcinoma model.

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Introduction

Cancer is caused by genetic damage and epigenetic factors. Recent epidemiologic studies have revealed that the incidence of breast cancer is increasing; it is now the third leading cause of cancer-related deaths [1]. Environmental and nutritional factors are involved in the development and progression of breast cancer [2]. Dietary polyunsaturated fatty acids (PUFAs) and their derivatives, such as eicosanoids and reactive oxygen species (ROS) formed during the metabolism of PUFAs also play a significant role in all stages of oncogenesis [3–5].

Melatonin (*N*-acetyl-5-methoxytryptamine) is a neurohormone synthesized nocturnally by the pineal gland in mammals [6] and is also present in a variety of edible plants, where its

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* Corresponding author. Tel.: +54 351 433 4020; fax: +54 351 481 0404.
E-mail address: Epasqual@cmefcm.uncor.edu (M. E. Pasqualini).

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concentration is much higher than in mammalian nocturnal production [7,8]. In mammals, melatonin triggers intracellular signal processes via the activation of the plasma membrane and cytosol receptors or nuclear-binding sites. More importantly, many melatonin actions are receptor independent, functioning as direct free radical scavengers [9]. Several studies have shown that melatonin has both antimutagenic and anticancer properties [10,11]. Melatonin stimulates the immune system through an increased synthesis of tumor necrosis factor (TNF)-α and through certain interleukins released by monocytes and lymphocytes, which may be responsible for some of its beneficial actions [12,13].

Melatonin also has two high-affinity G protein-coupled receptors, designated as MT1 and MT2 [14]. Both interact with several effectors systems, including adenylate cyclase, phospholipase C, and phospholipase A_2 [15]. The ability of melatonin to stimulate phospholipase C and A_2 suggests that this action involves the modulation of eicosanoid metabolism through cyclooxygenase (COX) and lipoxygenase (LOX) enzymes [16,17]. PUFAs such as arachidonic acid (AA) are a substrate of 15-LOX-2 generating 15 (S)-hydroxyeicosatetraenoic acid [15 (S)-HETE], whereas linoleic acid (LA; 18:2 ω-6) by 15-LOX-1 action generates 13(S)-hydroxyoctadecadienoic acid [13(S)-HODE] [18]. Both 15 (S)-HETE and 13(S)-HODE are known to exert pro- or anti-tumorigenic effects, depending on the type of cancer [19].

In a previous animal-feeding experiment that studied the effect on mammary gland adenocarcinoma of a diet supplemented with chia oil (*Salvia hispanica*) and rich in ω-3 fatty acids, it was demonstrated that tumor cell (TC) membranes were enriched with ω-3 PUFAs with a concomitant decrease in the AA-derivative eicosanoids released by 12-LOX activity as 12(S)-hydroxyeicosatetraenoic acid [12(S)-HETE] and COX-2 derivatives as 12 hydroxyheptadecatrienoic acid [12(S)-HHT]. Both eicosanoids are associated with an increase in cancer cell proliferation and lymphocyte migration [20].

Walnuts (*Juglans regia L.*) are rich in PUFAs such as LA and α-linolenic acid (ALA; 18:3 ω-3), as well as abundant phytomelatonin (~3.5–4.5 ng/g) [21].

Although the effect of the growth inhibition function of PUFAs and melatonin in breast cancer has been extensively studied independently, only minimal research has been dedicated to the role of both phytomelatonin and PUFAs from walnuts in breast cancer invasion and metastasis.

This research studies the effects of a diet containing phytomelatonin and PUFAs from walnut flour, as well as the synthetic form of melatonin on the growth of a murine mammary gland adenocarcinoma experimental model.

Materials and methods

Plant material and oil extraction

Walnuts were obtained from local markets in Argentina. Seeds were selected and ground in a stainless steel mill to a particle size of 2.4 to 4.8 mm. Lipid content was reduced with a Komet screw press (model CA 59 G, IBG Monforts, Mönchengladbach, Germany), with a 5-mm restriction die and a screw speed of 15g. The temperature of the process (50°C) was maintained with an electric resistance heating ring surrounding the press barrel and was controlled using a digital thermometer inserted into the restriction die [22].

Table 1
kcal/kg of Each Macronutrient and Total kcal/kg of CD, MD, and WD

Macronutrients	Diets (kcal/kg)		
	CD	MD	WD
Carbohydrates	2920	2920	3082.88
Proteins	640	640	445.20
Lipids	540	540	573.84
Total kcal	4100	4100	4102

CD, control diet; MD, melatonin diet; WD, walnut diet

Table 2
Experimental and Control Diet Composition Expressed as g/kg and mice melatonin daily intake expressed in ng/d

Diet components	Diets*		
	CD	MD	WD
Carbohydrates	730	730	770.72
Proteins	160	160	111.28
Lipids	60	60	63.76
Minerals	25	25	25
Vitamins	5	5	5
Fiber	20	20	20
Melatonin daily intake		≅1.5 ng	≅1.8 ng

CD, control diet; MD, melatonin diet; WD, walnut diet
MD: 6% corn oil, 16% casein, 33% sucrose, 40% cornstarch, 2% fiber, 2.5% salt mixture, and 0.5% vitamin mixture. Commercial melatonin (Rexall Sundown, Inc. Boca Raton FL33487) was administered in water at a concentration of 300 ng/L. Daily water intake per animal was approximately 4 to 5 mL
WD: 6% walnut oil, 8% casein, 8% nut flour, 33% sucrose, 40% cornstarch, 2% fiber, 2.5% salt, and 0.5% vitamins. Nut flour contained 7.5 ng/g melatonin. Daily diet intake per animal was approximately 3 g
* CD: 6% corn oil, 16% casein, 33% sucrose, 40% cornstarch, 2% fiber, 2.5% salt mixture, and 0.5% vitamin mixture. Corn oil is a commercial product provided by Arcor S.A (Argentina).

Tumor

We used a murine transplantable mammary adenocarcinoma tumor (M3), which was provided by Dra. Bal de Kier Joffé Elisa of the Angel H. Roffo Institute of Oncology, University of Buenos Aires, Argentina [23]. This M3 tumor is estrogen independent with a moderate metastasizing capacity and with a latency period of 6 ± 2 d after inoculation [23].

Weaning male and female BALB/c mice were used.

Transplantation of tumors and other animal studies were conducted in accordance with the guidelines of the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals; procedures were approved by the Institutional Committee for the Care and Use of Laboratory Animals of the School of Medicine, University of Cordoba, Argentina (http://secyt.webs.fcm.unc.edu.ar/../Reglamento-Animales-de-laboratorio) and the Animal Research Committee of the Institute of Oncology A.H. Roffo, Buenos Aires, Argentina [23]

Diets, feeding protocol, and M3 tumor inoculums

Three different isocaloric diets (caloric density 4100 kcal/kg) were used (Table 1). The final composition of each diet is detailed in Table 2. The nutritional composition of walnut flour per 100 g of dry solid is 50.9% carbohydrates, 39.1% proteins, 4.7% lipids, and 5.3% ashes [21].

Sixty post-weaned male and female BALB/c mice were randomly distributed among three groups (20 mice each: control: CD; walnut diet: WD; melatonin diet: MD). Food and water were provided ad libitum. Animals were kept in a light- and temperature-controlled room. After 3 mo of feeding with respective diets, mice were subcutaneously inoculated with 1 mm^3 of tumor tissue in the right flank. Thirty-five d after tumor inoculation, the experimental animals were sacrificed. At the end of the study, the tumor weight was measured (this parameter is similar to tumor volume). A magnifying lens was used to determine the number and sites of metastasis, as previously described [20,24].

Table 3
Fatty acid composition (%) of Corn and Walnut Oils*

Oils	Saturated fatty Acids			Unsaturated fatty acids					
				ω-7	ω-9			ω-6	ω-3
	16:0	18:0	20:0	16:1	18:1	20:1	22:1	18:2	18:3
Corn	11.23	1.41	0.39		31.90	0.36	0.46	53.85	0.36
Walnut	7.21	2.29	0.08	0.07	22.04	0.07		51.08	17.15

* Fatty acids of corn and walnut oils were determined as indicated in the Methods section. Values are the average of at least three determinations and the SE < 6% in all cases.

Table 4A
Unsaturated fatty acids profile of TC* from host Bearing M3 tumor fed with CD, MD, and WD

Diets	Unsaturated fatty acids (%)																
	ω-3					ω-5	ω-6			ω-7	ω-9						Others
	18:3	20:3	20:5	22:5	22:6	14:1	18:2	18:3	20:4	16:1	18:1	20:1	20:2	20:3	22:1	24:1	
CD	0.05	0.58	0.35	0.04	0.10	0.17	17.23†	0.10	0.14	4.03	19.95†	0.68	0.15	0.4	0.16	0.02	38.00
MD			0.15	0.17	1.35		1.14	0.60	0.47	2.63	10.12			1.33		2.78	21.63
WD	0.10		0.14	0.85	2.32		9.79	0.02	8.64*	2.11	16.45	0.74	0.71	0.14	0.17	0.72	8.15

CD, control diet; MD, melatonin diet; TC, tumor cell; WD, walnut diet

* Fatty acid composition of TC membrane was determined as indicated in the Methods section. Values represent the mean of at least three determinations from TC suspensions of 10^8 cells/mL (SEM < 6% in all cases).

† Significant differences between the treatments ($P \leq 0.05$).

For survival determination, a parallel assay was conducted. Groups of 8 to 10 animals of each diet were treated as previously described; these mice, however, were left to die naturally.

Tumor cell suspensions

TC suspensions (1×10^7 TC/mL) were prepared from primary tumors obtained from areas that were free of necrosis, blood clots, and connective tissue, with 0.01% pronase and 0.24% type 1-deoxyribonuclease in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO, USA). Samples were then washed twice, resuspended in Ca^{2+} and Mg^{2+}-free phosphate-buffer saline and stimulated with ionophore A23187 (2 M) (Sigma-Aldrich) for 15 min at 37°C. These suspensions were used for further studies [20,24].

Fatty acid analysis of oils and TC membranes of animals fed with control, walnut, and melatonin diets

Walnut and corn oils were subjected to alkaline saponification (1 N KOH). The unsaponifiable matter was extracted with *n*-hexane; fatty acid methyl esters (FAMEs) were obtained using 1 N sulfuric acid in methanol [25]. TC plasma membrane purification was carried out as previously described [26]. Briefly, TCs (1×10^7) were placed in homogenization solution (hypotonic Hepes–Manitol buffer; Sigma-Aldrich) and homogenized using a Polytron (7 sec at setting 7). The homogenate was treated with 10 mM of $CaCl_2$ and centrifuged at 3000*g* for 15 min. The supernatant was saved and centrifuged at 48 000*g* for 30 min. The pellet containing the plasma membrane fragments collected in deionized water and the lipids in the lower phase were extracted with chloroform: methanol: H_2O (3:48:47 v/v/v). FAMEs were obtained with toluene and sodium metoxide, which were maintained overnight at 4°C.

FAMEs were analyzed using a polyethylene glycol (Phenomenex, Inc., Torrance, CA, USA) capillary column (30 m × 320 μm id × 0.50 μm), on a Perkin Elmers Claurus 500 gas-liquid chromatograph (Santa Clara, CA, USA) equipped with flame ionization detector.

FAMEs were identified by comparing their retention times with those of authentic standards (Nu-Chek Prep, Inc., Elysian, MN, USA) [27].

Fatty acid (FA) percentages were quantified by calculating the basis of the peak area in reference to crescent concentrations of FA linear curves obtained from standards (Nu-Chek Prep) [27].

The membrane fatty acid unsaturation of TC was expressed as Double Bound Index (DBI) and was calculated according to the following equation:

$$DBI - \sum \frac{(UF \cdot DB)}{SF}$$

where *UF* is the percent composition of each unsaturated FA, *DB* is its respective number of double bonds and *SF* is the percent composition of saturated FAs [27].

Table 4B
Saturated fatty acid profile of TC* from host Bearing M3 tumor fed CD, MD, and WD

	Saturated fatty acids (%)									
Diets	13:0	14:0	15:0	16:0	17:0	18:0	20:0	21:0	22:0	24:0
CD		1.30	0.02	12.35	0.47	2.99		0.13	0.48	0.10
MD	0.05	0.56	0.13	21.40		22.39	0.49	0.65	10.91	1.59
WD	0.02	1.01	1.31	27.55	0.31	17.17	0.21	0.89	0.15	0.35

CD, control diet; MD, melatonin diet; TC, tumor cell; WD, walnut diet

* Fatty acid composition of TC membrane was determined as indicated in the Methods section. Values represent the mean of at least three determinations from TC suspensions of 10^8 cells/mL (SEM < 6% in all cases).

LOXs and COXs enzyme activities in tumor cells

The activities of 12-LOX and COX-2 with AA as the substrate and 15-LOX-2 with LA as the substrate were estimated as described previously, with slight modifications [26]. Determinations were made from 8 to 10 samples of TC suspensions (1×10^7 TC/mL) from each of the dietary treatment. TC metabolites were extracted using a STRATA C-18 cartridge (1 mL) (Phenomenex, Inc.) and detected by reverse-phase high-performance liquid chromatography (HPLC). Analysis was conducted with a C18 Phenosphere-Next column (5 μm; 4.6 × 250 mm) (Phenomenex, Inc.) in a Beckman System Gold Programmable Module Model 126. The metabolite separation was achieved using a timed program. A linear gradient from solvent A:methanol:water:acetic acid, 50:50:0.02 (v/v/v), pH 6.0 to solvent B:methanol, over 20 min. Ultraviolet Beckman System Gold Model 166 with a programmable detector linked to a computer for data processing was used. Ultraviolet absorbance analysis of the eicosanoids 12(S)-HHT, 12(S)-HETE, 15(S)-HETE, and 13(S)-HODE was read at 235 nm. Quantification of eicosanoids 12(S)-HHT from COX-2, 12(S)-HETE by 12-LOX, and 13(S)-HODE by 15-LOX-1 activities were obtained by using standard curves (Biomol International LP., Plymouth Meeting, MA, USA) and was expressed as ng/1×10^7 TC [19,20,24,28].

Walnut flour and plasma melatonin analysis

Melatonin extraction from walnut flour was carried out according to a previously described technique [29] with modifications. One g of sample (three replicates) was homogenized with 15 ml of methanol. The homogenate was centrifuged at 8000*g* (4°C, 35 min). The supernatant was collected and evaporated under vacuum. Residue was redissolved in 1 ml phosphate-buffer saline (pH 7.4, 20 mM). Two mL of chloroform were added and shaken for 2 min. After centrifugation (100 *g*, 5 min), the organic phase was evaporated and the residue was dissolved in 100 μL of HPLC mobile phase.

The extraction of plasma melatonin of mice was carried out using a previously described methodology [30]. Mice blood was collected by a cardiac puncture when mice were deeply anesthetized. Plasma was separated by centrifugation (200*g*, 15 min). One hundred μL of plasma from a pool of six mice taken from each of the three dietary conditions was mixed with ethanol (1:20 v/v), vortex mixed for 2 min, filtered through a 0.45-μm filter, and evaporated to dryness. The residue was resuspended in 400 μL 0.2 M acetic acetate buffer (pH 4.9) and 2 mL chloroform was added. After vortex mixture and centrifugation (850*g*, 10 min) the aqueous phase was discarded and the organic phase cleaned with 500 μL 0.1 N NaOH and evaporated under reduced pressure at 30°C. The residue was finally resuspended in 200 μL ethanol. Before injection, the samples were diluted 1:1 (v/v) with mobile phase.

Melatonin from walnut flour and plasma were analyzed by a HPLC system consisting of a Perkin Elmer Series 200 pump and Series 200 a fluorescence detector. The analytical column used was Nucleosil C18 (5 mm particle, 250 mm × 4 mm i.d.). The mobile phase consisted of a solution that contained 60% methanol and 40% of a solution containing 1.76 g sodium acetate, 46 μL

Table 4C
Total saturated, unsaturated fatty acids (%) and DBI of TC from host Bearing M3 tumor fed CD, MD, and WD

Diet groups	Saturated fatty acids	Unsaturated fatty acids	DBI
CD	17.85	82.15	3.71
MD	58.17	41.83	0.57
WD	48.95	51.05	1.94

CD, control diet; DBI, Double Bond Index; MD, melatonin diet; TC, tumor cell; WD, walnut diet

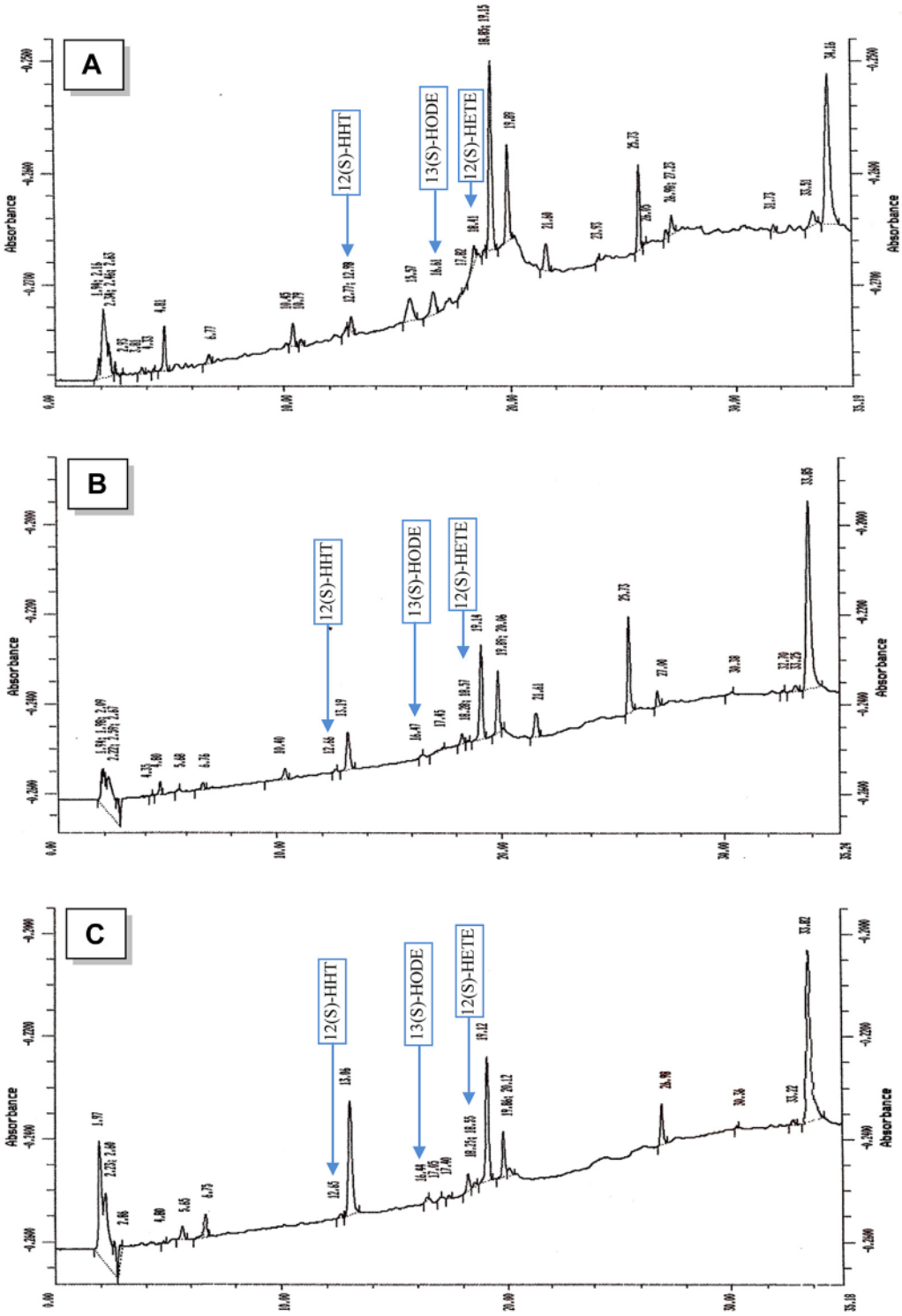

Fig. 1. Eicosanoid profiles derived from arachidonic acid and linoleic acid by lipoxygenase and cyclooxygenase enzyme activity released from M3 tumor cell of hosts fed different diets after stimulation with ionophore A 23287. (A) Control, (B) melatonin, and (C) walnut diets.

triethanolamine and 7.32 mL acetonitrile in 100 mL final volume made up with distilled water. The pH was adjusted to 4.9. Analysis was performed at room temperature at a flow rate of 0.5 mL/min. The fluorescence detection was carried out at 340 nm with excitation at 278 nm. The melatonin concentration in walnut flour and plasma were calculated on the basis of the peak area of melatonin, by reference of standard curves obtained by linear regression of standard melatonin (Sigma-Aldrich).

Evaluation of apoptosis

Apoptosis of TC suspensions (1×10^6 cells) was analyzed by flow cytometry (FC) (Coulter® XL EPICS® Flow Cytometer, Miami, FL, USA), using an Annexin V-fluorecein isothiocyanate (fitc) apoptosis detection kit (Sigma-Aldrich). Determinations were done in five to eight samples from each dietary treatment. The procedure is based on the binding properties of conjugated Annexin V-fitc to

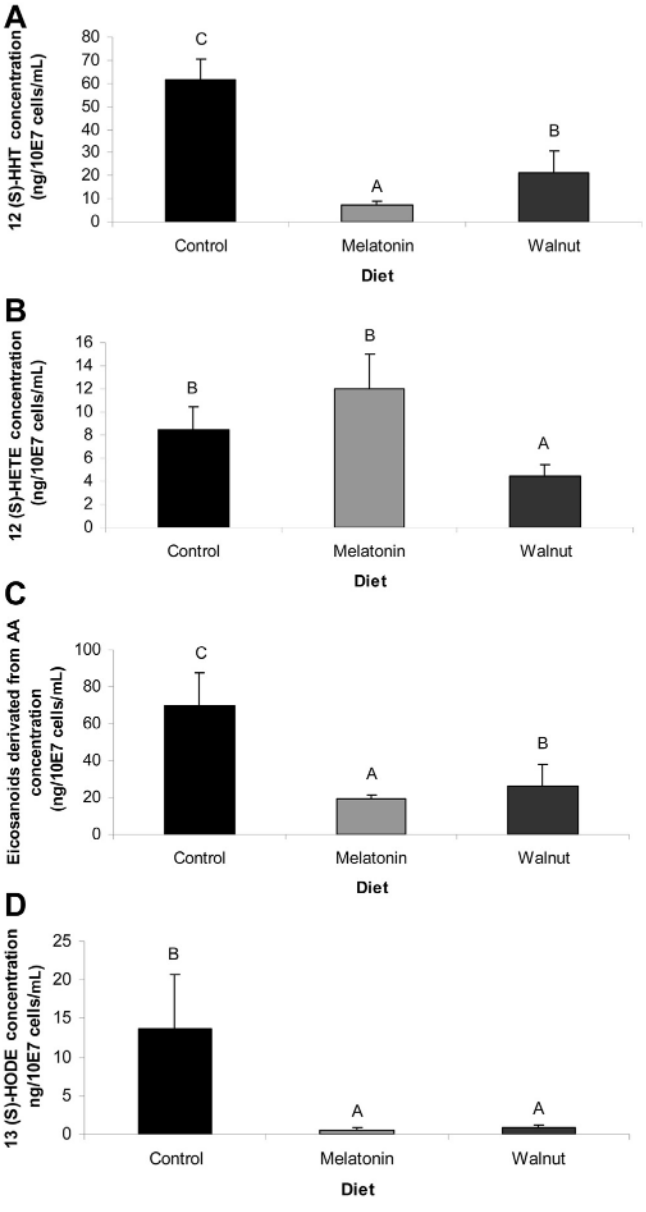

Fig. 2. Lipoxygenase and cyclooxygenase eicosanoids released from tumor cells: (A) 12(S)-HHT, (B) 12(S)-HETE, (C) sum of 12(S)-HHT and 12 (S)-HETE derived from arachidonic acid, and (D) 13(S)-HODE derived from linoleic acid. Values represent the means ± SEM of cyclooxygenase and lipoxygenase eicosanoids from 8 to 10 tumor cell samples. Different letters represent significant differences.

plasma membrane phosphatidylserine, in combination with propidium iodide staining, to evaluate both early and late apoptosis plus necrosis [31].

Tumor-infiltrating lymphocytes index

Tumor-infiltrating lymphocytes index (TIL) was assayed by analyzing CD3 complex expressed on mature T lymphocytes and natural killer (NK)T cells in TC suspensions (1×10^6 cells) by FC (Coulter® XL EPICS® FC) using a detection kit of CD3 fitc-conjugated hamster antimouse CD3 and monoclonal antibody (BD Biosciences Pharmingen, Philadelphia, PA, USA) [32]. Determinations were made using five to eight samples from each dietary treatment.

Statistical analysis

Data obtained were analyzed using an analysis of variance, and a generalized linear model with random-component gamma. Canonic link function (covariates) was used to evaluate the significance for diets, apoptosis, necrosis, eicosanoids, melatonin, TLI, tumor volume, and metastases [33]. All statistical analyses were performed using Stata Statistical Software: Version 12.1 (Stata Corp LP, College Station, TX, USA).

Table 5
Plasma melatonin content of host Bearing M3 tumor fed CD, MD, and WD

Diet groups	Plasma melatonin (Pg/ml)
CD	0.74 ± 0.01^a
MD	4.39 ± 0.48^b
WD	3.27 ± 0.61^b

CD, control diet; MD, melatonin diet; WD, walnut diet
Different superscript letters represent significant differences ($P < 0.05$)

Results

Fatty acid profiles of dietary oils and tumor cell membranes

Fatty acid composition of dietary oils is shown in Table 3. Values for the oils agree with those previously presented [34]. Table 4 summarizes the profile of main unsaturated and saturated FAs of the TC membranes from hosts fed with different diets. Table 4A shows that the proportion of LA (18:2, ω-6) was significantly lower in MD (1.14%) than WD (9.79%) and CD groups (17.23%; $P < 0.05$). Also, AA (20:4, ω -6) percentage was significant lower in CD (0.14%) and MD (0.47%) than WD (8.64%) groups ($P < 0.05$). With respect to ω-3 FAs, WD presented higher percentages of docosapentaenoic acid (22:5, ω-3) and docosahexaenoic acid (22:6, ω-3), 0.85% and 2.32%, respectively, than CD and

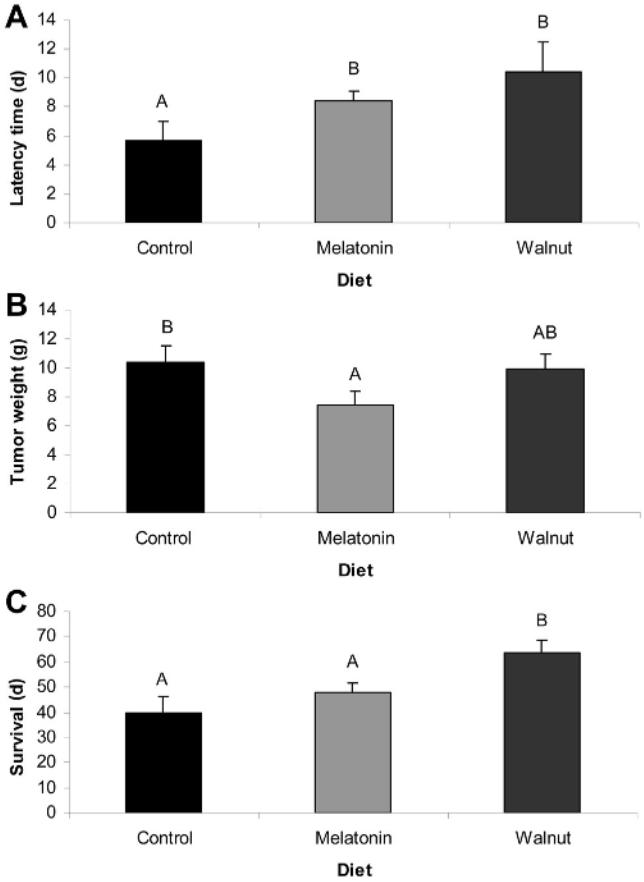

Fig. 3. (A) Latency time, (B) tumor weight, recorded during necropsy at 35 d after inoculation, and (C) survival of mice after inoculation of tumor of M3 hosts fed different diets. Values represent the mean ± SEM of 10 animals. Different letters represent significant differences ($P < 0.02$).

Table 6
Apoptotic TC Recorded in Tumor Suspensions (1 x 10^6 cells) from Host Bearing M3 Tumor Fed CD, MD, and WD

Diet groups	Apoptosis (%)
CD	22.74 ± 6.69[a]
MD	42.88 ± 7.83[b]
WD	43.36 ± 12.35[b]

CD, control diet; MD, melatonin diet; WD, walnut diet
Values represent the mean ± SEM of apoptotic cell (%) analyzed from 10 animals of each dietary condition. Different superscript letters represent significant differences ($P < 0.05$)

MD. Furthermore, with respect to ω-9 FAs, the CD group exhibited significantly higher levels of octadecenoic acid (octadecenoic acid (OA); 18:1, ω-9) (19.95%) than the WD (16.45%) and MD (10.12%) treatments ($P < 0.05$).

The degree of membrane FAs unsaturation (DBI) is shown in Table 4C. TC membranes of the CD group exhibited the highest DBI (3.71), whereas the MD mice had the lowest values (0.57).

Modulation of LOX and COX enzyme activities by PUFAs in tumor cells

Different dietary treatments modified LOX and COX eicosanoids derived from LA and AA after tumor cells stimulation with ionophore A 23187 (values expressed as ng/107 cells/mL), as shown in Figure 1. AA–COX derived-12(S)-HHT was significantly lower in MD and WD (7.16 and 21.49 ng, respectively) groups than in the CD group (61.34 ng) ($P < 0.05$) (Fig. 2A), whereas the release of 12(S)-HETE was greater in TCs from the CD and MD groups (8.39 and 11.97 ng, respectively) than in the WD (4.4 ng) group ($P < 0.05$) (Fig. 2B). The sum of LOX and COX eicosanoids derived from AA, 12(S)-HETE and 12(S)-HHT was significantly lower in the MD (19.14 ng) and WD (25.89 ng) groups than in the CD group (69.73 ng) ($P < 0.003$) (Fig. 2C). Values of LA–LOX-1-derived 13(S)-HODE metabolite showed that TC from the MD and WD groups (0.51 and 0.87 ng•10^7 cel•mL, respectively) released significantly lower amounts of this eicosanoid than TC from the CD mice (13.7 ng) ($P < 0.0001$) (Fig. 2D).

Melatonin content in walnut flour and plasma of mice fed different diets

The concentration of melatonin in walnut flour was 7.5 ± 0.9 ng/g. This value is similar to concentrations reported previously in studies on walnut seeds [29,35].

As expected, plasma melatonin values were significantly lower in the CD group (0.74 pg/mL) than in the MD and WD (4.39 and 3.27 pg/mL, respectively) groups ($P < 0.05$) (Table 5).

Effects of diets on latency tumor time, tumor weight, and animal survival

The latency time until tumors became palpable in the CD group was significantly lower (5.7 d; $P < 0.02$) than the MD and WD (8.4 and 10.4 d, respectively) groups (Fig. 3A). Tumor weight from the MD group was significantly lower (7.45 g) than the CD group (10.37 g; $P < 0.02$) and WD tumors presented an intermediate value (9.86 g) (Fig. 3B).

Mice from the WD group showed the highest significant survival time (63.66 d) compared with the MD and CD groups (47.87 and 40 d, respectively) ($P < 0.02$) (Fig. 3C).

No significant differences were found in the number and locations of macroscopic metastasis (two to five metastases in lungs and peritoneal tissue) between animals from the three groups (results not shown). This could be explained by the moderate metastatic capacity of the M3 tumor associated with the global gene expression profile related to cell adhesion, motility, invasion, and angiogenesis.

Effect of diets on tumor cell apoptosis

Early and late apoptotic cells in TC suspensions are shown in Table 6 and Figure 4. The control group showed the lowest percentage (22.74%) of TC suspensions, compared with the MD (42.88%) and WD (43.36%) groups.

Effects of PUFAs and melatonin diets on TIL index

To understand how different PUFAs and melatonin may influence TIL, the lymphocyte T and NKT percentage in tumor suspensions (1 × 10^6 cells) was analyzed by FC. CD showed a significantly lower TIL index (41.47%) than MD (68.56%) and WD (61.25%; $P < 0.05$) (Fig. 5).

Discussion

Clinical and experimental studies have shown that diet plays an important role in the promotion and prevention of cancer [36]. This study demonstrated that melatonin not only modifies the development of murine breast carcinoma by itself, it also acts in conjunction with PUFAs to influence tumor growth by

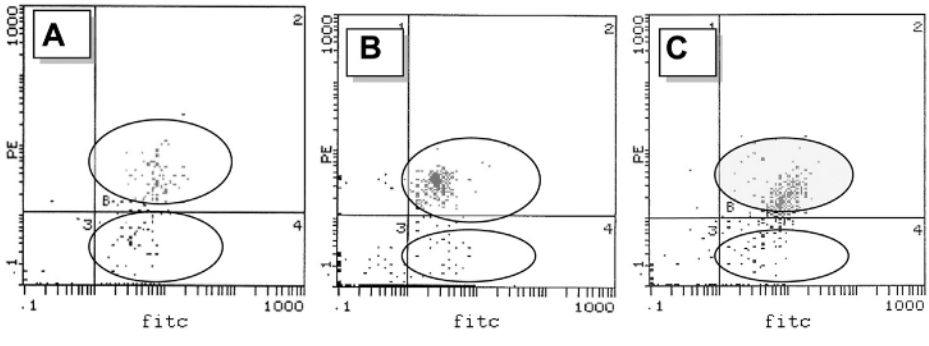

Fig. 4. Flow cytometry graphics of early and late apoptotic cell populations in tumor cell suspensions (1 × 10^6 cells) obtained from tumors of hosts fed under different diet conditions. Circles are shown in quadrant 2 (late apoptosis) and quadrant 4 (early apoptosis). (A) Control, (B) melatonin, and (C) walnut diet treatments. Fitc, fluorecein isothiocyanate.

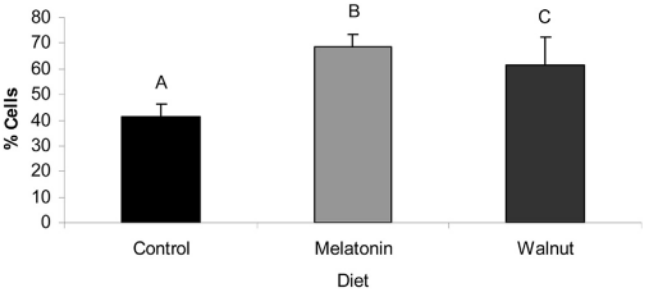

Fig. 5. Percentage of lymphocyte T and natural killer T cells in tumor suspensions (1×10^6 cells) obtained from tumors of hosts fed under different diet conditions analyzed by flow cytometry (A) Control, (B) melatonin, and (C) walnut diet treatments.Values represent the mean ± SEM. Different letters represent significant differences ($P < 0.05$).

modulating COX- and LOX-derived eicosanoids. Epidemiologic and experimental studies obtained in this study and by other researchers have shown that diets enriched in ω-3, ω-6, or ω-9 FAs or a combination of all three, differentially alter cell membrane fluidity, the activities of several proteins and enzymes, and the release of various eicosanoids which in turn, influence oncogenesis [18–20,24,37–40].

In this study, the FA composition of mammary gland TC membranes obtained from hosts fed with melatonin or with walnut flour showed a significant decrease in LA (18:2 ω-6) compared with those fed the control diet (Table 4A). This LA diminution could be due to the binding of melatonin to its membrane receptor MT1 or MT2, which is coupled to G-1 protein complex. The binding of melatonin to LA could lead to the blocking of LA incorporation based on the melatonin-MT1/MT2-G19 complex ability to inhibit intracellular production of cyclic adenosine monophosphate. This inhibition, in turn, blocks the FA transport protein channel (FAT and FATP) across the membrane [41]. This finding is supported by results from a previous study that demonstrated that rats fed a diet supplemented with phytomelatonin when inoculated with liver TCs (hepatoma 7288 CTC), showed a significant inhibition of LA uptake mediated by melatonin receptors [35]. Furthermore, walnut flour provides many different components that could be beneficial in the prevention of cancer, including tocopherols, sphingolipids, sterols, volatile compounds, and phenolic compounds [21], all of which have likely contributed to the observed effects.

Membrane TCs from CD (0.14%) and MD (0.47%) mice had significantly lower percentages of AA (20:4, ω-6) in comparison with WD mice (8.64%)(Table 4A). This effect may be attributed to a higher activity of phospholipase A2 in CD and MD TC membranes thus giving available free AA [42]. In part, the decreased levels of AA in TC membranes also could be due to altered activity of Δ^6 and Δ^5 desaturase as previously reported [43].

With respect to the LOX and COX activity evaluated by TC eicosanoids release, TC from the MD and WD groups generated significantly lower levels of 13(S)-HODE than the CD group (Fig. 2D). These results can be attributed to decreased uptake of LA substrate for 15-LOX-1 enzyme in melatonin treated animals, as expressed in a previous study [44].

13(S)-HODE has a mitogenic effect on certain cancer cells [45]. In light of this, the diminution of intracellular 13(S)-HODE production in MD and WD treatments could slow the activity of the enzyme that phosphorylates the mitogenic epidermal growth factor pathway (EGFR/MEK/ERK1/2 pathway), thus resulting in the inhibition of DNA synthesis and cell proliferation [45].

Moreover, the lower 12(S)-HHT (derived from AA–COXs pathway) (Fig. 2A) synthesized from melatonin-treated TCs suggests a melatonin selective inhibitory action on COX enzyme activity [46]. On the other hand, the lower levels of 12(S)-HETE in the WD group could be explained by the interaction between LOX and phenolic compounds present in walnut flour, which would result in a decreased enzyme activity [47].

In this study, commercial melatonin and phytomelatonin inhibited COX enzymes and induced apoptosis of TCs (Table 6). These results agree with those reported in a previous study where it was demonstrated that the inhibition of COX enzymes induced apoptosis in four human pancreatic cancer cell lines [48]. The higher AA found in the TC membrane of the WD group was well correlated with the longest survival registered in this experimental group (Table 4A and Fig. 3C). These results are in agreement with an earlier study [49].

The high percentage of apoptosis observed in TCs from MD and WD treatment (Table 6) also could be due to the immunosuppressive activities exerted by profuse tumor infiltration shown by T cells and NKT cells, and expressed as TIL index (Fig. 5) [50]. In this context, it is interesting to note that anticancer effects of melatonin are caused by its ability to activate lymphocytes, monocytes, and macrophages [51]. In contrast, the lower rate of apoptosis observed in TCs of the CD group could be due to its high OA content, which is incorporated in TC membranes. This assumption is supported by the finding that human breast cancer cell lines (MDA-MB-231) showed higher activity of OA-activated phosphatidyl inositol kinase, which promotes cell proliferation and inhibits apoptosis [52]. We have shown the deleterious effects of high OA in several growth parameters of different tumors [24,53].

In conclusion, the results of the present study suggest that host mice fed with phytomelatonin and FAs contained in walnut flour and the synthetic form of melatonin favorably modulate AA–COX eicosanoid system and membrane PUFA composition. This results in a synergistic antitumorigenic action on a model of mammary gland adenocarcinoma in mice. These beneficial actions may be due to an improvement of immune response and increased TC apoptosis, together with decreased TC generation of 12(S)-HHT and 13(S)-HODE metabolites. Because both walnut phytomelatonin and PUFAs can be incorporated through diet, this study emphasizes the important role of these nutraceuticals [54–56], which are nontoxic, safe, and natural and also have demonstrated anticarcinogenic potential.

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