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The effects of ω−6 and ω−3 fatty-acids on early stages of mice DMBA submandibular glands tumorigenesis

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ABSTRACT

The aim of this work was: to assess the impact of diets enriched in polyunsaturated fatty acids ω−3 and ω−6 families on the lipid profile of cell membrane and their effect on cycle regulation and apoptosis, evaluated by TP53 and Ki-67 expression in 9,10-dimethyl-1,2-benzanthracene (DMBA) induced tumor development in submandibular glands (SMG) in murine models. To generate tumorigenic changes, SMG mice in the experimental group were injected with 50 µl of 0.5% of DMBA. Both control (no DMBA) and experimental groups of BALB/c mice were fed with: chia oil (ChO), rich in ω−3 fatty acid; corn oil (CO), rich in ω−6/ω−3 fatty acid; and safflower (SO) oil, rich in ω−6fatty acid. Results demonstrate novel differential effects of ω−3 and ω−6 PUFAs on the regulation of early tumorigenesis events in murine SMG injected with DMBA. This knowledge may help to develop chemoprotective treatments, therapeutic agents and health promotion and prevention activities in humans.

1. Introduction

Salivary gland tumors include morphologically and clinically diverse group of neoplasms that occur at a rate of 2.5–3.0 per 100,000 per year in the Western world. The tumors of the major salivary glands, the submandibular glands (SMG) have a malignancy rate of 50%, and are thus more aggressive and worse prognosis of survival [1,2].

Various cancer theoretical models include factors such as tobacco, alcohol, genomic instability, alterations to DNA repair genes, diet, etc. [3,4]. Fatty acids, provided by the diet, are metabolized and incorporated into cell membranes and cause, in many cases, alterations to their physicochemical properties. In several studies, we have shown that fatty acids, particularly polyunsaturated fatty acids (PUFAs), when incorporated into cell membranes, modulate the production of diverse bioactive molecules such as eicosanoids [5,6].

The type and quantity of eicosanoids produced depends on the relative proportion of ω−3 and ω−6 PUFAs found in phospholipids of cell membranes, the cell type, and the enzyme phospholipase activation [7,8]. These bioactive molecules derived from lipids may cause mutations in the DNA. These mutations generate changes in the expression of various molecules, including those that regulate the cell cycle, influence the activity of transcription factors, the gene expression, and the signal transduction pathways that impact the processes of apoptosis and cell proliferation involved in cancer development [9]. In this context, it has been shown that the activity of PUFAs induce the accumulation, activation, and cellular relocation of multiple and diverse cellular molecules such as TP53, p21WAF1, GLI-1 in several cancers, including head and neck cancer [10]. The TP53 is a transcription factor that inhibits cell growth or promotes death by apoptosis [11]. Furthermore, clinical and experimental studies with malignant salivary gland tumors have shown that the rate of cell proliferation, the mutation of oncogenes, and the over-expression of receptors linked to cell growth, such as TP53 and 2/neu, were markers of early salivary gland tumor genesis [12]. Another marker associated with malignant lesions of the oral cavity, salivary glands, pharynx and larynx is Ki-67, which is strictly associated with proliferation and is confined to the nucleus. In several studies have used the immunocytochemical expression of Ki-67 as proliferative marker to define treatments and determine prognoses [13]. Both markers have been associated with dietary PUFAs as regulators of colorectal cancer development [14,15].

Although there are many studies on PUFAs and cancers such as breast, pancreas, colon, etc., there is little scientific literature on the

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relationship between head and neck cancers and PUFAs, particularly in cancers that occur in the SMG. Indeed, this type of cancer is very aggressive, with a poor prognosis, as it is generally diagnosed in its later stages. It is thus highly beneficial to determine dietary outlines that may prevent this cancer.

Taking this evidence into account, we hypothesize that lipid modification of the membranes, through the dietary addition of PUFAs, could induce variations in the activity of COX and LOX enzymes and eicosanoids. These could modify tumor development by influencing gene expression of TP53 and Ki-67 and apoptosis process in SMG. These molecular studies will enable the future design of new preventive strategies, gene therapies, and drugs to prevent the onset of cancer or inhibit its development. The aim of this study is thus to assess the impact of diets enriched in polyunsaturated fatty acids (PUFAs) of ω−3 and ω−6 families on the lipid profile of cell membrane and their effect on cycle regulation and apoptosis evaluated by TP53 and Ki-67 expression in 9,10-dimethyl-1,2-benzanthracene (DMBA) induced tumor development in submandibular glands (SMG) in murine models.

2. Material and methods

2.1. Experimental design

In order to evaluate the impact of different ω−6 and ω−3 PUFAs on membrane lipid profile of SMG murine, the animals were separated into two groups: Control (no DMBA injected) and experimental group (DMBA injected). The control group was used as a normal membrane lipid pattern, under the same time and same diet conditions as the experimental group, in order to demonstrate changes in phospholipids in the major salivary glands with the aging mouse [16].

Fig. 1 shows the experimental protocol:

2.2. Animals and diet

After weaning, BALB/c mice(male and female) with an average weight of 16.33 ± 1.20 g and 19.5 ± 0.76 g in control and DMBA groups, respectively, were randomized and housed in polycarbonate cages in groups of four, in a 12-h light and 12-h dark cycle at a constant temperature of 23 °C.

Animal studies were conducted in accordance with the guidelines set by the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and approved by the Institutional Committee for the Care and Use of Laboratory Animals at the Facultad de Ciencias Médicas (Universidad Nacional de Córdoba, Argentina).

Animals were fed with three different diets since weaning and throughout experimental time until finished, animals were fed with diets sufficient in essential fatty acids according to the requirements by mice and recommendations of the FAO. All diets were composed of% casein (16.0), sucrose (34.0), corn starch (39.0), fiber (2.0), salt mixture (2.5), and vitamin mixture (0.5), and supplied with either: 6% corn oil-*Zea mays* (CO, enhanced withω−6 and ω−3); 6% safflower oil-*Carthamus tinctorius* (SO, enriched in ω−6 PUFA); and 6% chia oil-*Salvia hispanica* (ChO, enriched in ω−3), according to previous experiments from our lab [6]. Food and water were provided ad libitum. Animals were inspected daily; food consumption and weight were recorded weekly. The chia seed oil was purchased from Nutraceutica Sturla S.R.L. (Argentina), safflower oil was donated by Argentina Bunge S.A. (Santa Fe, Argentina), and corn seed oil was purchased from Arcor S.A. (Argentina). As shown in Table 1, the fats provide by ChO contained high percentage of 18:3 ω−3 or by safflower oil (SO), which contained elevated quantity of 18:2 ω−6. Corn oil contained average levels of 18:2 ω−6 and low quantities of 18:3 ω−3.

Table 1
Fatty acids composition of Safflower (*Carthamus tinctorious L.*), Chia (*Salvia hipanica L.*), and Corn (*Zea mays*) oils. Fatty acids were determined as indicate in Materials and methods. Values are the average of at least three determinations (SEM was less than 6% in all cases).

Oil	Saturated Fatty Acids		Unsaturated Fatty Acids		
	14:0	16:0	ω−9 18:1	ω−6 18:2	ω−3 18:3
Safflower	7.09	2.61	14.27	75.80	0.24
Chia	6.47	2.51	6.71	21.36	63.0
Corn	–	11.23	31.90	53.85	0.36

2.3. Tumor induction by DMBA

All the animals were anesthetized with a solution of Isofluorane, through subcutaneous injection on the ventral surface of the neck at 90 days from weaning. The SMG of the experimental group were injected with one doses of 50 µl of 0.5% of 9,10-dimethyl-1,2-benzanthracene (DMBA) (SIGMA ALDRICH, USA) dissolved in acetone. The glands of the control group were injected with 0.9% acetone. The animals were sacrificed at 180days post-treatment with DMBA and their SMG was excised for biochemical and immune-histochemistry studies [17].

2.4. Plasma membrane separation and fatty acid profile

SMG cell suspensions (1×10^7) were placed in homogenization solution (hypotonic Hepes–Manitol buffer; Sigma-Aldrich), homogenized using a Polytron (7 s at setting 7), and were prepared using the same methods as our previous studies [18].

Fatty acid (FA) percentages were quantified by calculating the basis

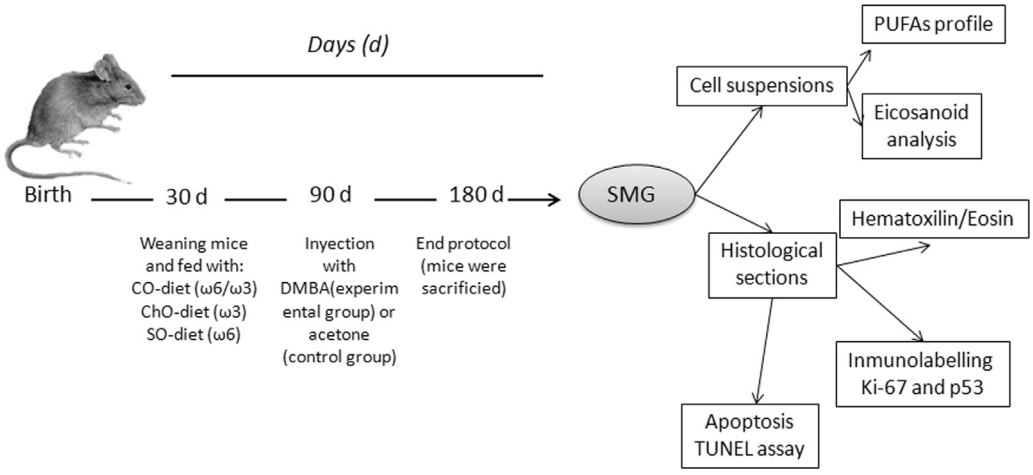

Fig. 1. A schematic experimental protocol.

of the peak area in reference to crescent concentrations of FA linear curves obtained from standards (Nu-Chek Prep) [18].

The membrane fatty acid instauration of SMG cells was expressed as Double Bound Index (DBI) and was calculated by the equation:

$$DBI = \sum (UFA*DB)/SFA$$

Where UFA (unsaturated fatty acid) is the percent composition of each UFAs, DB is its respective number of double bonds and SFA (saturated fatty acid) is the percent composition of SFAs.

2.5. PUFA metabolite analysis from SMG cell suspensions

We made determinations based on 5–8 samples of SMG cells (10^7cells/mL) each treatment. Cells were washed twice and suspended in phosphate-buffered saline with Ca^{2+} and Mg^{2+}. SMG cells were stimulated with calcium ionophore A23187 (2 μM) for 15 min at 37 °C. Metabolites were extracted using a STRATA C-18 cartridge (1 mL) and detected by reverse-phase high-performance liquid chromatography (HPLC) and following the protocols described by Comba et al. [7].

UV absorbance at 235 nm of the arachidonic acid(AA) metabolites,12(S)-HHT generated by the COX pathway,and12(S)-HETE, 15(S)-HETE and 5(S)-HETE generated by the LOX pathway, and the 13(S)-HODE from LA-LOX pathway. Quantifications of LOX/COX metabolites were obtained by commercial standard curves (Biomol International LP., Plymouth Meeting, MA, USA). The eicosanoids were expressed as percentage of 10^7 SMG cells. A C18Phenosphere-Next column (5 μm; 4.6 × 250 mm) (Phenomenex, Inc. USA) was used for high-performance liquid chromatography (HPLC) analysis

2.6. Histological analysis

For anatomopathological analysis of SMG, the tissues were fixed in 10% formalin buffered with hydrochloric acid-acetyl pyridine pH 7, processed for paraffin embedding, sectioned and stained with hematoxylin-eosin. The SMG sections were examined in an Olympus BX50 microscope and the histological images were digitalized with a SONY camera and Pro-plus software and morphologically described according to the World Health Organization (WHO) [19].

2.7. Immunocytochemistry

Immunocytochemistry of IgG anti-TP53 (CALBIOCHEM, USA) and anti-Ki-67 (DAKO, USA)was performed according Busamia et al. [20]. Immunolabeling was evidenced by3,3'-diaminobenzidine(DAKO, USA). The SMG sections were counterstained with Harris' hematoxylin and mounted in Entellan (Merck). The negative control was performed by replacing the primary antibody with phosphate buffered saline. Positive control samples were mouse mammary carcinoma. The immunostaining was considered negative when observed in 0–5% positive cells. The analysis of the samples was performed by a single blind examiner. The positive immunostaining was counted for each experiment and expressed relative to the total number of nuclei in an area of 5.10^{-3} mm^2. Values represent the median value of three independent samples. In each sample, five random fields were measured. The sections were analyzed by optical microscopy at a magnification of 400 X [15].

2.8. Apoptosis assays

The degradation of DNA was evaluated by TUNEL following manufacture protocol of Dead End Colorimetric Tunel System (Promega, Argentina) on tissue section of SMG embedded in paraffin. The number of apoptotic nuclei was counted for each experiment and expressed relative to the total number of nuclei in an area of 5.10^{-3} mm^2. Values represent the median value of three independent samples divided into five sections each. The sections were analyzed by optical microscopy at a magnification of 400 X.

2.9. Statistical analysis

The variation of amounts of lipids and immunolabeling of TP53 and Ki-67 and apoptotic nuclei were analyzed by the Kruskal Wallis test (non parametric ANOVA) with paired comparison *a posteriori* and a generalized linear model with random component gamma, canonic link function (covariates) was used to evaluate the significance for diets, eicosanoids, and control or with DMBA treatment [18]. A P-value of < 0.05 was set for rejection of the null hypothesis. The nonparametric methods are used because non-Gaussian distribution was proven. Data were analyzed with free R software (www.r-project.org).

3. Results

3.1. Tumor induction

The total number of animals at beginning of the experiment was 71 (CO n = 24 (14 controls; 10 DMBA)); ChO n = 25 (13 controls; 12 DMBA); SO n = 22 (10 controls; 12 DMBA), all survived until the time of sacrifice except in animals fed with SO, where three deaths were reported in both groups (3 controls and 3 DMBA). At 3–4 months post-DMBA injection, 80,7% (25/31 lives) developed similar characteristics, such as to pre-neoplastic changes in the neck region; and exhibited significant congestion in the region of the surgical incision. Hypertrophic lymph nodes were observed around the glands. The animals fed with the SO diet presented notorious pre-neoplastic morphologic changes (Fig. 2). And they did not shows external signs (alopecia, weight loss, cachexia, low mobility, peeling, etc.) of deficiency in essential fatty acids. No macroscopic or histological neoplastic changes were observed in the SMG of control animals.

3.2. Profile of Fatty Acid in SMG cell membranes

Animals fed with all diets and injected with DMBA showed significantly less SFA and unsaturated ω−7 and ω−9 fatty acids than animals in the control group; in contrast, most of ω−3 and ω−6 PUFAs increased in all DMBA-mice dietary groups with respect to the control group. A significant increase was observed between the mean percentages of Docosapentaenoic Acid (DPA) 22: 5 n-3, in the SO diet in animals treated with DMBA respect to Control. Interactions between diets and DMBA treatment were analyzed statistically, but non-significant interactions were observed (Tables 2, 3, and 5).

When analyzing the effects of the different diets in each group (control and DMBA) the following results were observed; A) a significant increase of this 18: 0 in the ChO diet animals injected with DMBA (14.97 ± 2.78; p = 0.0210, Kruskal Wallis Test; B) a highly significant increase of AA (p = 0.0595, Kruskal Wallis Test) in safflower diet (58.74 ±) and maize (61.37 ±) in relation to fed with chia diet (1.17 ±) in animals injected with DMBA.

A significantly lower DBI (2.08; p = 0.027) was observed with respect to the other two diets in animals injected with DMBA and fed with Chia diet. On the other hand, the SO (15.27, p = 0.0357 Kruskal Wallis Test) and CO (88.63; p = 0.0025 Kruskal Wallis Test) diets showed a higher DBI with respect to their corresponding controls of each diet (Table 4).

A minor non significant median value ω−6 /ω−3 ratio was seen in control (8.97) and DMBA (2.4) animals fed with ChO in comparison with other dietary treatments.

3.3. Eicosanoids

The percentage of 12(S)-HHT, 12(S)-HETE and 5(S)-HETE was significantly increased on DMBA-injected animals fed with ω−6 enriched diets (CO and SO) with respect to control groups. The 13(S)-HODE and

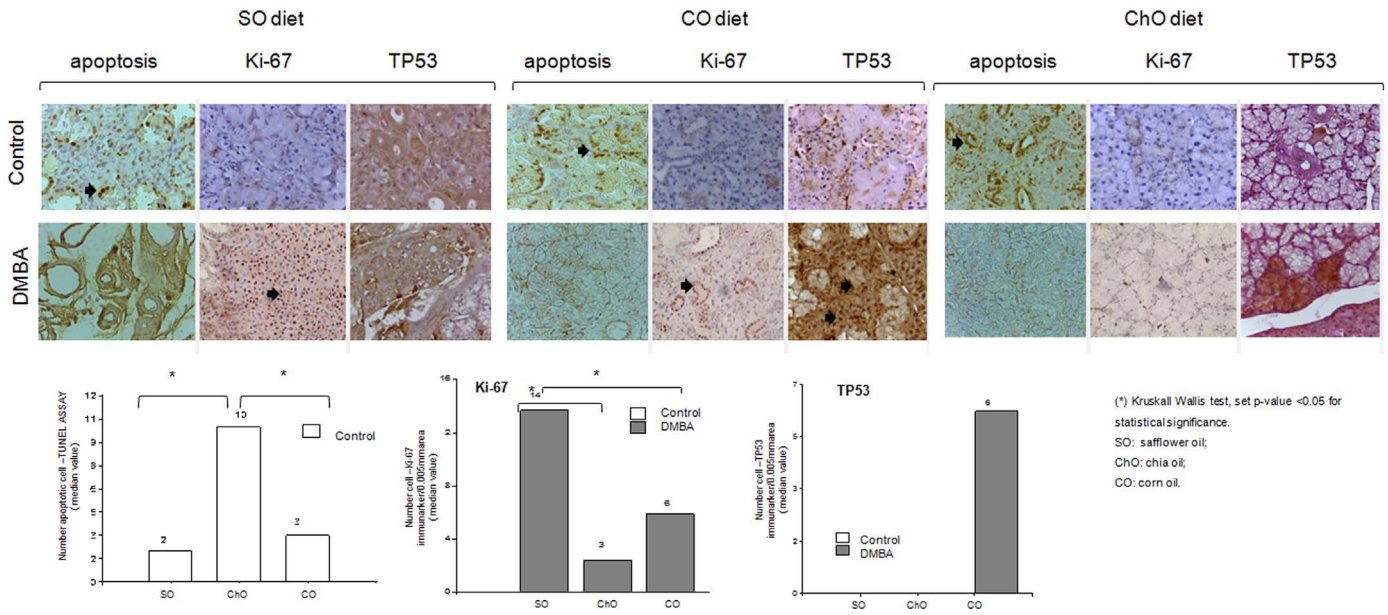

Fig. 2. Immunolabeling of mouse submandibular gland fed with different diets and control/DMBA group. The black arrow indicates positive immunolabeling of nuclei. The immunostaining was considered negative when observed 0–5% positive cells. The analysis of the samples was performed by a single blind examiner. The positive immunostaining was counted for each experiment and expressed. Barrs represent total number of nuclei in an area of 5.10^{-3} mm^2. Values represent the median value of 3 independent samples divided into 5 sections each. The sections were analyzed by optical microscopy at a magnification of 400 X. The symbols * indicate statistical significance, $p < 0.05$.

15(S)-HETE significantly increased in DMBA injected animals fed with CO diets compared to the control group (Table 5).

3.4. Apoptosis

The apoptotic cells evaluated by TUNEL were only observed in the control group; and the apoptotic nuclei were significantly higher on ChO fed mice compared to SO and CO diet (Fig. 2).

3.5. Immunolabeling

The Ki-67 and TP53 immunolabeling was only detected in the DMBA group. The SO diet showed significantly highest Ki-67 immunolabeling in relation to the CO and ChO groups (Fig. 2). On the other hand, the TP53 immunolabeling was only observed in animals fed with CO (Fig. 2);

Table 2
Saturate fatty acid percentages of mice SMG cell membranes were quantified by calculating the basis of the peak area in reference to crescent concentrations of FA linear curves obtained from standards. SO: safflower oil; ChO: chia oil; CO: corn oil. (*) Kruskall Wallis test. Bold, italicized letters, and highlighted in gray indicate statistical significance for p-value < 0.05 between control and DMBA for each diet. Each value is reported as the mean ± SE and median of the five independent experiments. All data is representative of five separate experiments.

Saturated Fatty Acids	***Diet***	**Control**			**DMBA**			***P-value(*)***
		Average	*SE*	*Median*	*Average*	*SE*	*Median*	
14:0	**SO**	**1.85**	**0.46**	**2.11**	**0.07**	**0.04**	**0.07**	***0.0357***
(myristicacid)	ChO	1.33	0.64	1.23	0.65	0.82	0.65	0.6000
	CO	0.77	1.33	0.001	0.04	0.02	0.03	0.6000
16:0	**SO**	**23.75**	**1.80**	**23.22**	**2.07**	**0.86**	**2.00**	***0.0357***
(palmiticacid)	ChO	28.03	1.22	28.15	15.55	19.02	15.55	0.8000
	CO	27.53	7.63	24.16	1.03	0.05	1.01	0.1000
18:0	SO	9.49	0.89	9.12	7.04	9.35	0.32	0.7857
(stearicacid)	ChO	18.02	9.58	13.23	14.97	2.78	14.97	0.9999
	CO	13.17	5.12	15.38	0.003	0.003	0.01	0.1000
24:0	SO	0.71	1.23	0.001	0.05	0.08	0.00	0.7143
(lignocericacid)	ChO	0.001	0.001	0.001	0.22	0.31	0.22	0.9999
	CO	1.32	1.14	1.96	0.001	0.001	0.001	0.1000

Table 3
Unsaturated fatty acid percentages of mice SMG cell membranes were quantified by calculating the basis of the peak area in reference to crescent concentrations of FA linear curves obtained from standards. SO: safflower oil; ChO: chia oil; CO: corn oil. (*) Kruskall Wallis test. Bold, italicized letters, and highlighted in gray indicate statistical significance for p-value < 0.05 between control and DMBA for each diet. Each value is reported as the mean ± SE and median of the five independent experiments. All data is representative of five separate experiments.

Unsaturated Fatty Acids		*Diet*	*Control*			*DMBA*			*P-value(*)*
			Average	*SE*	*Median*	*Average*	*SE*	*Median*	
	PA 16:1	**SO**	**5.04**	**0.86**	**5.2**	**0.03**	**0.07**	**0.002**	***0.0357***
ω-7		ChO	8.1	4.27	9.02	1.81	2.3	1.81 [a]	0.3000
		CO	3.2	3.12	1.87	0.00	0.00	0.00	0.1000
	OA18:1	SO	19.98	1.71	19.68	13.39	8.65	17.99	0.2500
		ChO	14.63	8.95	18.49	12.05	8.42	12.05	0.6000
ω-9		CO	19.52	1.61	18.88	11.83	0.19	11.8	0.1000
	24:1	SO	0.0006	0.000	0.001	1.25	1.12	1.74	0.1429
		ChO	0.001	0.000	0.001	0.00	0.00	0.00	0.1000
		CO	0.0007	0.000	0.001	0.19	0.11	0.12	0.1000
	LA 18:2	SO	19.65	2.96	20.86	14.59	13.49	11.09	0.7857
		ChO	9.81	4.36	7.87	17.83	4.00	17.83	0.2000
		CO	17.2	2.62	18.13	17.22	0.4	17	0.7000
ω-6	GLA 18:3	SO	0.001	0.000	0.001	0.09	0.11	0.04	0.6964
		ChO	0.4	0.69	0.001	25.69	36.34	25.69	0.6000
		CO	0.08	0.07	0.11	0.00	0.00	0.00	0.1000
	AA 20:4	**SO**	**0.0006**	**0.0006**	**0.001**	**58.74**	**6.96**	**57.99**	***0.0357***
		ChO	0.001	0.000	0.001	1.17	1.17	1.17	0.1000
		CO	0.0007	0.0005	0.001	61.37	3.08	61.08	0.1000
ω-3	ALA 18:3	SO	0.001	0	0.001	0.08	0.13	0.05	0.6964
		ChO	6.8	1.58	5.96 [a]	3.3	3.82	3.3	0.6000
		CO	0.03	0.03	0.02	0.02	0.01	0.02	0.9999
	EPA 20:5	SO	0.0003	0.0005	0	0.12	0.17	0.07	0.1425
		ChO	0.82	0.39	0.7 [a]	0.79	0.29	0.79 [a]	0.9999
		CO	0.08	0.13	0.001	0.36	0.08	0.35	0.1000
	DPA 22:5	**SO**	**0.0003**	**0.0005**	**0.000**	**5.45**	**10.82**	**0.95**	***0.0357***
		ChO	0.7	0.7	0.71	0.5	0.71	0.5	0.6000
		CO	0.14	0.25	0.001	0.08	0.07	0.1	0.9999
	DHA 22:6	SO	0.1	0.18	0.00	2.46	2.27	1.76	0.7142
		ChO	1.48	1.29	2.04	1.00	1.41	1.00	0.6000
		CO	0.54	0.93	0.001	4.03	0.06	4.01	0.1000

4. Discussion

In this work, we have demonstrated novel differential effects of ω−3 and ω−6 PUFAs on the regulation of early tumorigenesis in murine SMG injected with DMBA model.

In this study, a high percentage of the animals injected with DMBA in the SMG and fed with enriched diet in ω−3 and ω−6 PUFAs (75.4%) generated tissue modifications similar to initial tumorigenic stages (Fig. 2). These changes align with previous well-known activity of this polycyclic aromatic hydrocarbon [21], and were also observed in experiments of our group in Wistar rats SMG at seven days post injection with DMBA [17].

In this work, we observed that SFA, and ω−7, and ω−9 fatty acids were significantly decreased in animals treated with DMBA with respect to controls. Contrary almost ω−6 and ω−3 PUFAs were significantly increased in DMBA-mice in comparison to controls. These changes could be explained by the action of polycyclic aromatic hydrocarbons (PAHs) such as benzo(a)pyrene. Other research has observed that PAHs

Table 4
Percentage of saturated fatty acids (SFA), unsaturated fatty acids (UFA), and Double Bound Index (DBI = ∑ (UFA*DB)/SFA) of mice SMG cell membranes were quantified by calculating the basis of the peak area in reference to crescent concentrations of FA linear curves obtained from standards. SO: safflower oil; ChO: chia oil; CO: corn oil. (*) Kruskall Wallis test. Bold, italicized letters, and highlighted in gray indicate statistical significance for p-value < 0.05 between control and DMBA for each diet. Each value is reported as the mean ± SE and median of the five independent experiments. All data is representative of five separate experiments. DB is its respective number of double bonds.

Diet	Control			DMBA			P value(*)
	DBI	SFA	UFA	DBI	SFA	UFA	
SO	2.38	47.38	62.99	15.27	9.22	14.80	*0.0357*
ChO	1.33	43.59	97.83	2.08[a]	30.98	64.43	*0.0500*
CO	2.24	36.5	86.87	88.63	1.07	95.12	*0.0025*

affect processes including cell proliferation and communication, and induce apoptosis by remodeling cell membrane micro-domains due to intracellular alkalization and regulation of lipid biosynthesis [22].

A high percentage of AA was observed in SMG cell membranes in animals injected with DMBA in the same diets. These findings may be explained due to DMBA action, which induced an alteration of gene expression of Δ6 and Δ5 desaturase enzymes that would have led to an increase of this PUFA. These results are consistent with other studies on the modulation of c-erbB 1/EGFR and c-erbB-2/neugene expression in DMBA-induced mammary tumorigenesis in Sprague Dawley rats fed olive and corn oil-enriched diets, in which an increment in gene expression of pro-carcinogenic genes was observed in corn oil diet-fed animals (rich in ω−6) [23,24]. Previous studies from our laboratory, using diets enriched in ω−6 PUFAs, showed increased AA in mammary tumor cell membranes [10]. It is well-known that AA plays an important role in inflammatory processes, particularly in those associated with diseases such as cancer. Usually, the AA acts as substrate of enzymes involved in the production of eicosanoids with proinflammatory and pro-carcinogenic activity [10]. Other research on in mammary tumors of animals fed with corn oil and treated with DMBA have shown an increased incidence of tumors, suggesting that high ω−6 PUFA content stimulates their development [25]. In addition, several studies have recognized the aberrant metabolism of AA as an important factor in carcinogenesis, finding a COX-2 over-expression in premalignant and malignant oral cavity lesions in patients with oral cancers [26].

On the other hand, in concordance with our results, an experimental study in SMG of rats showed that the composition of phospholipids and triglycerides was modified in the animals fed with ω−3 PUFAs. This increment in the cell membranes of SMG could be explained by changes in the levels of DPA C22: 5 ω−3 and DHA C22: 6 ω−3 PUFAs related to increased Δ9-desaturase activity [27].

In relation to eicosanoid PUFAs derivate results, we observed that in animals injected with DMBA and fed with SO and CO diets rich in ω−6, the SMG cells increased the production of 12(S)-HHT, 12(S)-HETE and 5(S)-HETE with respect to the control (Table 2). This is consistent with our previous studies in cells from mammary tumor mice fed with the same PUFAs diets, showing that CO and SO cause modifications to the percentages of eicosanoids mentioned above [24].

Table 5
Eicosanoid composition of SMG of mice fed with different oils and treated/not treated with DMBA. (*) p-.value estimated by generalized model with random-component gamma. SO: safflower oil; ChO: chia oil; CO: corn oil. Bold, italicized letters, and highlighted in gray indicate statistical significance for p-value < 0.05 between control and DMBA for each diet. Each value is reported as the mean ± SE and median of the five independent experiments. All data is representative of five separate experiments.

Eicosanoid	Diet	Control			DMBA			P group(*)
		Average	SE	Median	Average	SE	Median	
12(S)-HHT	SO	0.48	0.61	0.28	0.00	0.00	0.00	*0.0001*
	ChO	0.42	0.14	0.44	0.00	0.00	0.00	
	CO	0.28	0.44	0.00	1.52	2.63	0.00	
13(S)-HODE	SO	0.69	1.26	0.09	0.00	0.00	0.00	*0.0001*
	ChO	1.53	1.11	1.78	0.31	0.44	0.31	
	CO	0.21	0.34	0.00	1.22	1.19	1.29	
15(S)-HETE	SO	0.69	1.02	0.30	0.00	0.00	0.00	*0.005*
	ChO	0.91	1.14	0.54	0.11	0.16	0.11	
	CO	1.22	1.81	0.17	0.50	0.61	0.33	
12(S)-HETE	SO	0.10	0.18	0.02	1.02	0.10	1.02	*0.0001*
	ChO	0.34	0.29	0.29	0.00	0.00	0.00	
	CO	0.51	0.40	0.48	0.60	0.61	0.58	
5(S)-HETE	SO	0.01	0.01	0.01	0.00	0.00	0.00	*0.0001*
	ChO	0.04	0.09	0.00	0.00	0.00	0.00	
	CO	0.17	0.16	0.19	0.75	1.30	0.00	

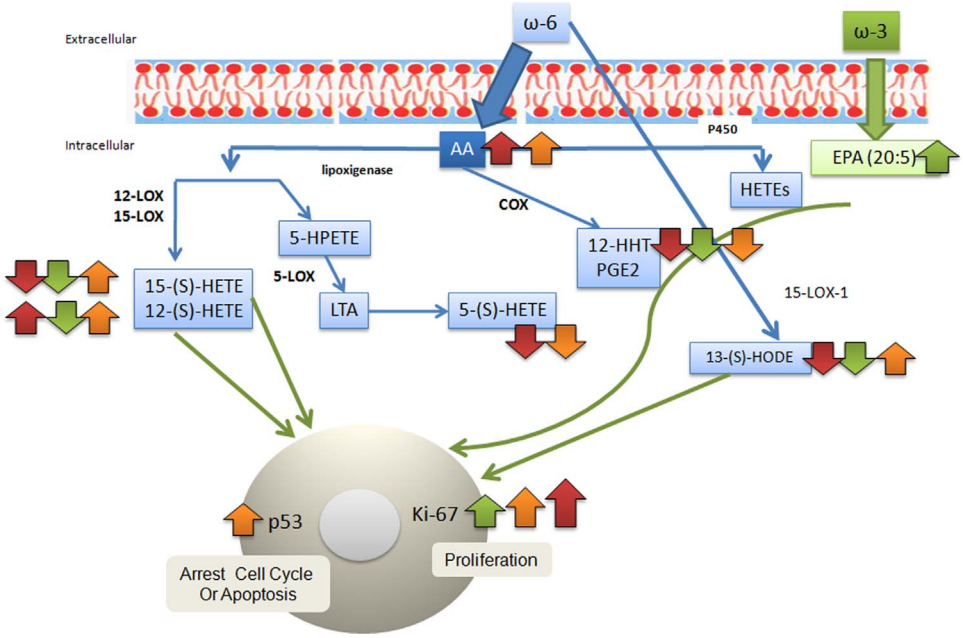

Fig. 3. Expression of SMG cells of mice fed with SO, ChO and CO diet and injected with DMBA. The arrows indicate PUFAs, Eicosanoids and Immuno markers that increased or decreased in relation to control group. Green arrow: ChO diet; orange arrow: CO diet; red arrow: SO diet. The drawn cell (below) shows the outcome of pathway of PUFAS. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Similarly, we also observed the highest positive Ki-67immunostaining in the same sample tissues of SMG belonging to DMBA-mice fed with ω−6 PUFA diets (Fig. 2). These are consistent with results of other researchers, who found high Ki-67immunostaining and high activity of 12(S)-HETE in undifferentiated squamous cell oral mucosa carcinoma, mucoepidermoid, adenoid cystic, acinic cell and salivary duct carcinomas, among others, with respect to the normal tissues [28,29]. A study on prostate tumor cell lines showed that tumor growth rate, final volume and PSA serum levels, and Ki-67 immunostaining increased in groups of animals fed with ω−6 [30].

In addition, we also observed high expressions of TP53 in DMBA-animals and fed with the CO diet. This could be explained by the high AA concentration observed in SMG cell membranes in these animals, which could trigger the TP53 gene expression and lead to the tumor cell cycle arrest [26].

In contrast to cell proliferation events, in our study, the score of apoptotic cells was significantly higher in SMG in control animals that were fed with the three diets with respect to DMBA groups. The control mice fed with ChO presented higher numbers of apoptotic cells than other dietary treatments. These results are consistent with our other studies, which have demonstrated that animals fed with ChO increased the number of apoptosis and diminished the number of mitotic cells [6]. Other authors have observed similar results, suggesting that the dietary intake of PUFAs ω−3 slows down tumor growth because of the change in the ω−6/ω−3 ratio, and promotes apoptosis and inhibits proliferation by direct competition of PUFAs ω−6 of enzyme activity of 5-LO-1 and COX-2 [30]. Several investigations have explained the basis for the protective effect of ω−3 PUFAs. They have suggested that DHA plays an important role in the metabolism of a carbon, influencing global methylation leading to a protective effect on diverse cancers [31]. In addition, it has been suggested that changes in the DNA methylation levels induce an imbalance of folate and Vit B12, modulated by ω−3 PUFAs. Other experimental studies on colon cancer in rats evaluated the effect of ω−3 PUFAs and suggesting a new protective role related to deregulate mechanisms of micro RNA associated with the induction of carcinogenesis [32].

An important issue is the ω−6/ω−3 ratio: in our study, we observed a non-significant minor value of the ω−6/ω−3 ratio in mice fed ChO, regardless of whether they had been treated with DMBA. Experimental in vitro or in vivo studies have demonstrated that the balance between PUFAs ω−6/ω−3 impact in tumor growth [29]. The increment of 15(S)-HETE, 12(S)-HETE and 13(S)-HODE in the corn diet could explain: this oil is enriched with ω 6 and ω 3 and its ratio stimulates the over expression of15 LOX 2, 12 LOX and 15 LOX 1 respectively, by generating a less oxidative environment and modulating LOXs activities [33,34].

In Fig. 3 we summarize the results and their relationship among diet compounds and the processes involved in early SMG tumorigenesis in DMBA-mice. We conclude that: a) animals fed with the ChO diet increased ALA and EPA; b) in contrast, AA significantly increased in mice fed with SO and CO diets; c). the 12(S)-HETE increased in SO and CO diets, 15(S)-HETE, and 13(S)-HODE increased in CO and ChO diets; d) Ki-67 was highest in mice fed with SO and CO with respect to ChO, and the increment of AA generates a high amount of 12(S)-HHT, 12(S)-HETE, and 5(S)-HETE, pro-carcinogenic eicosanoids.

5. Conclusion

The Chia oil (*Salvia hispanica*) rich in polyunsaturated fatty acids ω−3 causes changes in the fatty acid profile of the cell membranes of SMG reducing the carcinogenic action of DMBA respect to a diet rich in ω−6 fatty acids. This diet decrease the production of 12(S)-HETE, a pro-carcinogenic metabolite. This beneficial effect of ω−3 was shown through the decreased of cell proliferation in cells from the glands of mice fed chia diet.

Taken together, the results of this work show that natural nutrients such as PUFAs and their ω−6/ω−3 ratio could modify the action of carcinogenic substances and reduce the risk of head and neck cancer by increasing dietary intake of ω−3. Understanding the mechanisms behind this ratio may provide a new vision into the development of new prevention strategies or treatments for head and neck cancer.

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Author contributions

PME and BM contributed conception of the experiments, to experiments, data analysis and writing. SME, RG, GMI and MG contributed to the experiments, and revised the manuscript. All authors have approved the final version of the article.

Conflicts of interests

The authors declare no conflict of interest.

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