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Omega-3 Polyunsaturated Fatty Acids Supplemented Diet and its preventive effect on tumor growth in nude mice

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ABSTRACT

Cancer is one of the leading causes of morbidity and mortality worldwide according to the WHO. Strong evidence suggests that food and nutrition are important in modification of cancer process. There is increased evidence that specific dietary patterns or constituents such as n-3 PUFAs, may be associated with reduced risk of BC. Female athymic nude mice were fed since weaning to adulthood with a DHA+EPA (4%w/w) diet or with standard diet during 14-week. At week-7, tumor cell implantation with MDA-MB-231cell line took place, each mice received 5x106 tumor cells. When tumors became palpable, maximum length, width, and diameters were measured twice a week. Incidence of tumor development was observed in all mice (n=4, 100%) in the control diet group; instead, the n-3 PUFAs diet group, only two mice developed tumor (n=2, 50%), and the development time was longer compared to the control group. These differences were significant ($p < 0.001$). This study establish the potential effect as a preventive measure to BC. This evidence is consentient with epidemiological data about high n-3 PUFAs diet patterns in some populations may lower risk of BC, highlighting the importance of these components in our diet since childhood to promote the preventive effect. Being thus necessary, setting up recommendations for n-3 PUFAs supplementation from fish oil or, for a minimal dietary fatty fish intake/week, in order to attempt modulate carcinogenesis in populations at high risk, particularly those with a high prevalence of obesity.

KEYWORDS

Tumor growth, n-3 PUFAs, cancer prevention.

ABBREVIATIONS

BC: Breast Cancer.

EPA: Eicosapentanoic Acid.

DHA: Docosahexanoic Acid.

INTRODUCTION

Cancer is one of the leading causes of morbidity and mortality worldwide; and according to the World Health Organization[1,2]. This disease is not only polygenic, but multifactorial, altering cell proliferation and cell death[3]. Great progress has been made in the understanding of the cancer process; epidemiological, clinical, and basic studies have contributed to the identification of carcinogens and host conditions that modify susceptibility to cancer. However, strong evidence suggests that food and nutrition are important in modification of the cancer process. Breast cancer (BC) is one of the most important attributable cancers to modifiable risk factors such as obesity, altered body composition, poor physical activity, diet, among others[4,5]. By 2020, it could reach the first place in morbidity due to neoplasias, besides being the most prevalent cancer among women and the primary cause of global female cancer deaths. The great impact that BC has on health in women has conditioned health programs to focus largely on the early identifica-

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tion and prevention of this type of cancer[6]. There is increased evidence that specific dietary patterns or dietary constituents may be involved in the development of cancer or protect against it; however, some studies suggest that diets with high amounts on n-6 polyunsaturated fatty acids (PUFAs) are associated with alterations in lipid metabolism, related to tumor progression and cancer cell survival. In contrast, a high intake of dietary n-3 PUFAs, may be associated with a reduced risk of BC and regulation of cell survival and proliferation pathways. Epidemiological data and *in vitro / in vivo* studies have suggested beneficial effects of n-3 PUFAs on BC[7-10]. The aim of this study was to determine the effect of an enriched diet with n-3 PUFAs: Docosahexanoic Acid (DHA) and Eicosapentanoic Acid (EPA) affects tumor prevention and / or tumor growth parameters using athymic female nude mice as an *in vivo* BC model.

MATERIALS AND METHODS

Study: experimental.

Animals: Female athymic nude mice (Foxn1nu). Animals were maintained in microisolator cages within a pathogen-free isolation facility (environment with positive air pressure/ventilation in corridors and rooms, maintaining pressure gradients); temperature from 20 to 25 ° C, environmental relative humidity between 40 and 70% and subjected to 12 hours dark/light cycles. All procedures were performed in a unidirectional laminar airflow hood. The animals were divided in two groups: control group (n= 4) and n-3 PUFAs Diet group (n= 4) DHA + EPA (540mg + 360mg. respectively) They were fed immediately after weaning, either with the supplemented diet or with standard diet. All the animals completed the 14-week study protocol. Body weight (BW) was measured twice a week and diet consumption was determined every day. They did not receive any other surgical or hormonal manipulation. All protocols were elaborated according to the guide Care and Use of Laboratory Animals (Institute of Animal Laboratory Life Resources Commission Sciences National Research Council). And approved to the Institutional Committee for Biomedical Research in Humans and Animals of our institute: Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán (INCMNSZ).

Diets: The experimental diet was prepared fresh every day at the INCMNSZ bioterio with the intention of reducing organoleptic changes and alterations in the fatty acid. It was based on the Mazuri® Rat and Mouse Diet, Purina Mills, LLC. Experimental diet was supplemented with DHA + EPA (4% w/w). Diets were isocaloric with 3,41 kcal/g for the control diet and 3.43 kcal/g for experimental diet. EPA and DHA were supplied as ethyl esters by General Nutrition Center (Pittsburgh, Pensilvania USA). Fresh sterile diet was provided daily and autoclaved drinking water was provided *ad libitum*. It was verified that diet was pleasant to the palate and digestible. As well as the safety of preparation, verifying infections, evacuations and weight.

Cell Culture and Treatment: Cells from the human breast cancer triple negative cell line MDA-MB-231, obtained from the American Type Culture Collection. Cultured in RPMI 1640 (Gibco) and supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco), 100 IU/mL penicillin, 0.1mg/mL streptomycin (PS) and 0.3mg/ml (2mM) L-glutamine (Sigma). Cells were incubated at 37°C in a humidified incubator with 5% carbon dioxide.

Experimental Procedure: At baseline randomization method was used to assign the hole cage, each containing four 19-21 days (3 week) old mice to the control or supplemented diet. Mice received the respective diet for 6 weeks to allow dietary fatty acids to be incorporated into tissue lipids. At week 7, tumor cell implantation with MDA-MB-231 cell line took place, each mice received 5x106 tumor cells sterile suspension in PBS for inoculation (100 uL of cell suspension) into the right subscapular region using a 31GA needle.

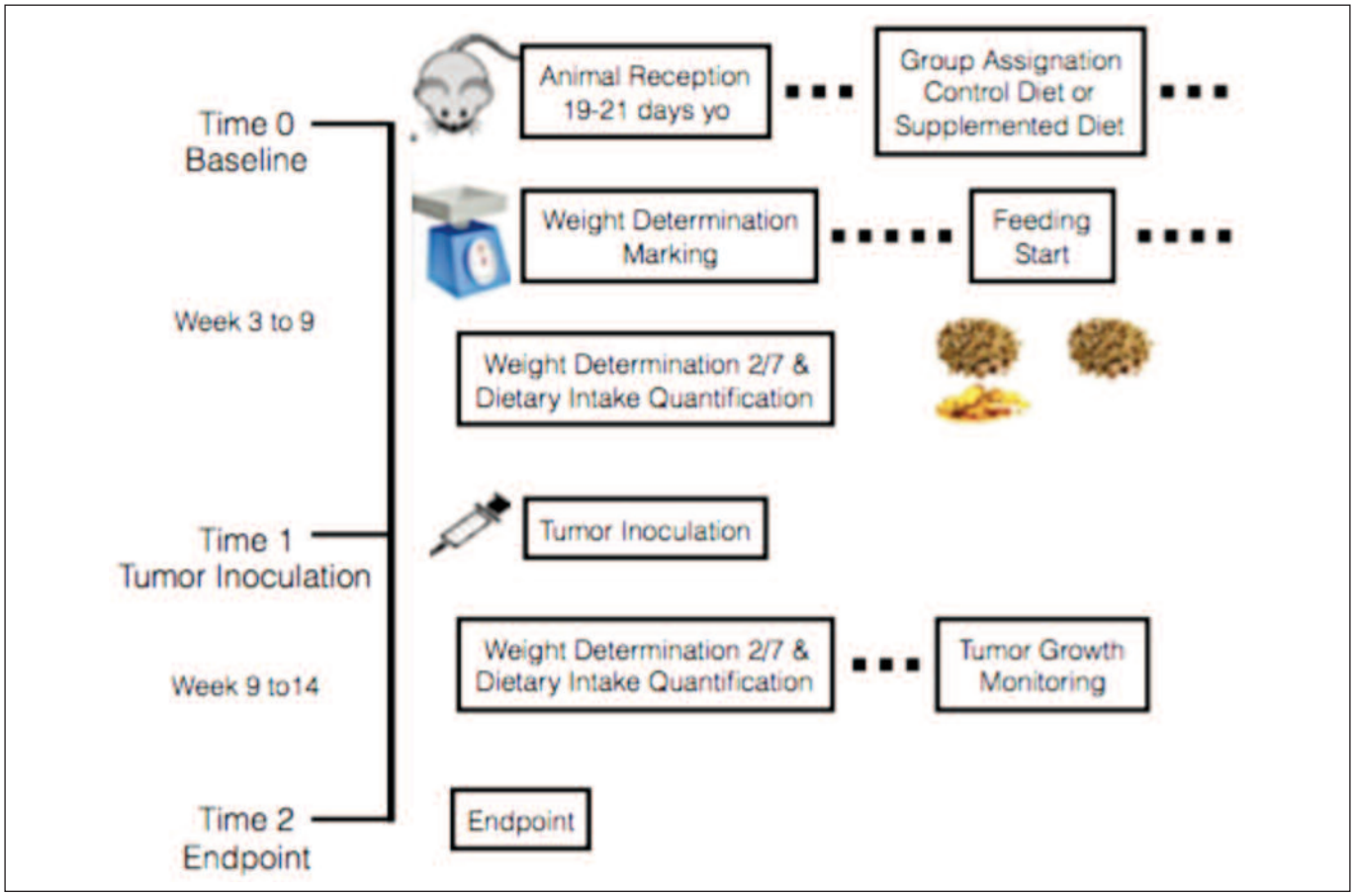
Selection of 5x106 tumor cells for inoculation was based on our pilot study showing 100% tumor development and a reasonable latency period for this cell line (data not shown). It is important to mention that inoculation technique was validated by achieving 100% of mice tumor development before starting this experiment. When tumors became palpable, their maximum length, width, and perpendicular diameters were measured with a vernier caliper twice a week, and tumor volumes, calculated until completion of the study as a sphere using L*W2/2 formula. The experiment was terminated 35 days after inoculation of tumor cells. When applicable, endpoint criteria were applied. Weight was measured twice a week and food amount was measured daily during the whole study (Figure 1). Sample size was determined in order to detect at least a 50% difference between groups.

Statistical Analyses: Descriptive statistics was performed using mean values and ± SEM. Dichotomous variables were expressed as frequencies and percentages. The volume of the control group tumors was compared with the volume of the experimental group tumors using Student's. The tumor volume of each group will be compared in their different times of tumor development through Student's t-test for paired samples. The probability curves of survival (tumor-free) were calculated by the Kaplan-Meier method and compared by the log-rank test, obtaining mean ± SE and CI95%. Values for $P < 0.05$ were considered significant data was analyzed using SPSS for Windows (version 20.00; SPSS Inc., Chicago, IL).

RESULTS

Eight mice were studied from their third week of life, moment from which the feeding with control diet or with the supplemented diet began (Time 0). A discrete increase in weight gain was seen between groups at week 12 (three weeks after MDA MB 231 cells inoculation = Time 1). Since that week, greater weight gain was observed in the control

Figure 1. Experimental Procedure.



diet group compared to n-3 PUFAs Diet group (33.85g ± 1.31g vs 27.47g ± 1.05g) as we can observe in week 14 (Figure 2). Incidence of tumor development was observed from day 11 after inoculation (n=2) in the control diet group, and the other 2 mice from this group developed tumor at day 14 (n=4, 100%). Instead, the n-3 PUFAs Diet group, only two mice developed tumor (n=2, 50%), and the development time was longer compared to the control group (day 17) (Day 11 *versus* Day 17) These difference were significant p value < 0.001 (Image 1). In addition, the group that consumed n-3 PUFAs diet, developed smaller tumors in comparison with the group of mice that consumed the control diet; this difference was statistically significant with a p value of 0.029, from week 9 until the time of sacrifice at week 14. (Figure 3). with a volume of 1.24 ± 0.72 mm3 vs 5.54 ± 1.54 mm3. The n-3 PUFAs diet group have significative more tumor-free days from inoculation day, we find significant differences between control diet group with a mean of 12.5 days ± 0.86 (95% CI 10.80 - 14.19) vs n-3 diet group mean of 26 days ± 4.5 (95% CI 17.1-35) p value of 0.008.

Figure 2. Weight gain from time 0 (3 week old mice) to time 2 (end point).

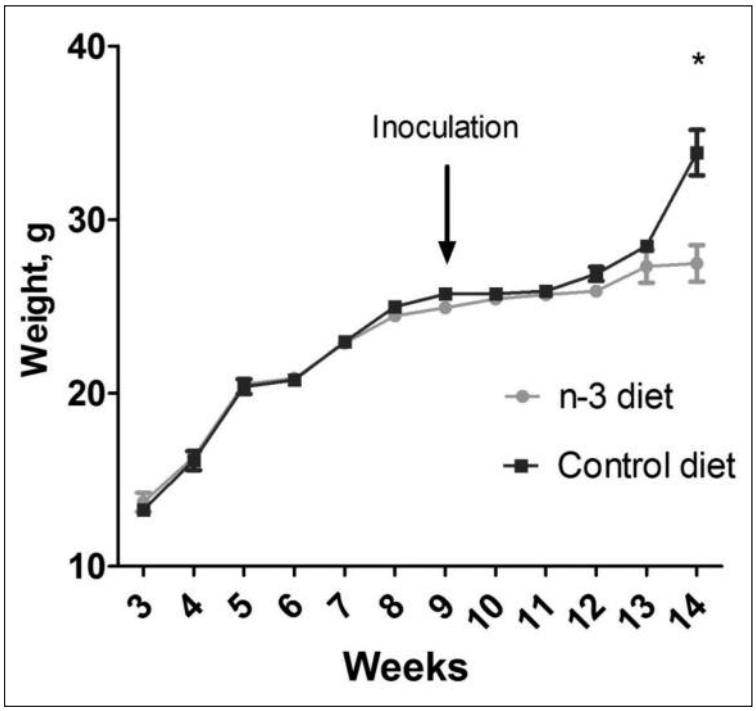
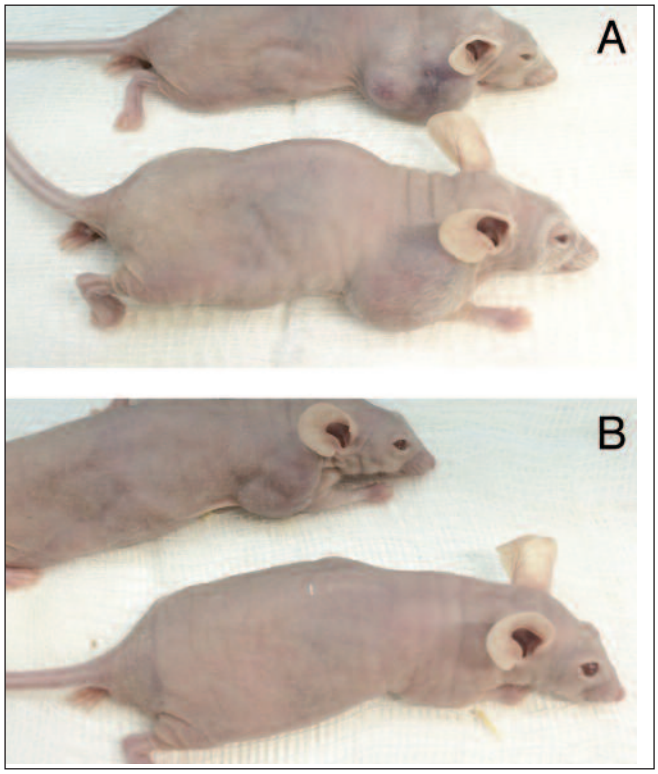
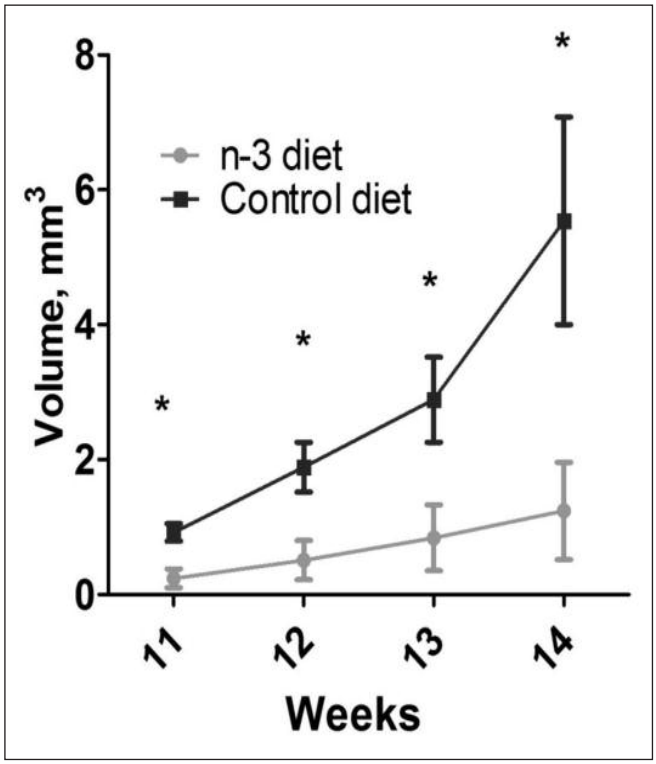


Image 1. Image 1. Tumor Incidence and Growth at week 14th (Time 2).



A. Control Diet Group. B. n-3 PUFAs Diet Group.

Figure 3. Volume progression from the first week the tumors were palpable (week 11) and measurable, to the end of the experiment (week 14).



DISCUSSION

BC remains one of the most threatening cancers between women despite the significant advancements in early detection and therapy[11]. The aim of this study, was to determine the effect of an enriched diet with DHA + EPA as a preventive measure in the development of BC. As n-3 PUFAs have been considered of great interest due to their ostensible potential to slow down BC cells growth once the disease is present. This evidence have been showed in several preclinical studies, where results showed: Reduced tumor volume and tumor burden in mice fed the fish oil diet compared to vegetable oils diets as well as inhibition of lung metastasis development; both outcomes with or without chemotherapy treatments[12-19]. The proposed and most accepted mechanisms described in the literature for this phenomenon are: their influence on cell plasma membrane composition, suppressing raft-associated cell signal transduction; antiinflammatory action, and/or affecting gene expression and signal transduction molecules, influencing cell proliferation, differentiation, apoptosis and metastasis[20-24]. Most of the existing evidence on the beneficial aforementioned effects of n-3 PUFAs in BC have been observed in experimental models, using n-3 PUFAs as a therapeutic or adjuvant measure[8]. Reason why our interest was focused on its use as a preventive and public health measure. The results of our study showed that DHA + EPA fatty acids, significantly prevented the incidence of tumor development or decreases the growth rate in a murine model when consuming an enriched n-3 PUFAs diet, since weaning up to adulthood. These results suggests that early life exposure to n-3 PUFAs diet may be a key factor for achieving the potential anti carcinogenic benefit. Previous studies have shown that DHA is a more potent inhibitor of BC than EPA[25], nevertheless, diet does not contain a single n-3 PUFAs, but several. And, since some BC risk factors are non modifiable, like age, sex, race, genetic - familial occurrence of neoplastic diseases; identification of modifiable factors and deep knowledge about nutritional aspects and lifestyle is needed to contribute to development of prevention strategies decreasing breast cancer incidence[26-29]. Since weight was no different between groups before or after cell line inoculation, but until when the tumors became palpable the difference in weight became evident, we assume there was no overweight or obesity inflammation associated in the development of tumors. Studies exploring the effects of the n-6:n-3 ratio intake, total amount, source, type, dosis and exposure time of n-3 PUFAs in cancer etiology is elemental for the development of these early prevention interventions. As well, further investigations will require to point at the role of n-6 PUFAs on their beneficial and / or harmful impact in BC risk, and measure it between different populations. It is important to take into account inter individual variations that can modify the effect of food components in human health[30-32].

CONCLUSION

In conclusion, this study establish the potential effect as a preventive measure to BC. This evidence is consentient with epidemiological data about high n-3 PUFAs diet patterns in some populations may lower risk of BC, highlighting the importance of these components in our diet since childhood to promote the preventive effect. Being thus necessary, setting up recommendations for n-3 PUFAs supplementation from fish oil or a minimal dietary fatty fish intake, in order to attempt modulate carcinogenesis in populations at high risk, particularly those with high prevalence of obesity worldwide[32-34].

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