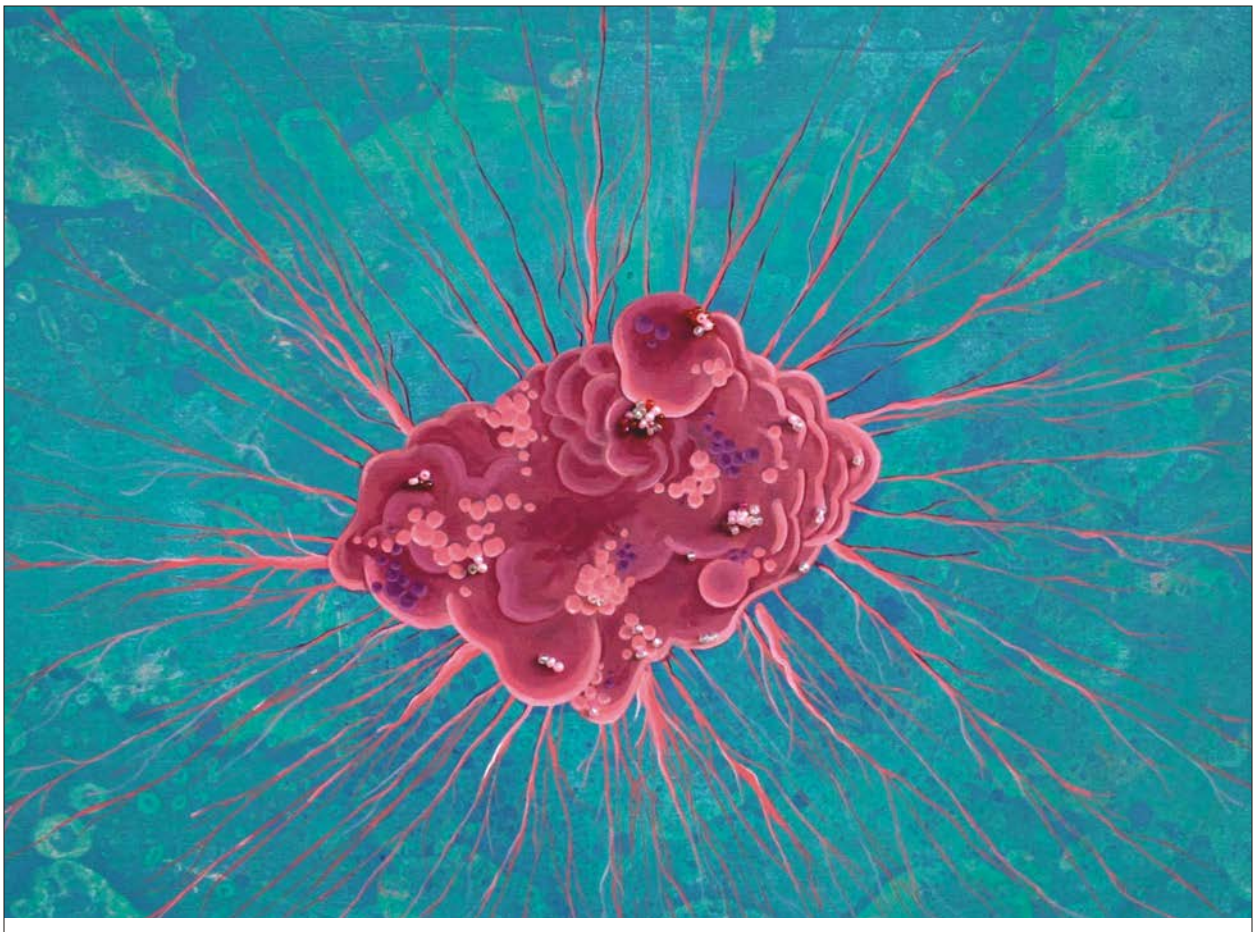

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The Influence of Olive Oil on Sprague Dawley Rats DMBA-Induced Mammary Tumors

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Abstract: The aim of the present study was to evaluate the possible protective role of olive oil on mammary carcinogenesis. Experimental studies can support epidemiologic data on the influence of some nutrients that can affect risk and prognosis of neoplastic lesions. In the present study, seventy two Sprague-dawley female rats 42 days old were equally divided in tree groups, being group C supplemented with olive oil (5%) and submitted to chemical carcinogenesis induction with 20 mg kg^{-1} of 7, 12-dimethylbenzanthracene (group B and C). At 150 days, all the animals were sacrificed and necropsy process was conducted. Animals from group A did not developed neoplastic lesions and group C showed significant differences on the number and volume of the neoplastic lesions when compared to animals from the group that was not supplemented with olive oil, it was also verified the absence of metastases in this group. The present data suggests a possible protective role of olive oil, due to its content of oleic acid and phenolic compounds, on growth and differentiation of mammary neoplastic lesions that should be confirmed on further investigation projects.

Key words: Breast, oleic acid, dietary fat, histopathology, chemical carcinogenesis

INTRODUCTION

Breast cancer is a serious public health issue affecting more than a million new cases every year worldwide. The identification of etiologic agents and their effect in the disease development is crucial to identify means to prevent the disease and improve the treatment and diagnostic methods. Epidemiologic and experimental studies identified some association between diet and breast cancer risk (Berg, 1975; Franceschi *et al*., 1995; Malin *et al*., 2003; Terry *et al*., 2001; Willett, 2001), however there is some controversy concerning the relation between dietary fat consumption as well as some specific fatty acids and the breast cancer risk (Berg, 1975; Landa *et al*., 1994; Martin-Moreno *et al*., 1994; Terry *et al*., 2003).

Excessive caloric consumption, common in industrialized countries, seems to have also a possible role on the disease development (Chlebowski, 2007; Doll and Peto, 1981; Fontana *et al*., 2006; Freedman *et al*., 1990; Iscovich *et al*., 1989; Miller *et al*., 1978;

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Shun-Zhang *et al.*, 1990). Nutritional imbalanced diets and an increased intake of lipids may have some influence on the disease burden (Welsch, 1992).

It is not clear whether the amount and the type of dietary fat can affect the risk of breast carcinoma development. This possible relation has been the subject of significant number of epidemiological and animal studies. The excessive consumption of dietary fat is recognized as a crucial factor in the increased risk of several common types of cancers (Holmes *et al.*, 2000). Studies made with animal models and humans, show that a high intake of n-6 polyunsaturated fatty acids (n-6 PUFA) stimulates several stages of development of mammary cancer (Goodstine *et al.*, 2003). In contrast, diets enriched with n-3 PUFA seem to prevent cancer (Keys, 1995; Landa *et al.*, 1994; Larsson *et al.*, 2004). Epidemiologic (Landa *et al.*, 1994; Martin-Moreno *et al.*, 1994; Trichopoulo *et al.*, 1995) and experimental (Cohen *et al.*, 2000) studies suggested that olive oil consumption is inversely associated with breast cancer. Olive oil is a dietary fat, frequently used in Mediterranean Countries and it is rich in oleic acid (OA, C18: n-9). The role of each fatty acid in animal tumor models deserve further investigation in order to solve the contradictory role of fat on carcinogenesis (Hardy *et al.*, 1997).

Olive oil it was already negatively associated with several chronic diseases including cancer and coronary heart disease (Keys, 1995). Recent data suggests that the components present in olive oil seem to have a more important role than it was previously thought (Lastra *et al.*, 2001). The high content of antioxidants, such as phenolic compounds and vitamin E (Owen *et al.*, 2000), strongly inhibits free radical production and can have a protective effect against carcinogens from several sources (Owen *et al.*, 2000, 2004). Because of the cultural role and the possible protective effects, further investigations on the association between olive oil consumption and breast cancer are needed. Experimental models can provide more information about the effect of nutritional factors on carcinogenesis. The studies of mammary carcinogenesis are among the systems were chemically induced rat models are of the most widely used for different reasons. The susceptibility of mammary gland to developed neoplasms is very high and these neoplasms closely mimic human breast disease (Costa *et al.*, 2002; Russo and Russo, 2000; Singh *et al.*, 2000). For these reasons it is purpose of this study to determine if there are some advantages in adding olive oil to evaluate possible effects on breast cancer prevention. Because this fat is generally expensive in most countries, it is important to develop extensive knowledge considering the real effect after absorption of all components of the mixture and their action in breast cancer and in possible development of metastases occurrence. This study do not pretend to expose any extensive histopathologic characterization of mammary lesions but rather evaluate the effects on tumor incidence and tumor grade of adding olive oil on a diet in the most used chemically induced breast cancer animal model.

MATERIALS AND METHODS

Animal Care

This study was conducted from January 2004 to April 2007. The experimental procedure was approved by the Veterinary Advice Commission published in the Portuguese legislation (DL 129/92) and it was conducted in strict adherence to animal care guidelines in compliance with guide for the care and use of Laboratory Animals (National Research Council, 1996). Seventy two female Sprague-Dawley rats (Charles River Laboratories, Barcelona) 42 days old were randomly assigned to three groups, housed in plastic cages maintained at 22±2°C, 55±10% humidity and with a 12 h light/dark cycle.

Table 1: Composition of the standard diet

Standard diet	Nutritional composition
Humidity	12%
Protein	15.5%
Fat	2.7%
Glucides	58.5%
Minerals	5.5%
Fibre	3.7%
Metabolizable energy	3000 Kcal kg^{-1}

Table 2: Nutritional composition of olive oil

Saturated fatty acids	**g kgG¹**
Myristic (C14)	0.1
Palmític (C16)	103.8
n-Heptadecanoic (C17)	0.9
Stearic (C18)	31.3
Arachidic (C20)	3.6
Behenic (C22)	0.9
Lignoceric (C24)	0.4
Monounsaturated fatty acids	**g kgG¹**
Palmitoleic (C16:1)	7.2
9-heptanodecanoic (17:1)	1.6
Oleic (C18:1)	760.6
Gadoleic (C20:1)	2.9
Polyunsaturated fatty acids	**g kgG¹**
Linoleic (C18:2)	60
Linolenic (C18:3)	7.2
Tocopherols	**g kg^{-1}**
α-Tocopherol	2.345
δ-Tocopherol	0.0238
Total polyphenols	141.8 ppm

Experimental Diets

All the animals received a standard food formula ISO9002 certified (Standard Panlab A04) *ad libitum* and had free access to tap water. Group C food was supplemented with 5% Portuguese Olive Oil (Gallo Virgin Extra Olive Oil gently supplied by Victor Guedes S.A.). The nutritional composition of Standard Panlab Diet and Olive oil are presented in Table 1 and 2, respectively.

Protocol for Chemical Tumor Induction

Group B and C animals received a single dose of 20 mg DMBA (Sigma-Aldrich, Lisboa, Portugal) solved in 1 mL olive oil by gavages at 50 days of age (Thompson, 2000; Gruenstein *et al*., 1966).

Other Procedures

Animal's body weight was recorded every two weeks. The weight homogeneity index (HW) was calculated at the beginning of the study, according to the formula HW = Ws/[(Ws+Wg)/2, being Ws is the lowest weight and Wg is the highest weight found in this group of rats. The body weight gain (WG) was monitored for a stipulated period of time, two weeks, considering the weight recorded in the beginning (Win) and the end (Wfin) of the considered period, according to the following formula:

$$WG = [(Wfin-Win)/Win]*100$$

Necropsy

All the surviving animals were humanly sacrificed after 150 days through inhalation of carbon dioxide and were submitted to necropsy process during which mammary and extra-mammary neoplastic lesions were searched for.

Collection and Evaluation of Tissue

The rate of neoplastic lesions in this experimental model was described through the ratio between the number of rats that revealed neoplasms and the number of rats still alive at the end of experiment.

For each group the occurrence (Oc) of mammary lesions was determined according to the different types of volume using the formula Oc (%) = No. tumors nx/No. of total mammary tumors found in each group at the end of the experiment, where x= tumors volume. The number of neoplastic lesions in each animal was divided into four classes, categorized as 0, 1-2, 3-5, 6-8, >9 tumors/rat. The number of neoplastic lesions in each animal was divided into four classes, categorized as 0, 1-2, 3-5, 6-8, >9 tumors/rat.

Tumors size was evaluated based on their volume ($V = 4/3\ \pi r^3$ where r is the average radius of several tumors in the same group) and then classified in the following categories: categorized as type A $\leq 0.033\ cm^3$ <type B $\leq 0.267 cm^3$ <type C $\leq 0.904\ cm^3$ type D $\leq 2.43\ cm^3$ and type E $>2.143\ cm^3$. The histological diagnosis of mammary tumors was based on the criteria outlined by the Consensus Conference Committee (1997).

Statistical Tests

All statistical tests were two-tailed and proven to be statistically significant at $p<0.05$. Significance tests for all pair wise comparisons were adjusted for multiple comparisons by multiplying the actual P value by number of comparisons made for the evaluation of statistical significance. The software package used was SPSS (SPSS Inc., Chicago, Ill).

Tumor occurrence (expressed as the percentage of tumour-bearing animals) was compared among the different groups by Chi-squared test association.

Survival distributions for the three groups were compared by log rank tests. The purpose of the analysis was to test whether the null hypothesis of distribution was equal in all groups. In addition to overall test of significance, pair wise comparisons between groups were also made. The overall weight gain of the animals of all groups was compared by use of single classification Variance Analysis ANOVA with repeated measures. The interest of this test was to verify the difference between weight gains over time among the groups. Pair wise comparisons among the groups were also conducted.

RESULTS

All the animals grew at a similar rate and gained weight during the experiment (Fig. 1), group C showed the higher weight gain however there were no significant differences in weight-gain between groups (Fig. 2). The weight homogeneity index was also higher in group C without significant difference between groups ($p>0.05$).

During necropsy there were not found any neoplastic lesions in animals from group A. The occurrence of neoplastic lesions was higher in group C (standard food+olive oil+DMBA) with 78%, as compared to group B with a 73%. Regarding total mammary tumor numbers/group, no significant statistic difference was found between group B and C ($p<0.05$) (Table 3).

A total of 53 mammary carcinomas were found in animals subjected to the olive oil diet against 33 found in animals submitted to induction protocol fed only standard food (group B). While the vast majority (84.85%) of the carcinomas had shown a pattern grade II in group B, grade III also presented 6% of the tumors in this group ($p<0.05$). In group C there were not grade III tumors and even with 60% having presented nuclear grade II there was a much higher percentage of grade I (39.62%) ($p<0.05$) when compared to group B (Table 4).

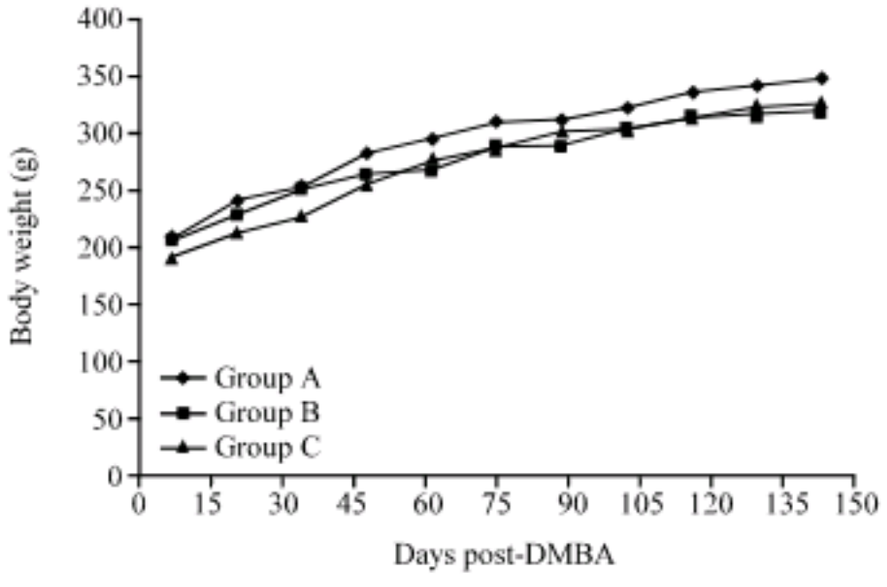

Fig. 1: Body weight evolution over a 150 days period

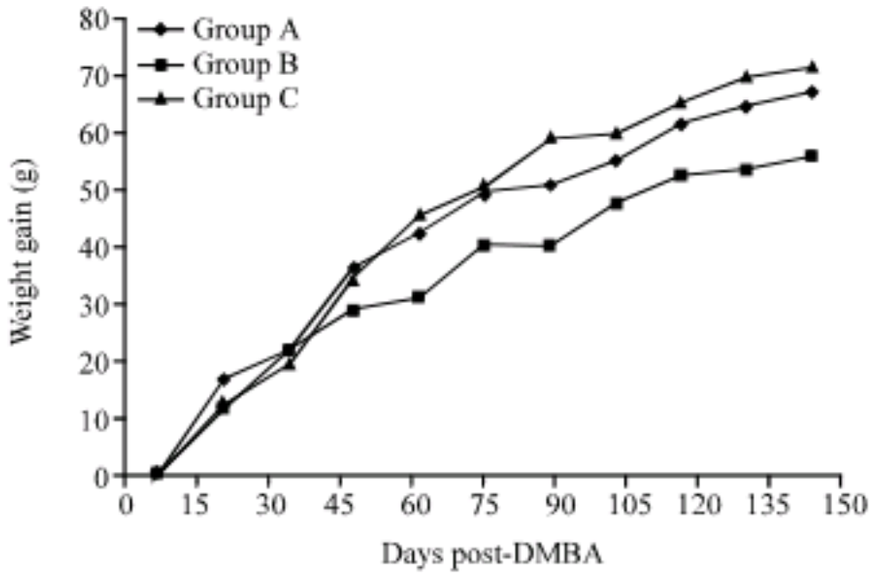

Fig. 2: Weight gain evolution over a 150 day period

Table 3: Animals distribution according to tumors number observed

Groups No.	0 Tumors	1-2 Tumors	3-5 Tumors	6-8 Tumors	>9 Tumors
A	22	0	0	0	0
B	6	9	7	0	0
C	4	7	4	1	2

Table 4: Total mammary tumors nuclear grade

		Nuclear grade (%)		
Groups No.	Treatment and diet	I	II	III
1	Standard food	0	0	0
2	Standard food + DMBA	9.09	84.85	6.06
3	Standard food + olive oil + DMBA	39.62	60.38	0

Most of the carcinomas exhibited a mixed structural pattern, with predominant cribriform and papillary pattern in both series ($p<0.05$).

The analysis of the architectural patterns shows that group B presents a cribriform variety in 33.3% of the total mammary carcinomas, 36% are of a papillary and cribriform type, 18% of the mammary carcinomas presented a comedo and cribriform pattern and 12% presented an association of papillary, cribriform and comedo pattern.

In group C the following results were obtained: 32% of the papillary types, 21% were cribriform, 42% were of a papillary and cribriform variety, 4% were cribriform and comedo or papillary and comedo in 2% (Table 5). The percentage values of carcinomas with a pure

Table 5: Total mammary tumors pattern grade

Groups No.	Treatment and diet	Pattern grade (%) Papillary	Cribriform	Comedo	Papillary+ Crifriform	Papillary+ Comedo	Cribriform+ Comedo	Papillary+ Crifriform+Comedo
A	Standard food	0	0	0	0	0	0	0
B	Standard Food + DMBA	0	33.3	0	36.36	0	18.18	12.12
C	Standard food + olive oil + DMBA	32.08	20.75	0	41.51	1.89	3.77	0

Table 6: Total mammary tumors pattern grade

Groups No.	Treatment and diet	Pattern grade (%) Papillary	Cribriform	Comedo	Papillary+ Crifriform	Papillary+ Comedo	Cribriform+ Comedo	Papillary+ Crifriform+Comedo
A	Standard food	0	0	0	0	0	0	0
B	Standard Food + DMBA	0	33.3	0	36.36	0	18.18	12.12
C	Standard food + olive oil + DMBA	32.08	20.75	0	41.51	1.89	3.77	0

cribriform pattern (33.33% in group B and 20.75% in group C) when compared with a the mixture pattern of cribriform more comedo type (18.18% in group B and 3.77% in group C) are different ($p<0.05$), the same happens in cribriform pattern and the mixture of papillary, cribriform and comedo pattern (12.12% in group B and null in group C). Also are different the comparison between papillary with cribriform type (36. 36% in group B and 41.51% in group C) and papillary more comedo ($p<0.05$).

The tumors dimensions also show a significant difference ($p<0.01$) between the two studied groups unto which the induction protocol was applied. As presented in Table 6 female rats treated with DMBA and consuming standard food presented a much higher percentage of large tumors (64%) in comparison to the ones that had a diet based on olive oil (32%).

DISCUSSION

The presently chosen experimental model, Sprague-dawley DMBA-induced mammary gland tumors, has been largely used due to the chemical induction efficiency and the specific susceptibility of this laboratory animal to breast neoplastic lesions which is consistent with results from the DMBA induction protocol followed (Russo and Russo, 1996). The induced breast cancer models also allow the observation of all the stages during the carcinogenesis process which can be especially useful to evaluate the effect of external conditions like diet, nutrients and therapeutic agents.

Data from present study had shown a possible positive effect of olive oil on several aspects of neoplastic lesions despite of some controversy on the neoplastic lesions number in group C (submitted to DMBA and supplemented with Olive oil) when compared with group B (only standard food and DMBA induction). The fact that there were not found extra mammary lesions in animals from group C and the lower architectural and nuclear grade verified in this animals suggests that olive oil and it's compounds can have a modulating effect on carcinogenesis process. This can be explained from some of the molecular mechanisms that justify the preventive role of Mediterranean-type diet, rich in this dietary fat. Firstly, antioxidants like phenolic compounds can affect carcinogen metabolism and reduce their bioavailability or their metabolic activation, this can also reduce the DNA

damage. Squalene and β-sitosterol, major components of a non-saponifiable fraction, have been proposed as potential anticancer agents (Katdare *et al*., 1997; Murakoshi *et al*., 1992; Nakagawa *et al*., 1985; Owen *et al*., 2004). In a study of how different varieties of olive oil types can influence animal mammary tumors induced by N-Methylnitrosourea. Cohen *et al*. (2000) proposed that the variations in fatty acid content and possible non-saponifiable characteristics of olive oil, may have important implications regarding breast cancer risk.

Olive oil can also reduce the hormone-stimulated growth due to its competitive inhibition of Δ6-desaturase and consequently affecting prostaglandin PGE2 production. Nevertheless, this competition may also affect eicosanoid production, these are responsible for increased growth, apoptosis inhibition, angiogenesis and metastasis, all tumor promoting factors (Stoll, 2002). Regarding the results of the present study, this can explain the lower nuclear and pattern grade of neoplastic lesions shown by group C animals. Considering pattern grade as a hallmark of tumor growth and nuclear grade, mainly evaluated by nuclear pleomorphism, these aspects may be measurements of cell differentiation and replication potential, possible signs of tumor aggressiveness and malignancy (Ivshina *et al*., 2006).

CONCLUSION

The potential protective role of olive oil for breast cancer proposed in several case-controlled epidemiologic studies from Spain (Landa *et al*., 1994; Martin-Moreno *et al*., 1994), Italy (Vecchia *et al*., 1995) and Greece (Trichopoulou *et al*., 1995) can be reinforced with the experimental data obtained in the present study. However, there can be pointed some limitations on the extrapolation of these results due to evident differences between animals and humans, including when considering food habits. In opposite to laboratory animals, people don't eat isolated food but meals and have complex dietary habits with several synergies between nutrients (Terry *et al*., 2001). Several interaction mechanisms and also other lifestyle factors like physical activity, smoking or alcohol consumption may affect the promotion or protective effects of specific nutrients. This complexity motivates the need to further investigation and confirmation the possible positive effects of moderate olive oil consumption, as part of a well balanced and healthy diet, in breast cancer clinical prognosis.

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