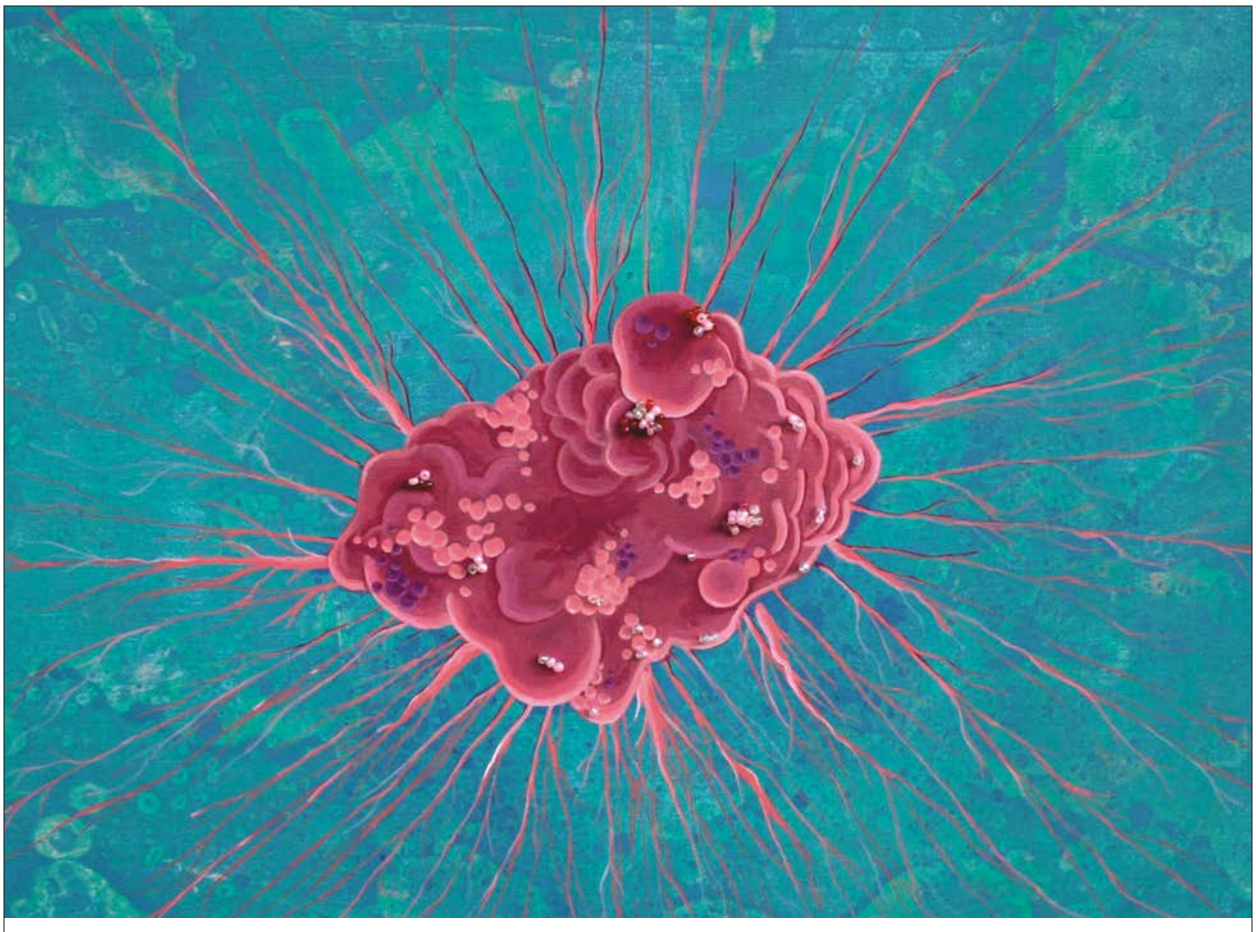

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Dietary Fiber and DMBA-induced Rat Mammary Carcinomas

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ABSTRACT

Dietary fiber had been previously associated with breast cancer risk but some controversies remain on the role of insoluble fiber type. The aim of the present study was to evaluate the effect of a fiber rich food in an experimental model of mammary carcinogenesis. Animals from study groups received a fiber rich food and were compared with two control groups receiving standard food, two groups received the administration of a common carcinogen and histopathological and blood analysis were conducted. Only four tumors were found on group receiving fiber and the carcinogen against the thirty three tumors found on group receiving only the carcinogen and standard food. Additionally, blood cholesterol and triglycerides were significantly lower in this group ($p<0.05$). Glycaemia was also lower but without significance. The experimental results had shown a possible protective effect of fiber on mammary carcinomas possible due to carcinogen binding, weight-gain prevention and insulin sensitivity. Further studies should address the effect of the several fiber types on mammary carcinogenesis process.

Key words: Cellulose, carcinogenesis, breast, cancer, nutrition, histopathologic study

INTRODUCTION

Laboratory animal studies have been essential to provide large evidence about the role of nutrition in the prevention of cancer. The most widely used model had been the Sprague-Dawley female rat and carcinomas are mostly induced using a chemical carcinogenic agent like 7, 12-Dimethylbenz(a)Anthrancene (DMBA) or N-methyl-N-nitrosourea (NMU). This animal model was considered quite appropriate due to its reproducibility, maintenance conditions, homogeneity and a low generation time, experimental conditions and requirements are well established and accepted (Costa *et al*., 2002; Russo and Russo, 1996; Russo and Russo, 2000).

Human and rat mammary carcinomas share some similarities in morphology and biologic behaviour. Elston and Ellis (1991) had developed a possible relationship between histological parameters that can be considered malignancy indicators, comparing both lesions originated in rats or humans.

With experimental studies, it also become possible to study the effect of a specific foods and/or nutrients on mammary carcinogenesis process (Amin, 2009; Khataibeh *et al*., 2006; Clarke, 1997; Rogers, 1997).

Breast cancer incidence had been increasing for several decades and data revealed that this disease had become the main cause of dead among women even in rural areas (Chatterjee, 2011; Laux, 2005). Despite controversial, dietary factors had been strongly associated with several cancers including breast cancer (Safdar and Khan, 2003).

Epidemiological data revealed a possible protective role of dietary fiber and fiber rich foods like fruits, vegetables, legumes and whole grains on the prevention of several chronic diseases, such as cardiovascular diseases (Lairon *et al.*, 2005; Larsson *et al.*, 2009; Liu *et al.*, 2002; Streppel *et al.*, 2008). Results had also shown a positive role of fiber on non-insulin dependent diabetes prevention (Lindstrom *et al.*, 2006; Meyer *et al.*, 2000; Schulze *et al.*, 2007). However, the protective role of fiber in breast cancer still remains open to discussion.

Park *et al.* (2009) suggested a protective role of dietary fiber in breast cancer risk and Patterson *et al.* (2010) concluded that a positive effect of fiber on breast cancer prognosis considering data revealed on observational studies. However, in most several cohort studies, there was not found any association between fiber consumption and breast cancer (Holmes *et al.*, 2004; Horn-Ross *et al.*, 2002; Lajous *et al.*, 2008; Terry *et al.*, 2002; Verhoeven *et al.*, 1997). Other studies had shown only modest correlations (Cho *et al.*, 2003; Giles *et al.*, 2006) and only a few found positive results strongly dependent of menopause status and other variables (Cade *et al.*, 2007; Mattison *et al.*, 2004; McEligot *et al.*, 2006).

Considering these conflicts the present study intended to evaluate the effect of fiber on mammary carcinogenesis process and histopathological characteristics associated with tumor malignancy.

MATERIALS AND METHODS

The experimental project was conducted from January to May 2009 and had last for six months. The histopathologic study had started in November 2009 and had been finished in March 2010. Animals came from Charles River Laboratories Barcelona and were kept in Experimental Pathology Institute in Coimbra University Medicine Faculty where experimental procedures had taken place. The histopathological study was conducted in Egas Moniz Cooperativa de Ensino Superior laboratory.

Animal care: The experimental procedure was approved by the Veterinary Advice Commission published in the Portuguese legislation (DL 129/92 and Dir 86/609/CEE) and in was conducted in strict adherence to animal care guidelines established by competent ethic commissions. Ninety-six female Sprague-Dawley rats 42 days old were randomly assigned to four groups, housed in plastic cages maintained at 22±2°C, 55±10% humidity and with a 12 h light/dark cycle.

Experimental diets: Animals from groups I and III received a standard food formula ISO9002 certified (Standard Panlab A04) *ad libitum* while those from groups II and IV received instead a fiber rich food also *ad libitum*. All animals had free access to tap water. The nutritional composition from both foods is presented in Table 1 and 2, respectively.

Protocol for chemical tumor induction: Animals from groups III and IV received 20 mg DMBA (Sigma-Aldrich, Lisbon, Portugal) solved in 1 mL olive oil by gavages after one week of quarantine, 50 days old.

Table 1: Standard food nutritional composition

Composition	Value
Humidity	12%
Protein	15.5%
Fat	2.7%
Glucides	58.5%
Minerals	5.5%
Fiber	3.7%
Metabolizable energy	3000 kcal kg^{-1}

Table 2: Fiber rich food nutritional composition

Composition	Value
Humidity	10%
Protein	12%
Fat	3%
Glucides	52.5%
Minerals	6.5%
Fiber	16%
Hemicellulose	18.9%
Cellulose	16.4%
Lignin	6.7%
Metabolizable energy	2200 kcal kg^{-1}

Blood analysis: Right before the administration of DMBA, 1 mL of blood was collected from each animal and the same procedure was conducted before sacrifice for biochemical parameters analysis.

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 they were all submitted to necropsy process. All the neoplastic lesions, mammary or extra-mammary found were measured and prepared for histological studies.

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:

$$Oc\ (\%) = \frac{\text{No. of tumors n}}{\text{No. of total mammary tumors found in each group at the end of the experiment}}$$

where, x is 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.

Tumor size was evaluated according to their volume ($V = 4/3\ \pi\rho^3$) where, r is the average radius of several tumors in the same group) and then classified in the following categories: categorized as

type A (volume ≤0.033 cm^3); type B (0.033 cm^3 < volume ≤ 0.267 cm^3); type C (0.267 cm^3 < volume ≤0.904 cm^3); type D (0.904 cm^3 < volume ≤2.143 cm^3) or type E (volume >2.143 cm^3).

Histopathological study: The tumors were dissected and the fragments collected for histology were fixed in neutral buffered formaldehyde, processed and embedded in paraffin, cut in microtome and stained with hematoxylin and eosin.

Data from the present study refers exclusively to mammary neoplastic lesions. The histological classification done considered the following elements: Type of lesion (benign or malignant, invasive or in situ), architectural pattern, cribriform areas and histological grade based on three parameters: tubular formation or Pattern Grade, nuclear pleomorphism or Nuclear Grade and Mitotic count (Elston and Ellis, 1991; Russo and Russo, 2000).

Statistical analysis: All statistical tests were two-tailed and conducted at 95% confidence level. 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 17 (SPSS Inc., Chicago, Ill).

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 through Mann-Whitney tests. The overall weight gain of the animals of all groups was compared by the use of single classification variance analysis ANOVA with repeated measures. The interest of this test was to verify the difference between weight gain over time among the groups.

RESULTS

As presented in Table 3, all animals gained weight however there was a significant difference between animals from Group IV and all the other groups ($p<0.05$). These group received a fiber rich food and had an average weight gain of 40.2%, significantly lower than average group I weight gain of 67.1% which received the standard food as well as lower than group II that also received a fiber rich food and had an average weight-gain of 51.1 ($p<0.05$). A large number of animals in group IV did not have any mammary lesion while in most animals from group III were found three to five tumors or one to two lesions (Table 4).

Animals from group IV also presented the lower number of mammary neoplastic lesions, significantly different from group III ($p<0.05$), only four were found against the thirty three found in group III. Despite fewer these neoplastic lesions were significantly larger when compared with

Table 3: Weight-gain and mammary lesions found within groups

Group	Caloric value (kcal 100 g^{-1})	Weight gain (%)	Surviving animals	Mammary neoplastic lesions
I	300	67.1±17.8	22	0
II	220	51.1±12.3	23	0
III	341	53.3±12.1	22	33
IV	220	40.2±21.9	21	4

Table 4: Comparison between the number of tumors found per animal within groups

Group	0 tumors (%)	1-2 tumors (%)	3-5 tumors (%)	6-8 tumors (%)	≥9 tumors (%)
I	100.0	0.0	0.0	0.0	0.0
II	100.0	0.0	0.0	0.0	0.0
III	9.1	31.8	40.9	0.0	0.0
IV	85.7	14.3	0.0	0.0	0.0

Table 5: Tumor volume comparison between groups

Group	Surviving animals	Mammary neoplastic lesions	Average tumor volume (cm^3)	Maximum : Minimum tumor volume (cm^3)
I	22	0	0	0
II	23	0	0	0
III	22	33	2.258±2.456	10.472 : 0.042
IV	21	4	6.516±6.616*	14.500 : 0.770

*p>0.05 when comparing average tumor volume between groups III and IV

Table 6: Tumor distribution according to tumor size categories

	Small tumors (%)			Large tumors (%)	
			Medium tumors		
Groups	Type A	Type B	Type C (%)	Type D	Type E
III	0.0	15.2	21.2	24.2	39.4
IV	0.0	0.0	25.0	25.0	50.0

Table 7: Carcinoma Architectural patterns frequency within groups

Architectural pattern	Group III (%)	Group IV (%)
Papillary	3	50
Cribriform	37	25
Papillary and Cribriform	42	25
Papillary, Cribriform and Comedo	5	0
Papillary and Comedo	3	0
Cribriform and Comedo	11	0

Table 8: Comparison of main histopathologic parameters within groups

	Pattern grade (%)			Nuclear grade (%)			Mitotic counts (%)				
Group	I	II	III	I	II	III	≤3	4-6	7-9	10-19	≥20
III	15.8	52.6	31.6	13.2	81.6	5.3	26.3	21.1	5.3	31.6	15.8
IV	100.0	0.0	0.0	50.0	50.0	0.0	0.0	0.0	0.0	0.0	0.0

those found in group III. The average tumor volume in group III was 2.258 cm^3 while in group IV was 6.516 cm^3 (Table 5). Tumors found in group IV were medium or large tumors, being 50% very large tumors while in group III 15.2% were even classified as small tumors and 39.4% were classified as type E or very large (Table 6).

The histopathological study conducted revealed additional significant differences in tumors from groups III and IV. As presented in Table 7, half of the tumors found in group IV had shown predominant papillary areas (Fig. 1) without being found any comedocarcinomas. Architectural patterns found in group III included comedocarcinomas mixed with cribriform areas (11%) and 42% had papillary and cribriform areas mixed (Fig. 2).

The few tumors found in group IV also showed less extent solid areas, all tumors were classified as pattern grade I while in group III 52.6% were classified as pattern grade II and 31.6% as pattern grade III ($p<0.05$). In what concerns to nuclear pleomorphism, higher in group III considering that 81.6% had scored intermediate nuclear grade and 5.3% scored high nuclear grade (Table 6). In group IV, half of the tumors presented low or intermediate nuclear pleomorphism (Fig. 3).

No mitosis was found in group IV tumors while 31.6% in group III had 10 to 19 mitosis in 10 high power fields. All these characteristics differed significantly within these two groups and results from this comparison are presented in Table 8.

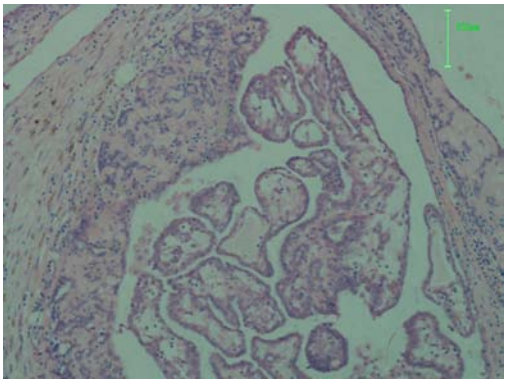

Fig. 1: Carcinoma of the rat mammary gland showing a papillary pattern sustained by thin connective tissue cores, neoplastic lesion fragment from Group IV animal. (Hematoxilin and Eosin, x100)

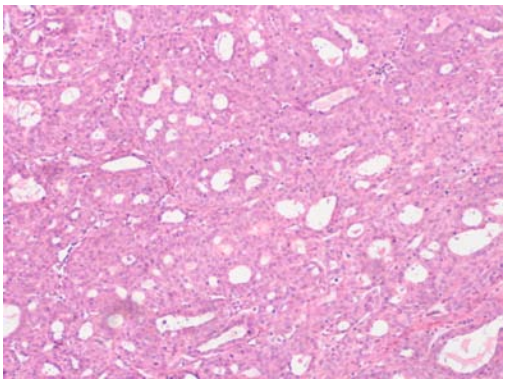

Fig. 2: Carcinoma of the rat mammary gland showing a papillary pattern sustained by thin connective tissue cores, neoplastic lesion fragment from Group III animal. (Hematoxilin and Eosin, x100)

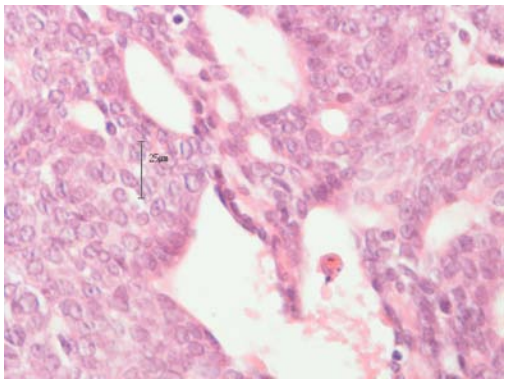

Fig. 3: Carcinoma of rat mammary gland, group IV animal showing a low solid areas extension and lower intermediate nuclear pleomorphism

Table 9: Comparison of glycaemia values within groups

Group	Average initial glycaemia (mg dL^{-1})	Average final glycaemia (mg dL^{-1})	p-value
I	119.00±14.3	154.5±30.8	<0.05
II	130.25±8.2	116.2±5.4	<0.05
III	123.10±17.4	165.8±100.2	<0.05
IV	140.00±30.7	121.5±32.5	<0.05

Table 10: Comparison of cholesterol values within groups

Group	Average initial cholesterol (mg dL^{-1})	Average final cholesterol (mg dL^{-1})	p-value
I	71.0±13.9	90.3±22.4	<0.05
II	77.4±8.7	71.7±5.6	<0.05
III	80.0±12.3	94.0±24.4	<0.05
IV	78.4±13.6	73.0±13.7	<0.05

Table 11: Comparison of triglycerides within groups

Group	Average initial triglycerides (mg dL^{-1})	Average final triglycerides (mg dL^{-1})	p-value
I	84.2±21.8	196.6±89.1	<0.05
II	92.3±19.1	84.8±22.6	<0.05
III	96.5±37.1	177.6±110.9	<0.05
IV	94.1±33.2	83.6±26.9	<0.05

In what concerns to blood parameters, glycaemia (Table 9), cholesterol (Table 10) and triglycerides (Table 11) increased and their values differed significantly between the beginning and the end of the study ($p<0.05$). Within groups, significant differences were found between groups III and IV in cholesterol and triglycerides values at the end of the study ($p<0.05$). Group III animals had shown the higher glycaemia and cholesterol values in the end of the study while group II had the lowest but tryglicerides were higher in group I and lower in group IV.

DISCUSSION

As described in last section results obtained in this experimental study revealed a clear benefit from fiber consumption on mammary carcinogenesis. Animals which received the fiber rich food had less mammary carcinomas and the few presented showed lower malignancy considering the histopathologic criteria used, the results obtained are in accordance to previous experimental studies conducted (Cohen *et al*., 1991; Zile *et al*., 1998).

Dietary fiber had been described as a complex portion of plant foods resistant to digestive enzymes (Caroline *et al*., 2003) but metabolically active. Fiber affects digestive tract motility as well as nutrient absorption and it can be fermented in large bowel leading to short chain fatty acids synthesis (e.g., propionic, butyric and acetic acids, among others) (Anderson *et al*., 2009; Lattimer and Haub, 2010; Papathanasopoulos and Camilleri, 2010).

In the present study, the fiber rich food was mainly composed by cellulose, an insoluble dietary fiber form. Soluble and insoluble dietary fiber fractions showed different effects on cancer risk on previous studies which could be explained by their different functions and properties which could also affect their effect on cancer development (Moore *et al*., 1998; Papathanasopoulos and Camilleri, 2010). Insoluble fibers like cellulose were addressed specially to be responsible for decreased digestive transit time. The ability to affect digestive motility could be responsible for decreased transit time which reduces exposure to several carcinogens. In fact, an *in vitro* study showed that dietary fiber is able to bind carcinogens with hydrocarbon structure like DMBA which

could affect significantly carcinogenesis (Gulliver *et al.*, 1983). This effect had also been shown by antioxidants present in Chlorella which had been proposed to act as modulators of DMBA-induced oxidative stress (Amin, 2008).

Some studies addressed also a special role of insoluble dietary fiber on the prevention of weight-gain and obesity which had been also associated with breast cancer risk (Isken *et al.*, 2010; Tucker and Thomas, 2009), mainly due to its effects on satiety and food intake (Samra and Anderson, 2007).

Like stated before, it had been also reported a potential protective role of this fiber type intake in type II diabetes although most benefits on glucose tolerance and insulin sensitivity (Pereira *et al.*, 2002) had been previously proposed to soluble fiber fractions. The mechanisms by which this could happen remained not clearly understood and in the present study, the animals that received the fiber rich food had lower average glycaemia values but not significantly different from the other groups. Some in vitro results revealed that high glucose concentrations could exert a proliferative effect in breast cancer cells (Fouladdel *et al.*, 2006) which reinforces the protective value of low glycaemia values.

Cholesterol values in groups fed with fiber rich food also decreased and differed significantly from Groups I and III ($p<0.05$) which reinforces the potential protective role of insoluble fiber. This cholesterol lowering effect could not be due to propionic acid because cellulose had been proven to be a poor fermentable fiber. However cellulose can bind cholesterol and lipid molecules reducing their absorption verified in experimental studies (Chau and Cheung, 1999; Chau *et al.*, 2008).

It is important to note that high levels of cholesterol and tryglicerides had been associated with breast cancer risk (Owiredu *et al.*, 2009). In the present study, animals fed with fiber rich food had also significantly lower triglycerides values at the end of the study which confirms previous experimental (Anderson *et al.*, 1994) and clinical data (Anderson, 2000; Slama *et al.*, 2011).

CONCLUSION

The protective role of insoluble dietary fiber on breast cancer risk had been subject of debate. Several controversial results pointed out a null or very weak association, with results showing that only soluble fiber consumption could exert a benefic effect. However, the present study, similarly with other animal studies suggest a protective role of a fiber rich diet on mammary carcinogenesis possibly due to its effects on reduced weight gain, carcinogen absorption and lower cholesterol and triglycerides levels. Mammary tumors found in fiber rich group subjected to carcinogen DMBA were significantly fewer and revealed lower malignancy. Wheat bran, whole grains and leafy vegetables are important insoluble fiber sources in human diet. Further studies should address the potential benefit from the consumption of these foods in mammary carcinogenesis.

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