INFLUENCE OF DIETS VARYING IN LIPID AND PROTEIN CONTENT ON THE HISTOPATHOLOGIC VARIETY OF MURINE SALIVARY GLAND TUMORS INDUCED BY 9,10- DIMETHYL-1,2-BENZANTHRACENE (DMBA)

INFLUENCIA DE DIETAS DE DIFERENTE CONTENIDO LIPÍDICO Y PROTEICO SOBRE VARIANTES HISTOPATOLÓGICAS DE TUMORES DE GLÁNDULAS SALIVARES MURINOS INDUCIDOS CON 9,10- DIMETHYL-1,2-BENZANTHRACENO (DMBA)

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ABSTRACT

The purpose of this study was to analyze the influence of diets varying in lipids and proteins on the histopathologic variety of murine salivary tumors induced by DMBA.

117 BALB/c mice were assigned to experiments one (E1: lipids, males) and two (E2: proteins, males and females), E1 comprising Soy oil (SO); Corn oil (CO, control); Fish oil (FO) and Olein (O) groups and E2, soy protein (SP) and casein (C) groups. Tumors were induced by DMBA and the animals were sacrificed at week 13ª post-induction. Tumor volume was calculated. Tumor sections were stained with H-E for histopathologic evaluation.

No significant association was found between tumor volume and dietary condition ($p>0.05$). In E1, FO animals developed mainly carcinomas (C) (58,8%), the sarcomas (S) and carcinosarcomas (CS) being especially of high-grade type (tumors <600 mm^3). In E2, SP animals developed mainly C (55.6%). Although no significantly different ($p>0.05$), S and C were more frequent in female and male mice, respectively. In both E1 and E2, the biggest tumors (>600 mm^3) were mainly high-grade S (87.5%-80%).

Dietary fat and soy protein appear to influence the tumor histopathology and thus its prognosis.

Key words: salivary glands - tumors - mice - dietary lipids - soy protein - histopathology

RESUMEN

El objetivo de este estudio fue analizar la influencia de dietas con diferente contenido de lípidos y proteínas sobre la variedad histopatológica de tumores salivales murinos inducidos por DMBA.

Se asignaron 117 ratones BALB/c a los experimentos uno (E1: lípidos, machos) y dos (E2: proteínas, machos y hembras). E1 comprendió a los grupos aceite de soja (AS), aceite de maíz (AM, control), aceite de pescado (AP) y oleína (O), en tanto que E2 incluyó a los grupos proteína de soja (PS) y caseína (C). Los tumores fueron inducidos por DMBA y los animales fueron sacrificados a la 13ª semana post-inducción. Se calculó el volumen tumoral. Los cortes de tumor fueron coloreados con Hematoxilina-Eosina para su evaluación histopatológica.

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No se encontró asociación entre volumen tumoral y condición dietaria (p>0.05). En E1, los animales del grupo AP desarrollaron principalmente carcinomas (C) (58,8%), en tanto que los sarcomas (S) y carcinosarcomas (CS) fueron de alto grado (tumores <600 mm^3). En E2, los animales del grupo PS desarrollaron principalmente C (55.6%). Aunque la diferencia no fue significativa (p>0.05), S y C fueron más frecuentes en ratones hembras y machos, respectivamente. Tanto en E1 como en E2, los tumores más voluminosos (>600 mm^3) fueron principalmente S de alto grado (87.5%-80%).

Los lípidos y la proteína de soja de la dieta parecen influenciar la histopatología de los tumores y, en consecuencia, su pronóstico.

INTRODUCTION

Spontaneous occurrence of salivary gland tumors in experimental animals, and especially rodents, is rare (1). However, salivary tumors may be induced by carcinogens like dimethylbenzanthracene, benzopyrene or methylcholanthrene - usually inoculated after the gland surgical exposure – or transplanted to the same or other animals (2, 3, 4).

Both chemically-induced or transplanted salivary gland tumors are useful experimental models to investigate their biological behavior when exposed to different agents (5). In this context, we have developed a model consisting of a non-surgical subcutaneous inoculation of DMBA in the submandibular area, which showed to be successful in 100% of cases (6).

Dietary components, lipids and protein sources among them, may modulate the biology of tumors originated from different organs, including the salivary glands (5,7,8). Thus, fatty acids belonging to n-3 and n-9 families behave as tumor protectors and promotors, respectively (6,9,10). In addition, phytochemicals like isoflavones (phytoestrogens) contained in soy protein demonstrated to protect against cancer, especially in hormone-dependent organs, due to their competitiveness with endogenous estrogens (11).

No references regarding the impact of diet on human and/or experimental tumor histopathology were found. So, the main purpose of this study was to analyze the influence of diets varying in lipids and proteins on the histopathologic variety of murine salivary tumors induced by subcutaneous injections of DMBA.

MATERIALS AND METHODS

Animals and dietary conditions

One-hundred and seventeen BALB/c mice of both sexes, ninety-seven males and twenty females, were employed. Forty-five days after weaning they were randomly assigned to experiments one (E1: lipids) and two (E2: proteins) in order to receive different dietary treatments. Female mice were employed in E2 because of the influence of phytoestrogens on hormone-dependent tumors. Animals belonging to E1 were divided into four groups: Soy oil (SO); Corn oil (CO, control); Fish oil (FO) and Olein (O), whereas those of E2 were assigned to two groups: soy protein (SP) and casein (C) (Table 1). Since no differences regarding the tumor biological behavior were found in a previous study between CO-fed animals and those fed a commercial formula, CO is considered here as the control group. Diets consisted in a semisynthetic formula containing: casein (16%); sucrose (34.9 %); corn starch (39%); fiber (2%); salt mixture (3.5%) and vitamin mixture (0.5%), supplemented in E1 with 5% of corn (n-6), soy (n-3, n-6), fish (n-3) oils and olein (85% of 18:1 n-9). In addition to the previously mentioned components, the dietary preparations for E2 contained soy protein isolate (16%) (SP) and casein (16%) (C), having the same lipid source: corn oil. Soy protein isolate had 3.71 mg/g protein of total isoflavones. The dietary formulae, with no significant difference in caloric value *per* gram, were prepared weekly and stored in sealed dark containers at –5ÚC. Food and water were provided *ad libitum* to the animals kept in a light and temperature controlled room. Food intake was similar in all groups.

Table 1: Distribution of animals according to dietary groups and sex

	Dietary groups					
	Experiment one (E1)				Experiment two (E2)	
Sex	Olein	Soy oil	Fish oil	Corn oil	Casein	Soy protein
Males	22	18	25	14	14	18
Females	—	—	—	—	—	20

Submandibular gland carcinogenesis

Two weeks after the animals began the specific diets, tumors were chemically induced by an injection of 9,10-dimethy-1,2-benzanthracene

(DMBA 95%, Sigma) under light anesthesia with ether (6). 0.5 mg of carcinogen was diluted in 200 ìl of corn oil, employed as a vehicle, and subcutaneously injected into the submandibular area. All the animals developed salivary tumors, mostly arising from the submandibular gland.

Mice were observed daily and tumor size recorded once a week by the same researcher. Thirteen weeks later, all the animals included in the study were sacrificed except those which had died spontaneously (n=3) because of cachexia. After a systematic necropsy, the salivary glands and tumors were examined. These glands and their associated tumors were dissected and three-dimensionally measured with a caliper. Volume, expressed in mm^3, was calculated by applying the built-in ellipsoid formula (12).

Histological procedures

Samples of salivary glands and their tumors were fixed in 10% neutral formalin solution, dehydrated and embedded in paraffin. Sections were stained with Hematoxilin and Eosin for histopathologic evaluation through light microscopy.

Tumor diagnosis was the result of observations and agreement of two well-trained pathologists with experience in salivary gland lesions.

Statistical analyses

For both E1 and E2 the variables studied were:

- Tumor histopathology: carcinomas (C), high-grade sarcomas (HGS), low-grade sarcomas (LGS), high-grade carcinosarcomas (HGCS) and low-grade carcinosarcomas (LGCS).
- Diet: CO (control), SO, FO and O for E1; C and SP (males and females) for E2.
- Tumor volume: <600 and >600 mm^3.
- Sex: males and females (in SP group).

The Pearson's chi square test was used to analyze the association between tumor volume and dietary condition and between tumor histopathology and sex. No statistical tests could be used to compare tumor histopathology and dietary condition due to the small number of animals in each subgroup (C, HGS, LGS, HGCS, LGCS).

RESULTS

Gross findings of experimental tumors

Numerous macroscopic variations were observed in these murine salivary gland tumors. They appeared as irregular hard masses varying from a few millimeters to 40 mm in diameter, cases in which the tumors surrounded the neck showing an expansive growth pattern. Others were fixed to the underlying tissues and sometimes they infiltrated the trachea, esophagus and neck muscles.

The overlying skin was frequently affected. Ulcerations covered by scabs or keratotic stuff as well as hemorrhage were also observed. One animal belonging to FO group showed an extensive macroscopically evident skin involvement.

When cut, tumors showed a whitish color and some cystic formations, unique or multiple, some of them filled with an oily content while others exhibited hemorrhage and necrosis focuses.

Histopathological findings of experimental tumors

Three histopathologic types, whose distribution is shown in Figure 2, were found: squamous cell carcinoma, sarcomas and carcinosarcomas. Sarcomas and carcinosarcomas were of both low and high-grade maligancy.

Squamous cell carcinomas: they were of highly differentiated keratinizing type, exhibiting several growth patterns. Cysts surrounded by neoplastic epithelium and solid nests with keratin pearl formation were also observed. Tumor cells showed atypia, mitoses or cytoplasmic keratinization (Figure 1-A). Squamous metaplasia was frequently evident within the duct-like structures and cystic lesions around the tumor.

Sarcomas: the low-grade type was characterized by a diffuse proliferation of regular and spindle-shaped cells with variable amount of intercellular substance, scant mitotic activity and pushed edges (Figure 1-B). The high-grade sarcomas had conspicuous atypia, pleomorphism, intense mitotic activity and necrosis. Tumor cells frequently showed rhabdomyoblastic features, the fibers containing several nuclei of different sizes. Multinucleated giant cells with large basophilic cytoplasms and hyperchromatic nuclei were also observed (Fig. 1-C). It was seen that this type of sarcoma infiltrated the neighboring tissues.

Carcinosarcomas: they had a biphasic appearance due to the presence of both epithelial and connective components. The first was characterized by nests of neoplastic squamous cells, the duct lumen with occasional keratinized

epithelial plugs. The sarcomatous component showed a fasciculated proliferation of spindle-shaped tumor cells with variable atypia (Figure 1-D). In some cases, distinct areas of chondro or osteosarcoma were also observed. The surrounding connective tissue exhibited focuses of fibromixoid appearance.

In spite of early clinical diagnosis, some tumors became undetectable at 9–12 week of evolution. Those salivary glands showed normal appearance during the macroscopic examination and were microscopically confirmed as non-tumoral cases (possible tumor remissions) (NT).

In both tumor and non-tumoral cases, mononuclear infiltrating edema, necrosis and hemorrhage, or cellular and sarcomatoid areas, was observed. The adjacent gland tissue showed lobular atrophy, squamous metaplasia in proliferative ductules or interlobular ducts and ductal segment dilatation.

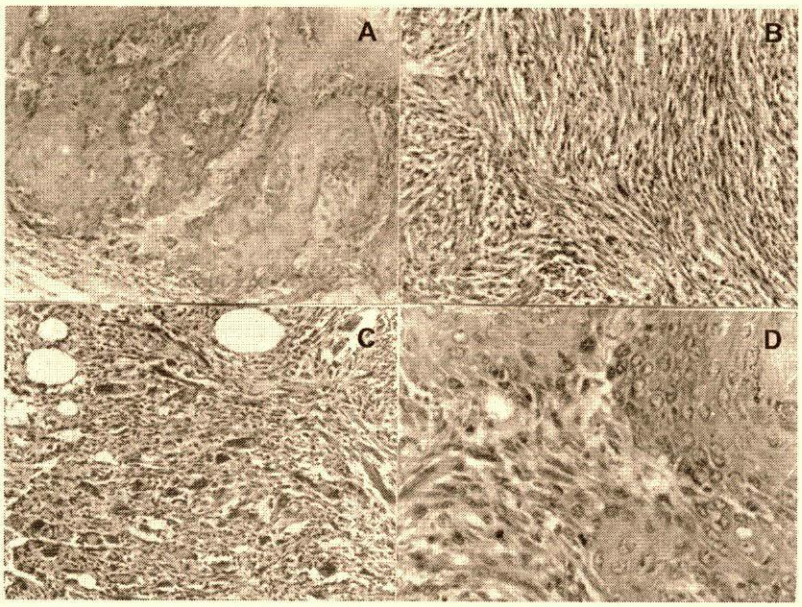

Figure 1: Histopathologic varieties of murine DMBA-induced tumors. H&E. Original magnification X850.
A. Epidermoid carcinoma formed by large solid nets of squamous cells with variable keratinization.
B. Sarcoma. Fused cells arranged in bundles.
C. High-grade sarcoma. Some cells show a rhabdomyoblastic phenotype.
D. Mixed mesenchymatic-epithelial neoplasia. Areas of squamous differentiation and of fusiform cells are combined.

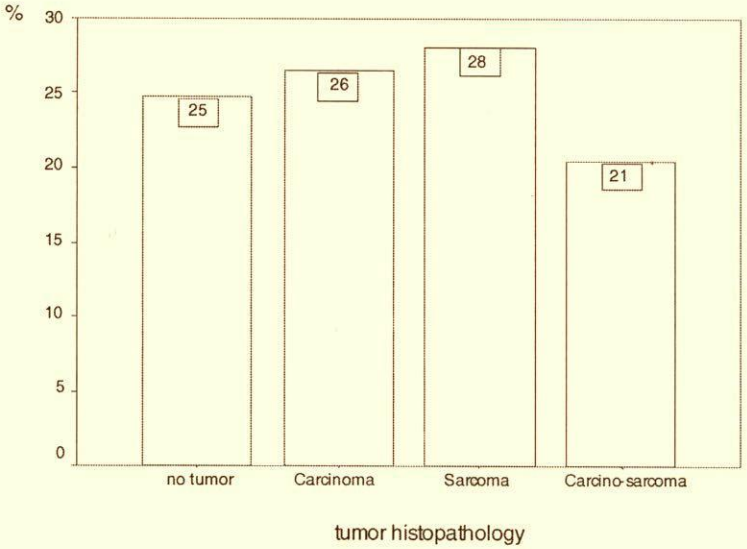

Figure 2: Percentage distribution of tumor histopathologic types considering all the animals.

Tumor histopathology by diet and volume

According to volume, tumors in E1 were distributed as follows: 67% (<600), 10% (>600) and 23% (NT, no tumor: after several weeks of evolution -9 to 13- some palpable masses became clinically undetectable; afterwards, those remissions were microscopically confirmed). When analyzed by dietary group, NT corresponded to 18% in CO group, 32% in FO, 28% in O and 22% in SO. Pearson's chi square test showed no significant association between tumor volume and dietary condition (p>0.05).

For E2, the percentages were the following: 62% (<600), 13% (>600) and 25% (NT). According to dietary group, NT corresponded to 73% in SP and 27% in CO. Pearson's chi square test showed no significant association between tumor volume and dietary condition (p>0.05).

In E1, FO group animals developed mainly carcinomas (58,8%) (tumors below 600 mm3). Among the sarcomas and carcinosarcomas in FO group, they were especially of high-grade type. Tumors above 600 mm^3 were mainly high-grade sarcomas (87,5%). Just one animal (12,5%) developed a low- grade carcinosarcoma. (Table 2).

Table 2: Tumor histopathology by volume and diet. Experiment one (E1).

Tumor histopathology	n and percentages	Dietary groups				
Tumor volume < 600		CO	FO	O	SO	Total
C	n	3	10	4	5	22
	C / diet (%)	13.6	45.5	18.2	22.7	100.0
	C / total tumors in dietary group (%)	30.0	58.8	28.6	41.7	41.5
	C / total tumors in all dietary groups (%)	5.7	18.9	7.5	9.4	41.5
HGS	n	2	3	2	0	7
	C / diet (%)	28.6	42.9	28.6	0.0	100.0
	C / total tumors in dietary group (%)	20.0	17.6	14.3	0.0	13,2
	C / total tumors in all dietary groups (%)	3.8	5.7	3.8	0.0	13.2
LGS	n	1	2	3	2	8
	C / diet (%)	12.5	25.0	37.5	25.0	100.0
	C / total tumors in dietary group (%)	10.0	11.8	21.4	16.7	15.1
	C / total tumors in all dietary groups (%)	1.9	3.8	5.7	3.8	15.1
HGCS	n	1	2	2	3	8
	C / diet (%)	12.5	25.0	25.0	37.5	100.0
	C / total tumors in dietary group (%)	10.0	11.8	14.3	25.0	15.1
	C / total tumors in all dietary groups (%)	1.9	3.8	3.8	5.7	15.1
LGCS	n	3	0	3	2	8
	C / diet (%)	37.5	0.0	37.5	25.0	100.0
	C / total tumors in dietary group (%)	30.0	0.0	21.4	16.7	15.1
	C / total tumors in all dietary groups (%)	5.7	0.0	5.7	3.8	15.1
Total	n	10	17	14	12	53
	C / diet (%)	18.9	32.1	26.4	22.6	100.0
	C / total tumors in dietary group (%)	100.0	100.0	100.0	100.0	100.0
	C / total tumors in all dietary groups (%)	18.9	32.1	26.4	22.6	100.0
Tumor Volume > 600						
HGS	n	2	1	2	2	7
	C / diet (%)	28.6	14.3	28.6	28.6	100.0
	C / total t umors in dietary group (%)	100.0	100.0	100.0	66.7	87.5
	C / total tumors in all dietary groups (%)	25.0	12.5	25.0	25.0	87.5
HGCS	n	0	0	0	1	1
	C / diet (%)	0.0	0.0	0.0	100.0	100.0
	C / total tumors in dietary group (%)	0.0	0.0	0.0	33.3	12.5
	C / total tumors in all dietary groups (%)	0.0	0.0	0.0	12.5	12.5
Total	n	2	1	2	3	8
	C / diet (%)	25.0	12.5	25.0	37.5	100.0
	C / total tumors in dietary group (%)	100.0	100.0	100.0	100.0	100.0
	C / total tumors in all dietary groups (%)	25.0	12.5	25.0	37.5	100.0

C: carcinoma;
HGS: high-grade sarcoma; LGS: low-grade sarcoma; HGCS: high-grade carcinosarcoma;
LGCS: low-grade carcinosarcoma
FO: fish oil;
CO: corn oil;
SO: soy oil;
O: olein.
n: number of animals
% of carcinomas by diet
% of carcinomas of total tumors in each diet
% of carcinomas of total tumors in all dietary groups

In E2, SP group animals developed mainly carcinomas (55.6%). In CO animals predominated both carcinomas (30%) and low-grade carcinosarcomas (30%) (tumors below 600 mm³). The biggest tumors, above 600 mm³, were mainly high-grade sarcomas (Table 3).

Table 3: Tumor histopathology by volume and diet. Experiment two (E2).

Tumor histopathology	n and percentages	Dietary groups		
Tumor volume < 600		CO	SP	Total
C	n	3	5	8
	C / diet (%)	37.5	62.5	100.0
	C / total tumors in dietary group (%)	30.0	55.6	42.1
	C / total tumors in all dietary groups (%)	15.8	26.3	42.1
HGS	n	2	1	3
	C / diet (%)	66.7	33.3	100.0
	C / total tumors in dietary group (%)	20.0	11.1	15.8
	C / total tumors in all dietary groups (%)	10.5	5.3	15.8
LGS	n	1	1	2
	C / diet (%)	50.0	50.0	100.0
	C / total tumors in dietary group (%)	10.0	11.1	10.5
	C / total tumors in all dietary groups (%)	5.3	5.3	10.5
HGCS	n	1	1	2
	C / diet (%)	50.0	50.0	100.0
	C / total tumors in dietary group (%)	10.0	11.1	10.5
	C / total tumors in all dietary groups (%)	5.3	5.3	10.5
LGCS	n	3	1	4
	C / diet (%)	75.0	25.0	100.0
	C / total tumors in dietary group (%)	30.0	11.1	21.1
	C / total tumors in all dietary groups (%)	15.8	5.3	21.1
Total	n	10	9	19
	C / diet (%)	52.6	47.4	100.0
	C / total tumors in dietary group (%)	100.0	100.0	100.0
	C / total tumors in all dietary groups (%)	52.6	47.4	100.0
Tumor volume > 600				
HGS	n	2	2	4
	C / diet (%)	50.0	50.0	100.0
	C / total tumors in dietary group (%)	100.0	66.7	80.0
	C / total tumors in all dietary groups (%)	40.0	40.0	80.0
HGCS	n	0	1	1
	C / diet (%)	0.0	100.0	100.0
	C / total tumors in dietary group (%)	0.0	33.3	20.0
	C / total tumors in all dietary groups (%)	0.0	20.0	20.0
Total	N	2	3	5
	C / diet (%)	40.0	60.0	100.0
	C / total tumors in dietary group (%)	100.0	100.0	100.0
	C / total tumors in all dietary groups (%)	40.0	60.0	100.0

C: carcinoma; HGS: high-grade sarcoma; LGS: low-grade sarcoma; HGCS: high-grade carcinosarcoma; LGCS: low-grade carcinosarcoma
CO: corn oil; SP: soy protein.
n: number of animals
% of carcinomas by diet
% of carcinomas of total tumors in each diet
% of carcinomas of total tumors in all dietary groups

In SP group and regarding sex, the Pearson's test showed no significant association with histopathology ($p>0.05$). However, it was observed that sarcomas and carcinomas were more frequent in female and male mice, respectively (sarcomas: 63.6% and 36.4%; carcinomas: 44.4% and 55.6%).

DISCUSSION

Tumors of different origin have arisen in the murine salivary glands of the present experimental model: carcinomas, sarcomas and carcino-sarcomas. From them, carcinomas were the most frequently observed histopathologic variety in tumors below 600 mm³ in volume, mainly in FO and SP dietary groups. Sarcomas -rare in human salivary glands- and especially their high-grade variant was predominant in the biggest neoplasias (above 600 mm³) irrespective of dietary group, showing an association of histopathology with tumor volume (13). El-Asfahani et al found that BALB/c mice developed mainly DMBA-induced epidermoid carcinomas; sarcomas were observed in a few cases (14).

When considering a group of animals fed soy protein with isoflavone content (E2), a sex difference regarding histopathology was observed. Sarcomas and carcinomas were more frequently found in females and in males, respectively, female mice having developed smaller tumors than males (15). In this respect, Turusov reported a higher incidence of DMBA-induced carcinomas in male than in female rats (16).

Since the salivary glands and their tumors are hormone-dependent organs, the difference observed in this study could also be attributed to the effect of phytoestrogens, but further research is necessary so as to confirm this explanation (17,18).

Different results -including ours- showed FO to act as a tumor protector. In the present study, it was observed that FO had a smaller initial diameter of palpable masses than O and CO (15). Regarding histopathology, FO was found in association with carcinomas. According to Turusov, carcinomas in rats are just locally aggressive and have less ability to produce metastasis in nodes and lungs than sarcomas (16). The apparent absence of metastases related to sarcomas in our study could be attributed to the fact that, according to the protocol, the

animals had to be sacrificed early. In addition, murine carcinomas in FO group had low volume and most of them were well-differentiated, the latter being a good indicator of favorable prognosis.

It could be concluded that the dietary fat may in somehow extend influence not only the tumor growth parameters but also their histopathology, which is related to the disease prognosis.

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