Chapter

Experimental Carcinogenesis with 7,12-Dimethylbenz(a)Anthrazene (DMBA) and Its Inhibition with Extra Virgin Olive Oil and a Diet of Mature Olives (*Picual* Variety)

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Abstract

7,12-Dimethylbenz(a)anthrazene (DMBA) is a carcinogen that induces carcinomas within a few weeks of application. Forty-four male hamsters were divided into four groups: DMBA dissolved in paraffin oil (DMBA-PO), DMBA dissolved in olive oil (DMBA-OO), paraffin oil and olive oil. Their mouths were swabbed daily with paraffin oil or extra virgin olive oil alternatively for the first two weeks, during the biweekly application of DMBA at 0.5% diluted in paraffin oil or olive oil for five weeks and daily until the twentieth week. The animals in the DMBA-OO and olive oil groups received an additional diet of mature *Picual* olives. The DMBA-PO carcinogen effect (35 carcinomas) is 100% and the inhibitory effect 0. The use of olive oil as DMBA solvent and the *ad libitum* diet with *Picual* olive has an inhibitory effect of 80%, with only three intraepithelial carcinomas and four verrucous carcinomas occurring and no invasive carcinoma.

Keywords: DMBA, carcinogenesis, olive oil, chemoprevention, squamous cell carcinoma

1. Introduction

One of the first models of experimental carcinogenesis in animals was carried out by Salley in 1954 [1]. After applying various carcinogens, including 9,10-dimethyl-1,2 benzanthracene on the oral epithelium of Syrian hamsters for 3 months, Salley was able to verify the existence of squamous cell carcinomas (SCC) and lymphatic metastases. Subsequently, several authors have standardized this model and repeated it in order to achieve new knowledge about DMBA and the process of experimentally induced carcinogenesis [2, 3].

7,12-Dimethylbenz[a]anthracene (DMBA) is a polycyclic aromatic hydrocarbon which may, on its own, induce premalignant lesions and carcinomas within a few weeks after it is administered in mucosae [4, 5]. Commonly, it has been used in combination with ethanol as a promoter. DMBA is released after the combustion of tobacco -especially with cigarettes- or from animal fat when meat is grilled, and is also found in smoked meat and fish. This substance is, therefore,

strongly involved in the carcinogenesis of oral, bronchiopulmonary and digestive tract malignancies [6–9].

In order to discover new drugs with cancer preventive effects, some authors have obtained promising outcomes at basic research level, specifically with substances such as salvinolic acid B [10] -derived from *Salvia miltiorrhiza*, used in fluorescence-, isothiocyanates [11] -synthetic derivatives of cabbages, squash, turnips and turnip greens-, *Buddleja incana* leaves, a tree that grows in Peru and Bolivia, Toona sinensis leaves [12], and olive oil extracts [9, 13–15]. In relation to the latter, and especially regarding its phenolic compounds, its antioxidant and cardiovascular protective properties are well known. In this sense, we have data stating that olive oil may act as preventive or inhibitor of carcinogenesis, and could even modify the nature of premalignant lesions that have already arisen, providing them a more benign and indolent behavior [14, 16].

2. Objective

To experimentally test the inhibitory effect on the carcinogenesis process of *Picual* variety extra virgin olive oil.

3. Methods

Forty-four male hamsters (*Syrian Golden*), 4-6 weeks old and weighing 60-80 g, were divided into four groups (two control and two experimental):

- Experimental DMBA-PO group (DMBA from Sigma Chemical Co.), 12 animals. The oral pouches were brushed daily with paraffin oil (PO) in the first two weeks. Then, a solution of 0.5% DMBA and PO was administered on Mondays and Fridays for five weeks; alternatively, PO was applied on Tuesdays, Wednesdays and Thursdays at the same time. Thereafter, animals received daily PO until the twentieth week. All hamsters were fed with standard feed, and *ad libitum* water. **Figure 1**.

- Experimental DMBA-OO group (DMBA from Sigma Chemical Co.), 12 animals. The oral pouches were brushed daily with OO in the first two weeks. Then, a solution of 0.5% DMBA and OO was administered on Mondays and Fridays every two days, for five weeks. Thereafter, animals received daily OO until the twentieth week. These hamsters were fed with standard feed, *Picual* variety ripe olives, and
  ad libitum water.

- Control DMBA-PO group, 10 hamsters. The oral pouches were brushed daily with PO for twenty weeks.

- Control DMBA-OO group, 10 hamsters. The oral pouches were brushed daily with extra virgin OO. Also, animals received diet with standard feed, *Picual* variety ripe olives, and *ad libitum* water for twenty weeks.

The animals in each group were sacrificed after twenty weeks. Then, a macroscopic description and histological analysis of the induced tumors in the oropharynx, esophagus and stomach were performed.

Figure 1.
The hamsters.

A carcinogenic effect of 100% was assigned to the total number of induced tumors in hamsters who received DMBA-PO. The inhibitory effect in the DMBA-OO group was established by the percentage difference over 100. An inhibitory effect >50% was considered significant in the DMBA-OO group.

This research work was examined and approved by the Ethical Committee for Animal Experimentation of the University of Seville (November 7, 2005). It met the requirements for experimentation with animals and was in accordance with the regulations in force in Spain and elsewhere the European Union.

4. Results

4.1 Macroscopically

The groups exposed to DMBA showed tumors of different characteristics. Nonspecific lesions and others more suggestive of malignancy were found in the oral pouches of the DMBA-OO group, with a predominance of the former. These findings included leukoplakia, denudation of the mucosa, ulcerations or tumors with a benign appearance. However, tumors in DMBA-OO group were less common and smaller than in DMBA-PO (**Figures 2A** and **B**).

On the other hand, the DMBA-PO group mostly showed malignant-looking neoplastic formations in the oral mucosa, such as ulcerated nodules, necrosis areas, exophytic and verrucous tumors, and areas with abundant vascularization.

In addition to the oral pouches, both DMBA groups presented tumors in the esophagus and stomach. Maximum and minimum measures of all lesions are shown in **Table 1**.

No visible lesions were found in the control groups which only received paraffin oil or olive oil.

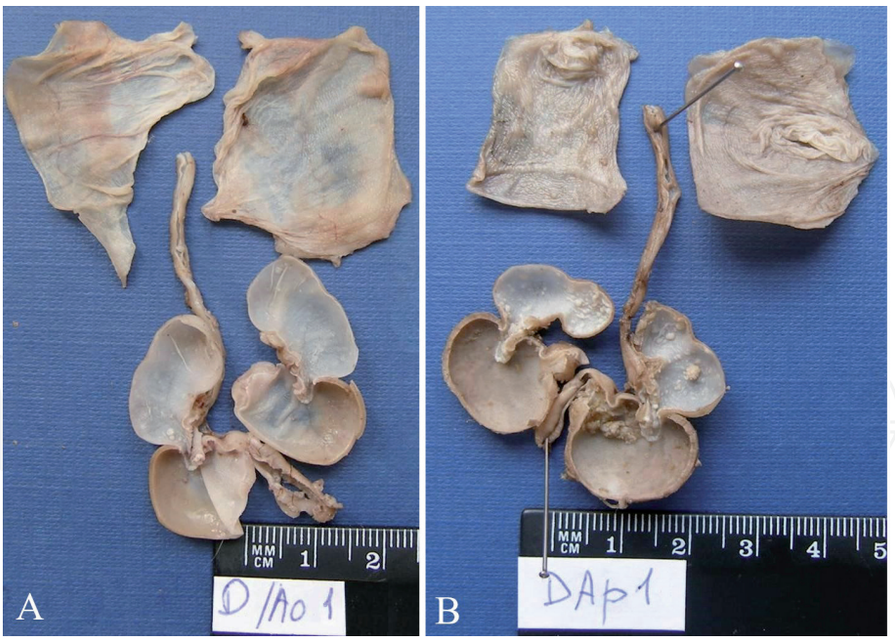

Figure 2.
Macroscopic comparison of the digestive tract of two animals belonging to the DMBA-OO group (A) and DMBA-PO group (B). (A) The oral pouches, esophagus and stomach showed few small lesions and benign appearance. (B) The oral pouches, esophagus, and stomach showed tissue retractions, larger tumors and apparently more malignant lesions.

Histological lesion	Location	Number of lesions		Mean diameter (Ø mm)	
		DMBA-PA	DMBA-OO	DMBA-PA	DMBA-OO
SQUAMOUS PAPILLOMA	Oral epithelium	1	11	1	0.14 (0.1-0.2)
	Esophagus	9	19	0.6 (0.2-1.2)	0.34 (0.2-1)
	Stomach	14	21	0.7 (0.3-1.2)	0.5 (0.2-1)
INTRAEPITHELIAL CARCINOMA	Oral epithelium	20	2	0.2 (0.1-0.4)	0.3 (0.3)
	Esophagus	0	1	0	0.3
	Stomach	1	0	0.6	0
VERRUCOUS CARCINOMA	Oral epithelium	1	2	1.7	4 (1-7)
	Esophagus	3	0	0.9 (0.8-1)	0
	Stomach	5	2	1.5 (0.8-2)	1.5 (1.2-1.8)
INVASIVE CARCINOMA	Oral epithelium	1	0	0.5	0
	Esophagus	2	0	3 (2-4)	0
	Stomach	2	0	5.3 (1.5-9)	0

Table 1.
Number, size and type of tumors in DMBA groups at 20th week.

4.2 Microscopically

The histological study at 20 weeks evidenced different types of lesions, demonstrating a complete carcinogenesis process in both DMBA groups: Squamous papillomas, intraepithelial carcinomas, verrucous carcinomas and invasive SCC.

SQUAMOUS PAPILLOMAS: Papillary projections lined with squamous epithelium were noted, showing hyperkeratosis and epithelial thickening. No atypia or mitotic activity was observed (**Figure 3**). Twelve papillomas were found among the groups exposed to DMBA (one in the DMBA-PO group and eleven in the DMBA-OO group). The differences regarding incidence of this kind of lesion were statistically significant (p .004).

INTRAEPITHELIAL CARCINOMAS (**Figure 4**): Twenty four intraepithelial carcinomas were identified. Twenty one occurred in the DMBA-PO group, and three in the DMBA-OO group. The differences observed between both groups were statistically significant (p .003).

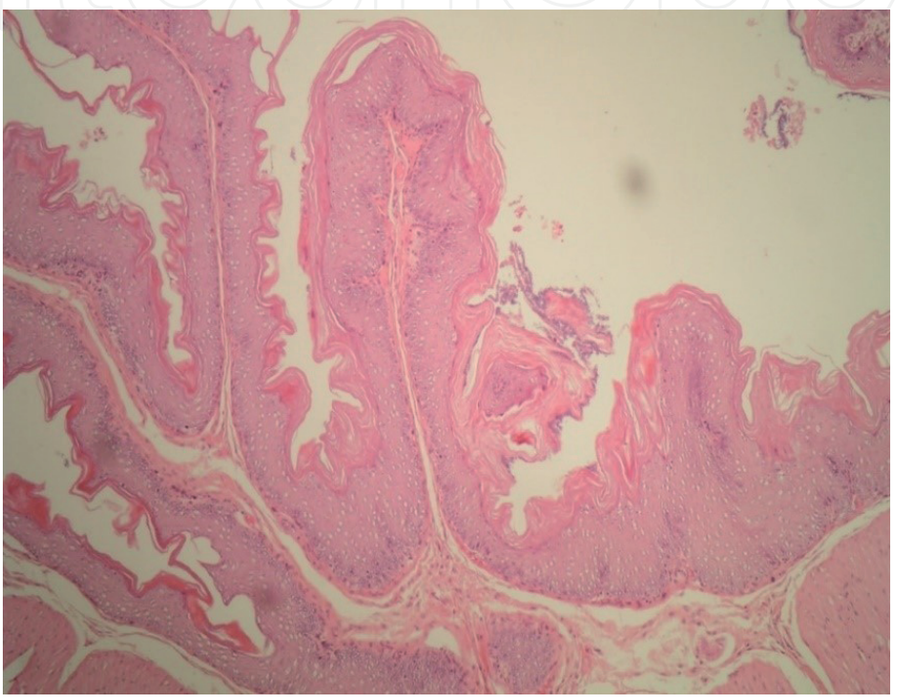

Figure 3.
Squamous papilloma is an exophytic lesion which shows typically papillary growth and highly differentiated epithelium.

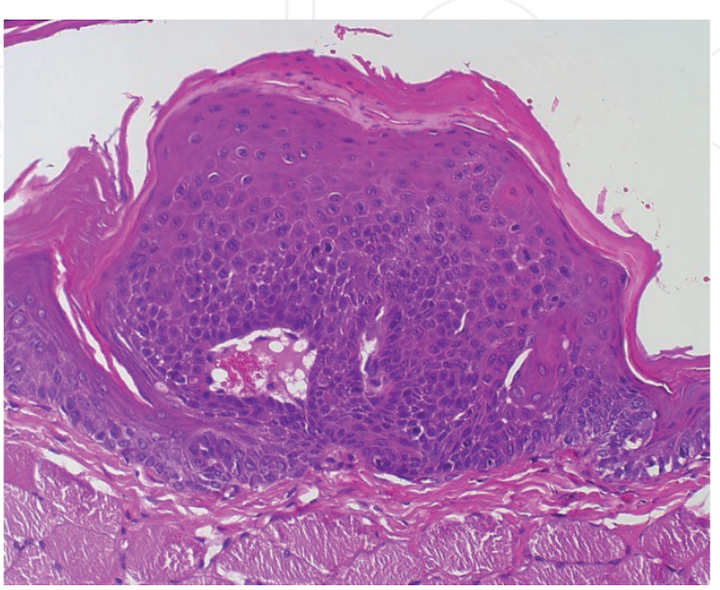

Figure 4.
Intraepithelial carcinoma is classically characterized by full-thickness with hyperkeratosis and parakeratosis, hypercellurality, nuclear atypia and mitotic figures. The epithelium-stroma interface is preserved.

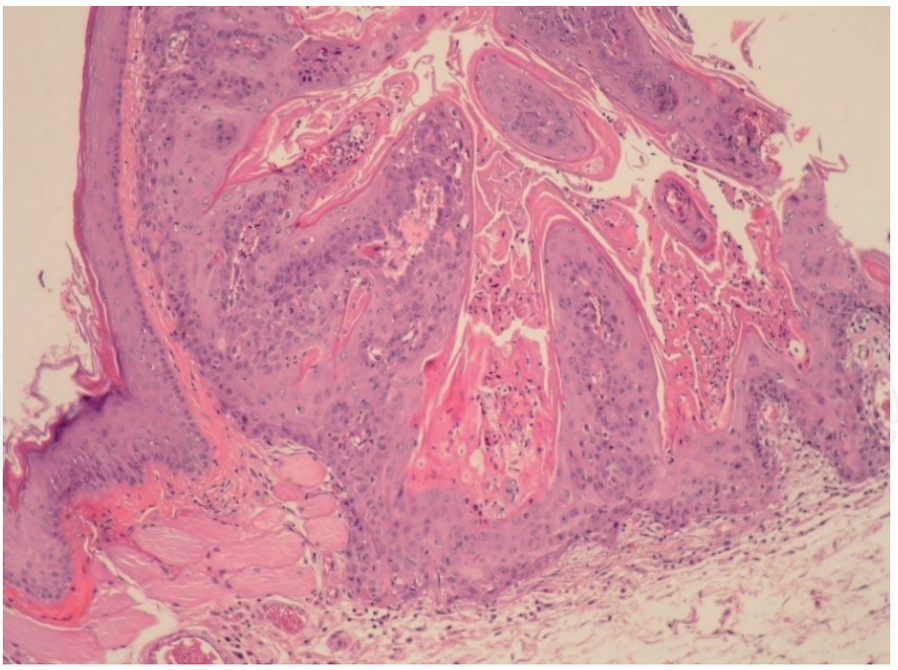

Figure 5.
SCC, verrucous carcinoma. Verrucous carcinoma is warty-appearing, highly differentiated, and shows hyperkeratosis. There is minimal atypia, abundant eosinophilic cytoplasm and normal mitotic figures. No invasion of the stroma by isolated neoplastic cells was observed.

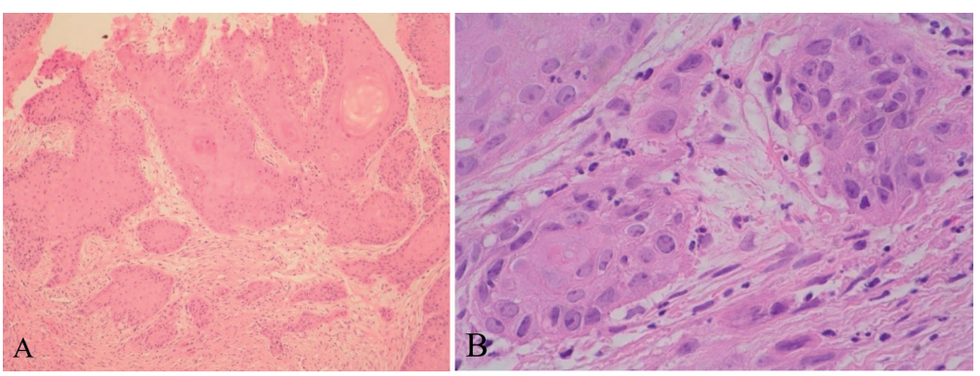

Figure 6.
Invasive SCC (A). The SCC is composed of infiltrating islands or nets of malignant cells, which form an irregular growth pattern (B).

Lesion		DMBA-PA (%)	DMBA-OO (%)
INTRAEPITHELIAL CARCINOMA	Carcinogenic effect	21 (100)	3 (14.3)
	Inhibitory effect		(85.7)
VERRUCOUS CARCINOMA	Carcinogenic effect	9 (100)	4 (44.4)
	Inhibitory effect		(55.6)
INVASIVE CARCINOMA	Carcinogenic effect	5 (100)	0
	Inhibitory effect		(100)
TOTAL CARCINOMAS	Carcinogenic effect	35 (100)	7 (20)
	Inhibitory effect		(80)

Table 2.
Carcinogenic and inhibitory effects of DMBA-PO/DMBA-OO, according to lesion subtypes.

VERRUCOUS CARCINOMAS: Several exophytic lesions with papillomatosis and infiltrative growth (**Figure 5**). Thirteen verrucous carcinomas were found, nine in the in the DMBA-PO group and four in the DMBA-OO group. This was not statistically significant (p .523).

INVASIVE CARCINOMAS (**Figure 6**): Light microscopy revealed epithelial proliferations that, like cords, invaded the adjacent stroma. In addition, the proliferating cells showed marked atypia and mitotic activity. Five invasive carcinomas were found in the DMBA-PO group.

The carcinogenic effect in the DMBA-PA group (35 carcinomas) corresponded to 100%, while in the DMBA-OO group (7 carcinomas), it was of 20%. According to the observed results, inhibitory effect seen in the DMBA-OO group was 86% for intraepithelial carcinoma, 56% for verrucous carcinoma, and 100% for SCC (**Table 2**).

No tumors were observed in the control animals.

5. Discussion

This research work about carcinogenesis is based on an experimental model of induced SCC after the administration of DMBA at 0.5% -dissolved in mineral oil- into the oral pouches of the hamster. We think, like Nagini and Kowshik [3], that the DMBA carcinogenesis model in hamster oral pouches is characteristic and highly representative of the "cancer induction". In addition, it is advantageous for the reproducibility of lesions, facilitates experimental research, and can be used as a test for chemotherapy and preventive agents. Also, in this work, the olive oil inhibitory effect on carcinogenesis has been studied alone -extra virgin olive oil applied before, during, and after DMBA, and *ad libitum* diet with ripe olives ripe of the *Picual* variety, from the olive harvest-, and combined -as a solvent for DMBA- [16].

The carcinomas produced in the upper gastrointestinal tract were SCC, similar to SCC of the oral mucosa in humans. These results coincide with those obtained in other experimental works [17, 18].

In oral carcinogenesis, using DMBA in hamsters, some authors have described the development of precancerous lesions and, subsequently, their progression towards intraepithelial carcinoma and invasive carcinoma after a few months. At 8 weeks, precancerous lesions usually appear. At 12 weeks, these evolve to intraepithelial carcinoma; eventually developing into invasive carcinomas at 18 weeks. This phenomenon, although slower, also occurs in humans [19]. The results obtained in our work resemble those of oral cancer progression described in the literature.

As in the field of experimental carcinogenesis, research on cancer prevention has continued to grow in recent decades, focusing on agents proposed for this purpose, although with few results yet. This is the case of the mediterranean diet, which is largely based on extra virgin olive oil, and that has been explored in the prevention of breast cancer [11, 13], and colorrectal cancer [9]. In the present work, the combination of olive oil as dissolvent, extra virgin olive oil applied before, during, and after DMBA application, and *ad libitum* diet with *Picual* variety olives, have been used as a preventive agent of DMBA carcinogenesis.

Menéndez et al. have shown that extra virgin olive oil polyphenols can inhibit erbB-2 malignant transformation of human breast cancer epithelial cells [14]. Owen et al. pointed out the importance of phenolic compounds isolated from olive oil as antioxidants and their anticancer potential [20].

In this sense, olive oil is composed of 99% different fatty acids, the most important being oleic acid, a monounsaturated fatty acid, with a richness of 60-80%,

and other fatty acids -palmitic, stearic, palmitoleic, linoleic, and linolenic-. The remaining 1% is made up of vitamin E and natural antioxidants. The most important antioxidants are phenolic compounds, present in the mesocarp of the olive and in extra virgin olive oil, which are mainly responsible for the antioxidant properties and which are not present in any other vegetable oil. For this reason, the diet added to the standard feed that the hamsters received was ripe olives from the tree, recently harvested and not spoiled. The variety of olive richest in phenolic compounds is the *Picual* variety.

Keys et al. demonstrated an inversely proportional relationship between the incidence of cardiovascular diseases and the adoption of eating habits established in seven countries in the Mediterranean area [21]. It seems that this "cardiovascular protection" resides in the creation of an anti-atherosclerotic plasma profile, which is defined by a decrease in total cholesterol and low-density lipoprotein (LDL) cholesterol levels, as well as by an increase in high-density lipoprotein (HDL) cholesterol. Some studies have attributed these properties to the high content of oleic acid -monounsaturated grade acid of the omega-9 series- of olive oil [22].

Analyzing our results, we can affirm that the combination of olive oil as a solvent for DMBA, extra virgin olive oil applied before, during, and after DMBA administration, and *ad libitum* diet with *Picual* olives has shown the capability to reduce the malignant progression of lesions already started, and modify the malignant phenotype of some neoplasms, making it less aggressive.

It is possible that in the DMBA-OO group, -COOH groups and unsaturated bonds of the vegetable oil could absorb or react with carcinogen, decreasing the effective concentration of the carcinogen. The antioxidant effect and anticancer properties of extra virgin olive oil expressed by some authors are reinforced [18, 19].

The study of the lesions at 20 weeks showed a total of 59 neoplasms in the DMBA-PO group and 58 in the DMBA-OO group, so there were no differences in the absolute incidence of tumors. However, clear differences were observed regarding the type of neoplasms and malignancy. Eighty-eight percent of the tumors in the DMBA-OO group corresponded to benign squamous papilloma-type tumors, compared to 41% that developed in the DMBA-PO group; the rest were carcinomas.

In addition, hamsters that did not eat ripe olives and did not receive extra virgin olive oil, developed 21 intraepithelial carcinomas, 9 verrucous carcinomas, and 5 invasive carcinomas; while animals that received the olive oil as a solvent for DMBA, extra virgin olive oil -before, during, and after DMBA-, and *ad libitum* diet with *Picual* olive developed 3 intraepithelial carcinomas, 4 verrucous carcinomas, and no invasive squamous carcinoma.

6. Conclusions

The inhibitory effect of extra virgin olive oil (*Picual* variety) on the experimental chemical carcinogenesis is higher than 50% for carcinomas, especially for intraepithelial carcinoma and invasive squamous carcinoma.

Furthermore, the tumors originated in animals who received DMBA mixed with olive oil were predominantly benign, specifically of the squamous papilloma subtype.

Therefore, these data suggest that the extra virgin olive oil and the diet with ripe olives extracted from the harvesting of the tree may modulate the experimental carcinogenesis with DMBA, originating very well differentiated and not very aggressive tumors.

Conflict of interest

The authors declare no conflict of interest.

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