Chemical carcinogenesis by DMBA (7,12-dimethylbenzanthracene) in female BALB/c mice: new facts

Carcinogenese quimica por DMBA (7,12-dimethylbenzanthracene) em camundongos femeas BALB/c: novos fatos

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Abstract

Polycyclic aromatic hydrocarbons are known carcinogens used in rodent experimental models. In this study, the carcinogen DMBA (7,12-dimethylbenzanthracene) was administered by gavage, diluted in corn oil, to female BALB / c mice at hebdomadary doses of 1 mg per animal for 1, 3, 6 or 9 weeks. Animals were weighed and monitored weekly until death. Remaining animals were euthanized at the age of 53 weeks. At necropsy, representative fragments of neoplasms were collected and routinely processed for histopathological analysis. Of all mice that received DMBA, 68.57% developed some type of tumor. Of the 70 mice treated with various doses of DMBA, 22 (31.43%) developed mammary tumors. The adenoacanthoma was the most commonly (18.75%) diagnosed histological type of breast cancer. Lung (15.71%), lymphoid tissue (11.43%), stomach (7.14%) and skin (2.86%) were also primary sites of tumor development. One third (33.33%) of the mice receiving 1 mg of DMBA developed lung cancer. Therefore, the administration of DMBA was shown to be an efficient model of carcinogenesis in mice, especially for the study of breast cancer, when using the highest dose, and lung, when using the lowest dose. Carcinogenesis models have been used for several purposes in cancer research. These results represent new facts for a classic carcinogenesis model.

Keywords: Carcinogenesis. DMBA. Mice. Lung neoplasms. Breast neoplasms.

Resumo

Hidrocarbonetos policíclicos e aromáticos são carcinógenos usados em modelos experimentais em roedores. Neste estudo, o carcinógeno DMBA (7,12-dimetilbenzantraceno) foi administrado por gavagem, diluído em óleo de milho, para camundongos BALB/c em doses hebdomadárias de 1 mg por animal por 1, 3, 6 ou 9 semanas. Os animais foram pesados e monitorados semanalmente até a morte. Os animais remanescentes foram eutanasiados com a idade de 53 semanas. Na necroscopia, fragmentos representativos das neoplasias foram colhidos e rotineiramente processados para exame histopatológico. De todos os animais que receberam DMBA, 68,57% desenvolveram algum tipo de tumor. Entre os 70 camundongos tratados com diferentes doses de DMBA, 22 (31,43%) desenvolveram neoplasias mamárias. O adenoacantoma foi o tumor mamário mais comumente diagnosticado (18,75%). Pulmões (15,71%), tecido linfoide (11,43%), estômago (7,14%) e pele (2,86%) foram também locais primários de desenvolvimento de neoplasias. Um terço (33,33%) dos camundongos que receberam 1 mg de DMBA desenvolveram neoplasias pulmonares. Portanto, a administração de DMBA foi considerada um modelo eficiente de carcinogênese em camundongos, especialmente para o estudo de neoplasias mamárias, quando a maior dose é utilizada, e de neoplasias pulmonares, quando utilizada a menor dose. Os modelos de carcinogênese química têm sido usados para diversos estudos na pesquisa em câncer, os resultados aqui apresentados mostram novos fatos para um modelo clássico de carcinogênese.

Palavras-chave: Carcinogênese. DMBA. Camundongos. Neoplasias pulmonares. Neoplasias de mama.

Introduction

Breast cancer is the second most common malignancy worldwide, second only to lung cancer and the first among women. Approximately, 22% of new cancer cases worldwide are breast cancer. Although breast cancer is considered to have a

relatively good prognosis if early diagnosed and treated appropriately, in Brazil, the median survival rate after five years is considered low compared with 73% in developed countries (BRASIL, 2009). Due to the high frequency with which it reaches the human population, the scientific community has made many efforts in trying to diagnose this disease early and treat it more effectively, but many studies have yet to be developed in order to elucidate mechanisms, prognostic factors, treatment and predictive factors and thus, establish better control of breast cancer. In this sense, several research groups have attempted to develop experimental models of mammary carcinogenesis, so that various stages of this process are better understood and provide advances in prevention and cancer control. Several models of radiation carcinogenesis (WINCEWICZ et al., 2010) and by chemical agents (CHEUNG et al., 2010; JEE et al., 2011; MATSUOKA et al., 2010) have been presented, using different concentrations in order to induce the appearance of tumors, like studies with 2-acetylaminofluorene (REIGH; STUART; FLOYD, 1978), 3-methylcholanthrene (SHI et al., 2003), N-*methyl*-N-*nitrosourea* (MAFFINI et al., 2008) and 7,12-dimethylbenzanthracene (MEDINA, 1974). Currently. the most used carcinogens are DMBA and N-*methyl*-N-*nitrosourea* (MEDINA; KITTRELL, 2005).

The main objective of this study was to establish a mouse model of mammary carcinogenesis so that it could be used in research to treat and prevent the development of breast tumors. The DMBA is a polycyclic aromatic hydrocarbon, a group that contains the majority of chemical carcinogens (CURRIER et al., 2005) and requires biotransformation in the liver and mammary gland (TAMULSKI; MORREAL; DAO, 1973). To obtain a greater induction of mammary gland tumors, the carcinogen is administered to young animals when these glands were undifferentiated, and then they were submitted to a high rate of cell proliferation during normal mouse growth (RUSSO; RUSSO, 1998).

Materials and Methods

Animals

Ninety female BALB/c mice from the animal facility of the Department of Pathology, School of Veterinary Medicine and Animal Science of the University of São Paulo were used. The animals were kept in a room with ventilation (16-18 air changes / hour), relative humidity (45 – 65%), controlled temperature (20 – 24°C) and light / dark cycle 12:12, given water and balanced diet *ad libitum.* The study has been approved by the Committee on Bioethics of the School of Veterinary Medicine and Animal Science of the University of São Paulo, Proc. nº. 1099/2007.

Carcinogenesis model

The carcinogen 7,12-dimethylbenzanthracene (Sigma-Aldrich, USA) was used, diluted in corn oil and administered by gavage to female BALB/c mice. Each animal received 1 mg per week until completing the total dose of 1, 3, 6 or 9 mg. The experiment began in the eighth week of life of animals, when they were weighed and examined by inspection and palpation weekly. Of the 90 animals, 20 were used as controls, receiving equally, by gavage, corn oil without the carcinogen. A total of 18, 15, 18 and 19 mice received, respectively, 1, 3, 6 or 9 mg of DMBA. All animals were euthanized when reaching inadequate physical condition, or at the end of 53 weeks (Figure 1).
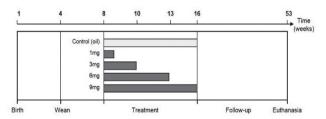

Figure 1 - DMBA carcinogenesis protocol used in the experiment
Source: (AVANZO, 2011)

After being anesthetized with sodium pentobarbital (250 mg/kg), euthanasia was performed by section of the abdominal aorta. Immediately after euthanasia, necropsy

was performed, and representative fragments of tumors and organs (lungs, liver, kidneys, spleen, and stomach) were collected. These samples were fixed in methacarn (60% methanol, 30% chloroform and 10% acetic acid) and routinely processed for embedding in paraffin. Histological sections were stained with hematoxylin and eosin to be examined under a light microscope.

Histopathological study of tumors

All mouse tumors were classified according to IARC Scientific Publication no. 111 (TURUSOV; MOHR, 1994).

Statistical analysis

Statistical analysis was performed using the GraphPad Prism (version 5.0, GraphPad Software Inc. USA). Two-way ANOVA was used to assess changes in body weight in relation to the rates during the experiment. To assess survival, Kaplan-Meier and long-rank test were used. The significance level was set at $p < 0.05$.

Results

Body weights

There was a significant variation in body weight ($p < 0.0001$) during the first 9 weeks of the carcinogenesis model (Figure 2). This significant change also occurred when doses were compared among them, and when the interaction of the doses and the time (weeks) was evaluated ($p < 0.0001$). Animals that received the dose of 9 mg presented the lowest body weight at the 9th week.

Survival

The longest survival time was observed at doses of 1 and 3 mg, with no difference in mortality rates between these two groups. The highest mortality rate was observed in the group exposed to 9 mg of DMBA. The first tumor appearance occurred at the 10th week in the 6 mg and at the 16th in the 9 mg group. Although the lesions appeared earlier in the first group (6 mg), the peak of mortality occurred earlier at 9 mg; the differences between these groups ranged from 6 to 14 weeks. In both groups, the experiment was completed at 40 weeks. The survival differences were statistically significant when all groups were compared with control ($p < 0.001$) and between 1 and 6 mg ($p < 0.001$), 3 and 6 mg ($p = 0.0017$) and 6 and 9 mg ($p = 0.0034$); the difference was not significant only between groups 1 and 3 mg ($p = 0.4414$) (Figure 3). The control group remained without any physical or behavioral changes throughout the experiment.

Neoplasms diagnosed in BALB/c mice treated with different doses of DMBA

At necropsy, tumors were observed in breast, lung, lymphoid tissue, digestive tract and skin, varying according to the dose and locations (Table 1). One of
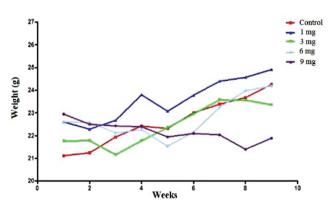

Figure 2 - Body-weight variation along the experiment for the control group and the 1, 3, 6 and 9mg DMBA administration groups

Source: (AVANZO, 2011)

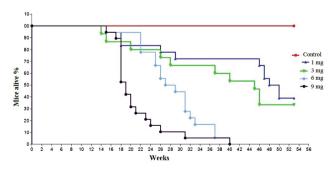

Figure 3 - Kaplan-Meier curve of the control group and the 1, 3, 6 and 9mg DMBA administration groups

Source: (AVANZO, 2011)

Table 1 –   Tumor incidence in different organs in control and DMBA-exposed groups – Sao Paulo – 2011

Neoplasias	Control	1mg	3mg	6mg	9mg	TOTAL
Breast	0.00%	11.11%	20.00%	38.90%	52.63%	22.23%
	(0/20)	(2/18)	(3/15)	(7/18)	(1019)	(22/90)
Lung	0.00%	33.33%	26.70%	0.00%	5.16%	12.23%
	(0/20)	(6/18)	(4/15)	(0/18)	(1/19)	(11/90)
Lymphoid tissue	0.00%	0.00%	13.30%	16.67%	15.80%	8.89%
	(0/20)	(0/18)	(2/15)	(3/18)	(3/19)	(8/90)
Digestive tract	0.00%	0.00%	6.70%	5.56%	15.80%	5.56%
	(0/20)	(0/18)	(1/15)	(1/18)	(3/19)	(5/90)
Skin	0.00%	0.00%	0.00%	0.00%	10.52%	2.23%
	(0/20)	(0/18)	(0/15)	(0/18)	(2/19)	(2/90)
TOTAL	0.00%	44.44%	66.67%	61.11%	100.00%	53.33%
	(0/20)	(8/18)	(10/15)	(11/18)	(19/19)	(48/90)

the animals had a tumor in the dorsal region near the column, developed hind limb paralysis, and was then euthanized.

During the experiment, among all mice that received DMBA, 68.6% developed some type of tumor. Of the 70 mice treated with various doses of DMBA, 22 (31.43%) developed mammary tumors. Adenoacanthoma was the most commonly diagnosed histological type of breast cancer. Lung (15.71%), lymphoid tissue (11.43%), stomach (7.14%) and skin (2.86%) were also primary sites of tumor development. One-third (33.33%) of the mice receiving 1 mg of DMBA developed lung cancer.

All doses of DMBA used in this study were able to induce breast tumor, which began to appear at the 10th week after the first dose of carcinogen. However, the vast majority of tumors appeared from the 16th week on.

Breast tumors were more frequent, with higher incidence in animals exposed to 6 and 9 mg and the non-breast occurred mainly in the final phase of the experiment. The lung was the second most frequent primary site, with higher incidence in the lowest dose. The histological classification of tumors observed is presented in table 2.

Among the histological types of tumors observed, adenoacanthoma was more frequent among breast, which included, besides, a cystic papillary adenocarcinoma, carcinosarcoma, undifferentiated tumor and fibroadenoma. In most of the animals, the tumors were highly infiltrative, with high mitotic activity, hemorrhage and extensive necrosis. Papillary carcinoma was the most frequent among lung tumors, which included, in addition, a solid mixed carcinoma and undifferentiated tumor. Squamous cell carcinoma was the only tumor type seen in the stomach. Images of tumor histological types observed are presented in figures 4 and 5.

Table 2 –   Cancer incidence in different tumor types in control and DMBA-induced groups – Sao Paulo – 2011

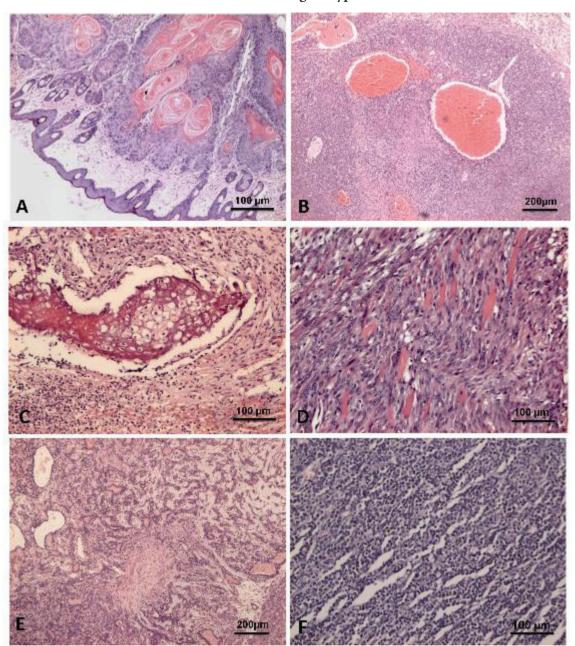| Neoplasias	Number of tumors	%
Breast	22	45.83
Adenoacanthoma	9	18.75
Cystic papillary adenocarcinoma	7	14.58
Carcinosarcoma	2	4.17
Fibroadenoma	1	2.08
Undifferentiated tumor	3	6.25
TOTAL 22	100.00	
Lung 11	25.00	
Lung papillary carcinoma	7	14.58
Mixed solid carcinoma	4	8.33
TOTAL 11	100.00	
Digestive tract	5	10.42
Gastric squamous cell carcinoma	4	8.33
Esophagus squamous cell carcinoma	1	2.08
TOTAL 5	100.00	
Lymphoid tissue - Lymphoma	8	16.67
Skin– keratoacanthoma	2	2.08
TOTAL 48	100.00	

Tumor histological types

Figure 4 – Breast cancer developed in DMBA-induced groups. A. Adenoacanthoma, HE, bar=100 μm. B. Cystic papillary adenocarcinoma, HE, bar=200 μm C. Carcinosarcoma with cartilaginous metaplasia, HE, bar=100 μm. D. Carcinosarcoma with muscular invasion, HE, bar=100 μm. E. Fibroadenoma, HE, bar=200 μm. Undifferentiated tumor, HE, bar=100 μm
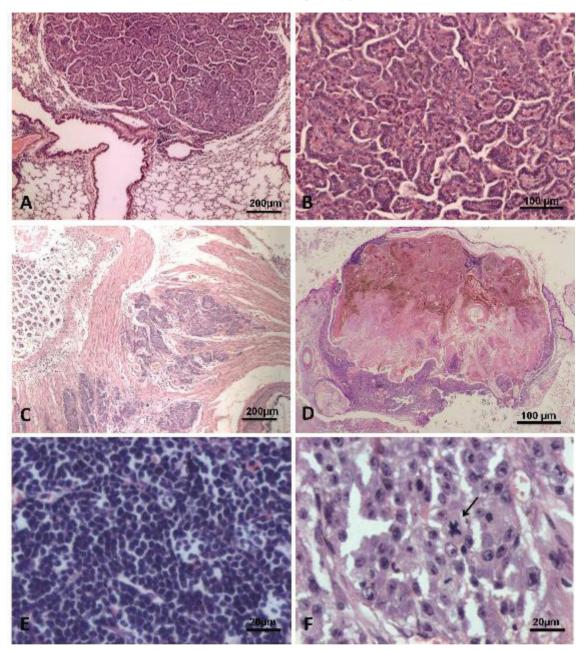
Source: (OLIVEIRA, 2012)

Tumor histological types

Figure 5 – A. Lung papillary carcinoma, HE, bar=200 µm. B. Lung papillary carcinoma, HE, bar=100 µm.
C. Gastric squamous cell carcinoma, HE, bar=200 µm D. Keratoacanthoma, HE, bar=100 µm
E. Lymphoma, HE, bar=200 µm. F. Atypical mitosis (arrow), HE, bar=20 µm
Source: (OLIVEIRA, 2012)

Discussion

The main objective of this study was to establish a model for the study of mammary carcinogenesis in BALB/c mice. The carcinogen DMBA has been chosen because it was used in many studies of mammary carcinogenesis (MEDINA, 1974; HASLAM; BERN, 1977; LANE et al., 1985; BARROS et al., 2004; CURRIER et al., 2005; MEDINA; KITTRELL, 2005; WIJNHOVEN et al., 2005; LU et al., 2006). Besides mammary tumors, in our study, neoplasms of diverse histogenesis have also been found, and we considered it important to report these findings, representing new facts for this carcinogenesis model.

All doses of DMBA used in this study were able to induce breast tumor, which began to appear at the 10th week after the first dose of carcinogen. However, the vast majority of tumors appeared from the 16th week. Likewise, other studies with the same strain of mice reported the appearance of tumors between the 10th and 17th weeks (LANE et al., 1985; MEDINA; KITTRELL, 2005). Two other studies reported, in mice, different incidences of mammary tumors with the dose of 6 mg of DMBA, 40% in one study (WIJNHOVEN et al., 2005) and 75% in another (CURRIER et al., 2005). Higher incidence of mammary tumors were observed in rats, reaching 100% after 13 weeks of the beginning of administration (BARROS et al., 2004). In this study, we found 45.83% of breast cancers among all tumors, and the incidence was greater with increasing dose.

It has been noted that the development of mammary tumors in non-DMBA treated mice is common, mainly in the final phase of the study (MEDINA; KITTRELL, 2005); this aspect has also been shown in our study. Currier et al. (2005) reported non-mammary neoplasms in DMBA model, including lung (15%), skin (10%) and lymphoid tissue (5%), although with lower incidence rates than those observed by us of 15.71%, 2.86% and 11.43%, respectively. It is known that diet has an influence on the development of tumors (EL-BAYOUMY et al., 2006; MEDINA; LANE; SHEPHERD, 1983), as well

as genetic characteristics of the strains involved and the climatic and environmental aspects associated with different countries, which may explain those differences.

The increased incidence and number of breast cancer was correlated with increasing dose of DMBA. Among the mechanisms that explain the development of mammary tumors by DMBA, there is the theory of activation of receptor/transcription factor AhR (aryl hydrocarbon receptor / transcription factor), a member of the family Per-ARNT-Sim (PAS), of transcription factors that influence the development, circadian rhythms and hypoxia responses (TROMBINO et al., 2000). The administration of DMBA results in increased AhR activity and induction of CYP1A1, CYP1A2 and other enzymes involved in xenobiotic metabolism (HOFFMAN; GAY, 1981). These enzymes convert the DMBA in epoxide mutagenic intermediates, the 7-hydroxymethyl-12-methylbezantracene (7-HMBA), 12-hydroxymethyl-7-metilbezantracene (12-HMBA) and 7,12-dimetilbezantracene that form DNA adducts. In the liver, the three metabolites above can be found, whereas in the mammary gland only monohydroxy (7- HMBA and 12-HMBA) are formed (TAMULSKI; MORREAL; DAO, 1973). These metabolites activate the AhR, mainly that complex with the heat-shock protein 90 (hsp90) and probably with other proteins; this has been only suggested but not described. When, for some reason, these two proteins dissociate, the AhR is free to translocate to the nucleus and dimerize with cofactor ARNT (aryl hydrocarbon receptor nuclear translocator), associating with specific transcription regulatory sequences in DNA. Once in the nucleus, they induce the expression of genes encoding growth factors and proto-oncogenes, including c-erb-2, c-myc, c-fos, c-Jun and H-ras (TROMBINO et al., 2000).

A higher expression of AhR, c-myc, cyclin D1 and Rb protein was found in DMBA-induced mammary tumors in mice. These proteins activate Wnt, NF-

kB signaling pathway and the prolyl isomerase Pin-1 pathway, suggesting that tumor development is related to the cell growth and anti-apoptotic pathways. The crucial role of the P450 system seems clear, specifically the CYP1A1 and CYP1A2, and the transcription factor AhR in DMBA carcinogenic mechanism in all tissues. However, genes involved in each type of neoplasm may vary and require further study. Furthermore, the possible reversal dose-effect observed when lung tumors are compared with other histological types must be considered.

In our study, the most frequently diagnosed breast tumor was the adenoacanthoma, which was different from other studies in which the simple adenocarcinoma was the most prevalent, followed by adenoacanthoma (LANE et al., 1985). It is important to state that the adenoacanthoma is not a mammary histotype frequently found in humans.

In order to study mammary carcinogenesis, doses of 3, 6 and 9 mg of DMBA are more suitable, since the 1 mg dose resulted in a low incidence of breast tumors. However, the choice of dose depends on the purpose of the study. Considering, for example, investigations into the chemopreventive activity of an active principle, the lower dose is recommended (SPORN; SUH, 2002).

We conclude that the DMBA resulted in an effective model of mammary carcinogenesis primarily, but also for stomach and lymphoid tissue neoplasms in higher doses (3, 6 and 9 mg), with incidence varying proportionally with increasing dose. In lower doses of 1 and 3 mg, DMBA may be useful in a rodent model to study lung carcinogenesis.

Acknowledgments

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