Original Article

Chemical Carcinogenesis in Rat (*Rattus norvegicus*) Submandibular Gland Using DMBA

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Abstract

Objective: This paper aims to investigate the neoplasms produced after the injection of 9,10-Dimethyl-1,2-benzantracene (DMBA) in rat submandibular glands. **Material and Methods**: Twenty eight 3-month old male rats (*Rattus norvegicus*), approximate weight of 300g, were used. The animals were divided into four groups of seven subjects each. After anesthesia and proper preparation, all animals were injected with 0.1ml of 2% DMBA in the left submandibular gland. By the end of the fifth, tenth, fifteenth and twentieth weeks, the animals were sacrificed by lethal doses of anesthetics. **Results**: The results in the fifth week presented seven cases of chronic sialadenitis. After ten weeks, one case of ductal cell atypia was evident, along with two cases of squamous cell carcinoma and four cases of chronic sialadenitis. Between the fifteenth and twentieth weeks, the cases were diagnosed as follows: three cases of hyperemia; three cases of squamous cell carcinoma; one case of sarcoma and seven cases of carcinosarcomas. **Conclusions**: This study allowed the investigation of glandular carcinogenesis after DMBA injection, from the beginning of inflammatory changes to the neoplastic manifestation of tumors. Salivary carcinogenesis can provide beneficial material for the study of rare human salivary gland neoplasms like sarcomas, carcinomas and carcinosarcomas.

Keywords: Carcinogenesis. Salivary gland neoplasms. Rare tumors. 9,10-Dimethyl-1,2-benzanthracene.

Introduction

According to the Head and Neck Classification of Tumors, released in 2005 (World Health Organization -WHO), the global incidence of all salivary gland tumours varies from 0.4 to 13.5 cases per 100,000 individuals. When only malignant neoplasms are considered, the frequency is between 0.4 to 2.6 cases per 100,000 individuals. Salivary gland tumor incidence is different between ethnic groups and can be related to ionizing radiation. Regarding the site, most tumours occur in the parotid gland, followed by submandibular, minor and sublingual glands. Females are more affected than males.[1]

Animal carcinogenesis bioassay, targeting

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the salivary glands, was first reported in 1942, by Steiner.[2-5] The carcinogen of choice, according to the literature, is a component of the polycyclic aromatic hydrocarbon (PAH) group named as 9,10-Dimethyl-1,2-benzantracene (DMBA). It is suitable for use as a pellet or diluted for injection. The animals used for research are rats and mice.[6-8] As stated by Cataldo et al.[2] and Enzmann et al.[9] rats are the animals of choice in experimental salivary gland carcinogenesis.

Although the investigative technique of carcinogenesis in animal salivary glands is not a new research field, the methodology differs among researches. In attempt to produce salivary gland neoplasms, we carried out a chemical induction in rat salivary glands using DMBA injection. The present study was aimed to review and compare the histopathologic data presented by the consulted authors with our results. The occurrence of rare tumors was discussed taking into account the updated human salivary neoplasm classification.

Material and Methods

Animals

Twenty eight 3-month old male rats (*Rattus norvegicus*), approximate weight of 300g, were used. The animals were divided into four groups of seven rats each and maintained in cages at room temperature. They were fed with water and food *ad libitum*, provided by São José dos Campos Dental School – UNESP. The research protocol of this study was approved by the Ethical Research Committee of this Institution (038/2004-PA/CEP) and followed the guidelines for proper handling of laboratory animals of the Brazilian Association for Experimental Laboratory Animals (COBEA).

Surgical Procedures

All animals were anesthetized with a solution of Xylazine Chloride (Rompum, Bayer, Sao Paulo, Brazil), as a muscular relaxant, associated with Ketamine base (Dopalen, Agribrands do Brasil, Divisão Vetbrands Saúde Animal, Jacarei, Brazil) as a general anesthetic, in a 1:0.5ml proportion. The solution was used following a dosage of 0.1ml per 100g of rat body weight. All animals were shaved in the neck. After antisepsis, one oblique ventral neck incision, followed by dissection was performed in each animal. The left submandibular gland was injected with 0.1ml of 2% DMBA in acetone (Figure 1). The skin was closed with 3-Ø silk suture. No control group was investigated. The drug DMBA is a well-documented complete carcinogen and the purpose of the experiment was a neoplasm production.

By the end of the fifth, tenth, fifteenth and twentieth weeks, the animals were sacrificed by lethal doses of anesthetics. The submandibular salivary glands were removed and fixed in 10% buffered formalin. After fixation, they were embedded in paraffin, sectioned at 5 micrometers and stained with hematoxylin and eosin for microscopic examination.

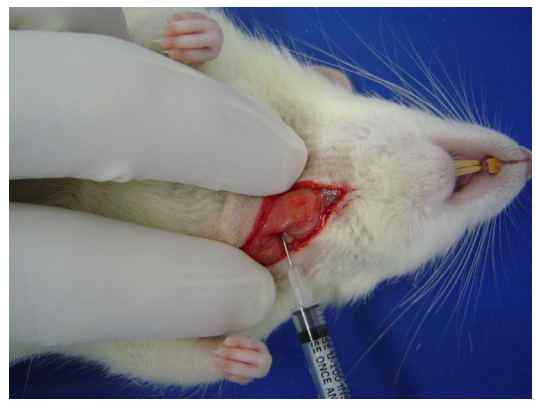

Figure 1 – Injection of 0.1ml of 2% DMBA/acetone in the left submandibular salivary gland

Results

Clinical Examination

The enlargement of the left submandibular salivary gland was first noticed after ten weeks of the DMBA injection. There were no signs of skin lesions or ulceration during the experiment (Figure 2). Some animals, in the last group, experienced walking limitations due to neoplasm's dimensions.

Histopathologic Examination

The results of the fifth week presented seven cases of chronic sialadenitis. After ten weeks, one case

of ductal cell atypia was evident, two cases of squamous cell carcinoma and four cases of chronic sialadenitis were also seen. In the fifteenth week, the cases were diagnosed as one case of hyperemia, two cases of squamous cell carcinoma and four cases of carcinosarcoma. The results of the twentieth week group revealed two cases of hyperemia, one case of sarcoma, one case of squamous cell carcinoma and three cases of carcinosarcoma.

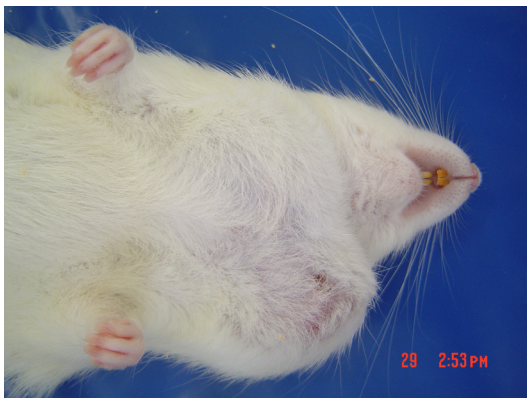

Figure 2 – Animal exhibiting an enlargement in the left side of the neck after 20 weeks

The non-neoplastic lesions were not striking microscopically. Sialadenitis revealed a severe acinar atrophy, dilatation of the ducts and a stroma composed, almost exclusively, by neutrophils (Figure 3). Hyperemia cases were constituted by stromal oedema, vascular proliferation and few inflammatory cells. The single case of duct atypia consisted of hyperplastic ducts exhibiting scarce cellular atypia. Scattered inflammatory infiltrate and necrosis completed the case.

The sole case of sarcoma presented spindle cells arranged in compact fascicles. The cells were fusiform with a hyperchromatic nucleus in some fields. Other images showed pleomorphic cells with a moderate amount of mitotic figures.

Basically, all carcinomas resembled squamous cell carcinomas. They were composed by well-differentiated malignant cells arranged in a solid parenchyma and associated with a single large structure sometimes hyperplastic, sometimes atrophic and filled with keratin. The stroma exhibited variable amounts of duct-like components and invasion by some strands of malignant neoplastic epithelial cells forming keratin pearls (Figure 4). Other clusters of moderate to poor-differentiated cells showed abnormal mitosis and darkly stained nuclei (Figure 5).

In the diagnosed carcinosarcomas, the carcinomatous fields were similar to those in squamous cell carcinomas. The sarcoma fields exhibited moderate differentiation and resembled fibroblastic cellularity, sometimes blended with the epithelioid component (Figure 6).

In summary, we were able to diagnose 3.6% of cellular atypia, 3.6% of sarcoma, 10.7% of hyperemia, 17.9% of squamous cell carcinoma, 25% of carcinosarcoma and 39.4% of chronic sialadenitis.

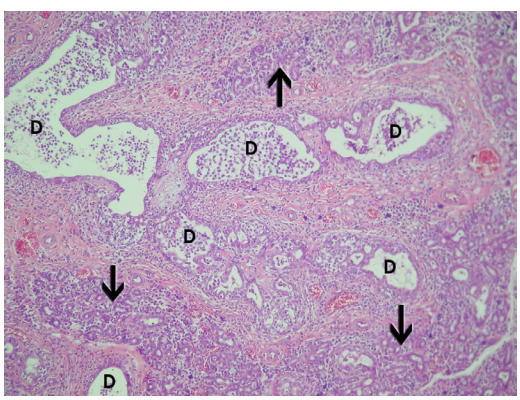

Figure 3 – Sialadenitis. The ducts are enlarged and filled with neutrophils (D). The acinar component disappeared, giving place to a mainly neutrophilic inflammatory infiltrate surrounding ductal remnants (arrows). Hematoxylin & Eosin. 100x

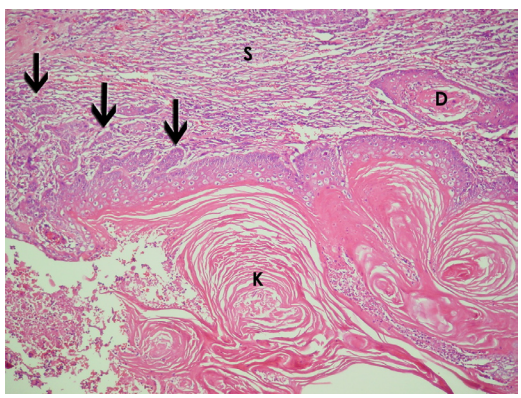

Figure 4 – Carcinoma. The epithelial component displays an abundant formation of keratin (K) filling a cyst-like lumen. The stroma (S) shows nests of tumor infiltration (arrows) and a duct-like structure (D). Hematoxylin & Eosin. 100x

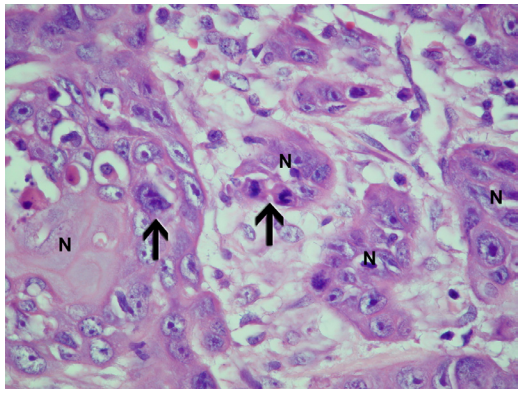

Figure 5 – Epithelial cell nests (N) presenting pleomorphism and abnormal mitosis (arrows). Hematoxylin & Eosin. 630x

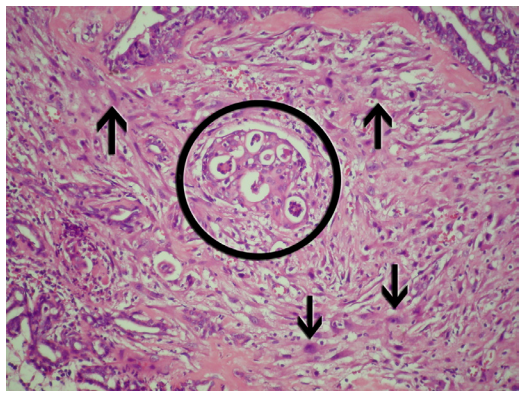

Figure 6 – Centrally, a cluster of a cribriform ductal component surrounded by a sarcomatoid tissue. The sarcomatoid cells are markedly pleomorphic (arrows). Hematoxylin & Eosin. 200x

Discussion

Carcinogenesis is a multistep process characterized by genetic changes followed by cell malignization and tumor development. These changes involve genetic damage, disruption of cell division genes and critical lack of control in cells growth. According to Fassoni et al.,[10] DMBA is a well-known carcinogen responsible for all the stages of oncogenesis. This concept was also shared by us.

In 1910, Löwestein tried to induce tumors in animal salivary glands. However, as he did not use PAH, he was not able to produce neoplasms. Steiner in 1942, using pellets of various PAH, was able to induce carcinomas and adenocarcinomas in rats and mice.[2,4-5] Standish[5] was interested in the histhologic changes after salivary gland DMBA induction. But only Cataldo et al.[2] elucidated the natural history of squamous cell carcinomas after implantation of DMBA in rat salivary glands. The subsequent authors used Cataldo and coworkers' methodology as a gold standard. The use of DMBA injection or pellet implantation revealed similar findings among the researches, although the methodology of one author was different from the other. As the literature data were not reliable or were lacking in information, we decided to inject 0.1ml of 2% DMBA in acetone, after an oblique neck incision from the mandible to the left shoulder of each rat. This incision allowed us to expose directly the rat's submandibular gland.

Regarding carcinogenesis in rat salivary submandibular glands using DMBA pellets, Cataldo et al.[2] defined four phases after studying the methodology of Standish.[5] The degenerative phase showed inflammatory reaction, disappearance of acini and persistence of ductal components. The second phase, called proliferative phase, was composed of a proliferation of duct-like structures sometimes associated with hyaline-like connective tissue. The metaplastic phase presented metaplasia of cystic structures. Those were composed of stratified squamous epithelium and proeminent production of keratin. The last phase, named as malignant neoplastic phase, presented squamous cell carcinomas arising from the wall of epidermoid cysts. The neoplasms seemed to be very invasive. The authors also quoted that the experiment took twenty weeks and, from the sixteenth to the twentieth week, the stroma showed atypical fibroblasts.

Although some findings of our experiment resembled the data presented by Cataldo et al.,[2] we were able to observe some singularities in regard of DMBA injection. First of all, we preferred the term sialadenitis to name the first phase. Our second phase disclosed two cases of squamous cell carcinoma and one case of cellular atypia among four cases of sialadenitis. These findings allowed us to conclude that the metaplastic phase was concomitant to the neoplastic phase and not one separate from the other. The third and fourth phases were markedly tumoral, despite of some lesions diagnosed as non-neoplastic. We were able to diagnose the following types of neoplasms: sarcoma; carcinoma and carcinosarcoma.

The salivary glands malignancies found in our experiment are very rare in human pathology. Squamous cell carcinomas, sarcomas and carcinosarcomas account, respectively, for less than 1%,[11-12] 0.3%[13] and 0.16 to 0.2%[14-15] of salivary tumors.

Our study points to a carelessness by other

researches in the evaluation of the encounter of stromal and epithelial components. Cataldo et al.[2] were not able to name the neoplasms accordingly because, as stated by Alvarez-Canãs e Rodilla[14] carcinosarcomas were first reported in 1967. However, the authors that followed Cataldo et al.[2] described carcinomas and/or sarcomas but never named the neoplasms regarding the association of both patterns. This issue was not investigated by Schmutz e Chaudhry,[16] Wigley e Carbonell,[17] Zaman et al.[18] and Ogawa et al.[19] Takai et al.[20] coined the term "collision tumors" for the mixed neoplasm encounter in mice. Watanabe at al.[21] referred to the occurrence of two cases of carcinosarcoma after twenty-four weeks of DMBA pellet implantation. Although they were able to diagnose correctly the tumors, their paper was shallow when discussing carcinosarcomas.

We understand that further investigations must be carried out in our experiment to disclose, for instance, the immunohistochemical profile of the neoplasms. It is fair to say that a new focus must be driven to animal salivary gland neoplasm induction. As human salivary gland neoplasms, such as squamous cell carcinomas, sarcomas and true mixed tumors are rare, experimental carcinogenesis could provide enough material for researches to study and understand these uncommon lesions.

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