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Histological Evaluation of the Effect of Bee Venom on Squamous Cell Carcinoma of the Tongue

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ABSTRACT

Background: Bee venom (apitoxin) composition contains numerous biologically active peptides, including melittin (main component), apamin, peptide mast cell degranulation and enzymes and non-peptide components such as histamine. While the effect of bee venom, according to the literature, would be beneficial in rheumatic diseases, evidence of its action on tumor pathology is inconclusive. In this paper, the potential chemopreventive effect of apitoxin on tumor lesions of the tongue was studied. **Materials and Methods:** Two groups of rats were used. Both groups were subjected to the carcinogenic action of 4-NQO. One group served as a control, and the other group (experimental) received subcutaneous bee venom that was applied weekly. The incidence of dysplastic lesions and tongue squamous cell carcinoma (TSCC) in both groups was determined microscopically. In addition, peritumoral mast cell density was determined at 30 weeks of exposure to a carcinogen. **Results:** The results showed no significant reduction in the incidence of TSCC. The peritumoral mast cell density found in dysplastic lesions and TSCC was lower than in the normal tongue tissue. **Conclusions:** The findings of this study indicate poor action of bee venom as a preventive drug in the emergence of TSCC. The lack of recruitment of mast cells indicates that every tumor is influenced by its particular microenvironment.

INTRODUCTION

Globally, oral cavity cancer ranks fifth among the different known malignancies. In the United States, Siegel *et.al.* (2015) reported that there are 14,000 new cases per year of lingual cancer, and it is estimated that approximately 50% will die as a result thereof [1, 2]. In addition to smoking and drinking alcohol, which remain the two major carcinogenic factors leading to lingual cancer, human papillomavirus infections (HPV) and low socioeconomic status are implicated as risk factors leading to the development of this cancer [3, 4].

The current treatments of surgery, radiotherapy and chemotherapy, alone or in combination, have not shown progress in increasing survival. The primary treatment is associated with high mortality and loss of organ function, while 50% die of recurrences [5].

On the other hand, there is growing evidence on the effects of new substances in certain cancers, including synthetic compounds and products of animal, vegetable or mineral origin. Among the animal substances being studied are components of some poisons such as those from scorpion [6] or bee [7].

Bee venom (apitoxin) contains in its composition biologically active peptides, including melittin (main component), apamin, peptide mast cell degranulation, enzymes (phospholipases, hyaluronidase) and histamine as a non-peptide component [8].

While the effect of apitoxin, according to the literature, would be beneficial in diseases of rheumatic origin, evidence of its action on tumor pathology is inconclusive, although some studies have reported a strong apoptotic action of bee venom in lung cancer [9] and liver [10] tumors.

Based on this previous evidence, the present work was designed to study the possible chemopreventive effect of apitoxin in tongue tumor lesions, and a secondary objective was to examine migration of mast cells in the peritumoral area.

MATERIALS AND METHODS

Twenty Wistar male rats were used for this study. The experimental procedure was carried out in compliance with ethical principles for animal research, according to the revised protocol

approved by the Institutional Committee on Care and Use of Experimental Animals (CICUAL) of the Faculty of Medicine of the Cuyo National University.

The rats were separated randomly into two groups: 10 animals were assigned to the control group (group 1), and 10 were assigned to the experimental group (group 2).

To achieve the development of lingual squamous cell carcinoma, nitroquinoline (4-NQO) was used. The substance 4-NQO was obtained as a powder (Sigma, St. Louis, MO, USA, cat. # N8141) and was dissolved in drinking water at a final concentration of 0.02 g / l (20 ppm). Both groups were subjected to the action of 4-NQO dissolved in drinking water. The water was changed once a week, and food was provided *ad libitum*. Common clinical controls were measured, including weight, diet and change in hair color.

On the other hand, rats in the experimental group (group 2) were not only given water with 4-NQO but were also provided with a dose of subcutaneous apitoxin every week. Bee venom was diluted (Apismillifera 3X, equivalent to 1 mg / ml) at 0.5 mg / kg.

Getting tongue tissue samples:

The animals in both groups were sacrificed 30 weeks after the start of the administration of specific treatments. The tongue was removed and photographed for macroscopic analysis. The whole tissue was separated into two sections: the anterior segment (in front of the V lingual) and posterior segment (behind the lingual V). Each tissue sample was set in paraformaldehyde 10% solution.

Sections of tongue samples were embedded in paraffin and then cut and stained with hematoxylin-eosin (H&E) in accordance with the standard protocol.

In addition, Toluidine blue staining was also performed to identify mast cells in the tissue sections.

Macroscopic evaluation:

The samples were classified as clinically normal tissue (TN), white lesion (WL), exophytic lesion (EL) or ulcerated lesion (UL).

Microscopic or histopathologic evaluation:

The histological analysis was performed using light microscopy (Zeiss Axiostar). Tongue tissue sections were grouped according to pathological diagnosis: a) normal (TLN), b) epithelial dysplasia (D) and c) squamous cell carcinoma (SCC).

Quantification of mast cells:

In samples stained with toluidine blue, mast cells showed purple granules in the cytoplasm.

Mast cells in the peritumoral zone were quantified by counting the positively stained cells on each tissue section and were expressed as cells/mm2 tissue area.

Statistical analysis:

Data are expressed as the means ± SD. Statistical significance comparing different sets of mice was determined by analysis of variance (ANOVA) and Tukey post-hoc test using InStat GraphPad software (InStat Software, San Diego, CA). A P value < 0.05 was considered statistically significant.

RESULTS

Macroscopic findings in the evaluation were as follows:

In group 1 (control), 20% of lingual organs indicated no evidence of clinically detectable pathological changes (normal tongue tissue:NTT) in both the lingual surface of the dorsal posterior third and the anterior lingual area. In the remaining 80%, lesions were detected as having different sizes ranging from small well-defined white papules to extensive exophytic formations.

The clinical examination in the experimental group (10n) found that 60% of animals had normal tongues, while 40% of the remaining animals showed tumor lesions (figure1) on the tongue (20% were indurated or white lesions and the other 20% were exophytic lesions).

One of the animals developed a large exophytic and ulcerated tumor mass, which compromised the lower lip and extended to the floor of the mouth.

Histopathology evaluation:

In the experimental group subjected to the effect of 4- NQO and the application of subcutaneous apitoxin, microscopic examination (figure2) found a 40% incidence of squamous cell carcinoma. Pleomorphic tumor cells and submucosal invasion forming abundant keratin nests were observed. Histological examination of the excised lesion of the lower lip repeated this pattern while accentuating disorganization and extension into the muscle layer of the lip.

In the control group, 20% of the samples examined microscopically showed benign papillomatous formations and cellular alterations compatible with a diagnosis of epithelial dysplasia (D) without invasion of the basement membrane, but with abundant inflammatory reaction in the subepithelial connective tissue. The incidence of squamous cell carcinomas (SCC) induced by 4- NQO was 60% with extensive invasion of underlying connective tissue, cells with hyperchromatic nuclei, nucleus / altered cytoplasm and foci formation or keratin pearls in connective tissue.

Mast cell counts, characterized by the presence of positive cytoplasmic granules by toluidine blue, showed a significant reduction in cell density in the peritumoral area located both in dysplastic lesions and in those with a diagnosis of tongue squamous cell carcinoma (figures 3 and 4).

DISCUSSION

In a previous study, Curiee S. *et al.* (1992) performed an experimental study in which bee venom was injected at three different doses in the footpad of mice, and immediately afterwards, breast cancer tumor cells were injected at the same site; the results indicated increased survival of the treated group [11]. However, the present results show no significant preventive effect of bee venom in the development and progression of dysplastic lesions to carcinoma squamous cells. In our case, apitoxin was applied subcutaneously in a distant tumor site to examine a potential systemic effect of the poison because applying apitoxin directly on the tongue may be impractical.

Pirut *et al*. reported effects of a peptide from scorpion venom (BmKn-2 and derivatives) in a culture of cancer cells [12]. They used the poison in cell culture of oral squamous cancer (line

HSC-4) and obtained cell growth arrest and induction of apoptosis. However, bee venom, applied in the manner described in this research, showed no significant inhibitory effect of cell growth as to prevent or delay the development of SCC of the tongue.

To determine the behavior of some cells with immune activity, in this paper, we evaluated the density of peritumoral mast cells (PMC). PMCs are frequently observed in various tumors and have been linked alternately with rejection or promotion of growth and spread of tumor [13].

Sharma B *et al*. [14] and Telagi N *et al*. [15] described in oral squamous cell carcinoma an increase in the number of mast cells and capillary blood, suggesting that these cells in particular play a role in angiogenesis, thus favoring dissemination.

In contrast, Debta P *et al*. reported mast cell infiltration in cancer from several sites in the oral cavity, including the tongue [16]. They concluded that, along with eosinophils, mast cell infiltration is a favorable prognostic indicator.

In this paper, we describe a reduction in the density of PMC which is in line with the findings of HH Oliveira-Neto *et al.* [17], who also found a lower density in squamous cell carcinoma and premalignant lesions of the oral cavity compared to normal controls. They attributed this phenomenon to a failure in the migration of mast cells. In the same way, the apitoxin used in this study did not increase the recruitment of mast cells, as expected due to its pro-inflammatory action.

The significance of these findings suggests the possibility that each tumor is influenced by its particular microenvironment, behaving differently in modulating the immune response to tumor cells according to their location.

Although the preventive effect of apitoxin on the occurrence of squamous cell carcinoma of the tongue has not been demonstrated, there is still a need to evaluate the effect of apotoxin on other developmental stages of this neoplasm to further examine its potential usefulness or reject the use of the drug for this specific purpose.

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Conflicts of interest: The authors declare that they have no conflict of interest.

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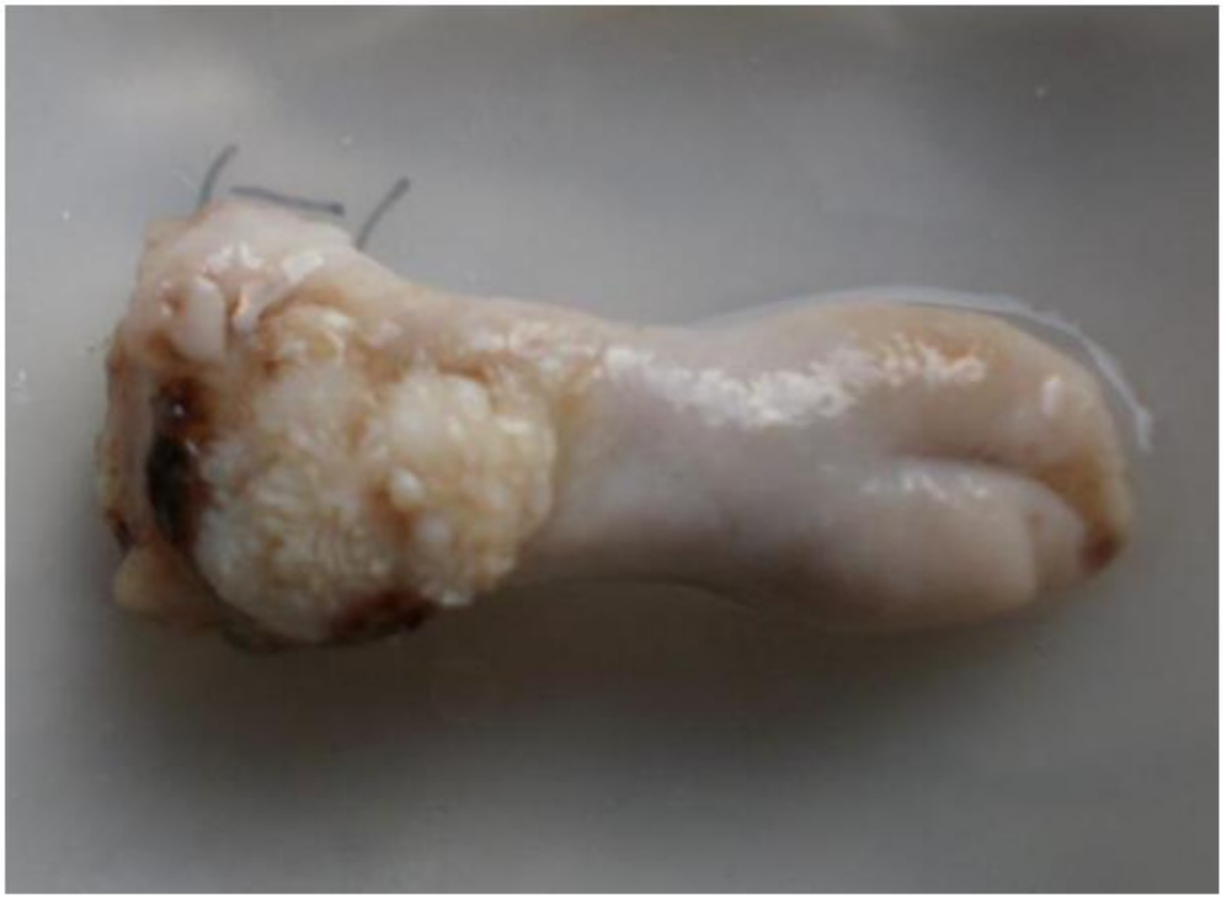

Figure 1 displays a tongue body, belonging to the experimental group (4-NQO + apitoxin), showing a macroscopic tumor lesion.

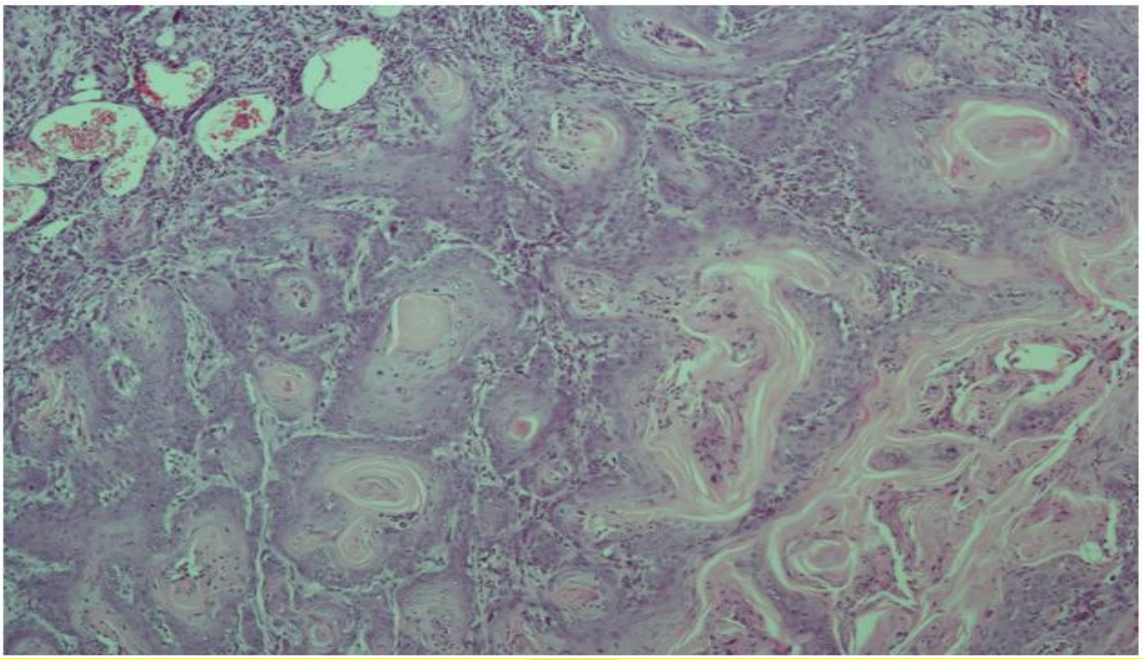

Figure 2 shows the histopathological changes found in the samples, compatible with a diagnosis of tongue squamous cell carcinoma (Hematoxylin-eosin x100).

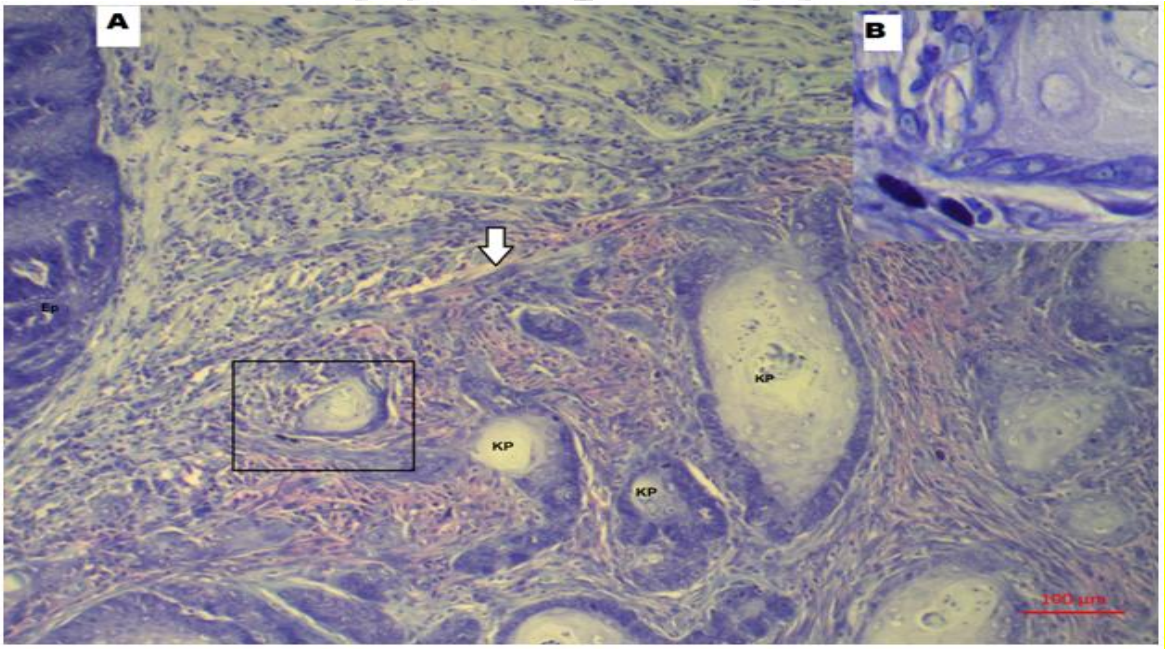

Figure 3 (A): Photomicrograph (100x toluidine blue) illustrates several keratin pearls (kp) of a moderately differentiated squamous cell carcinoma. White arrow shows tumoral border. Magnified square section (B) shows an inactive mast cells group.

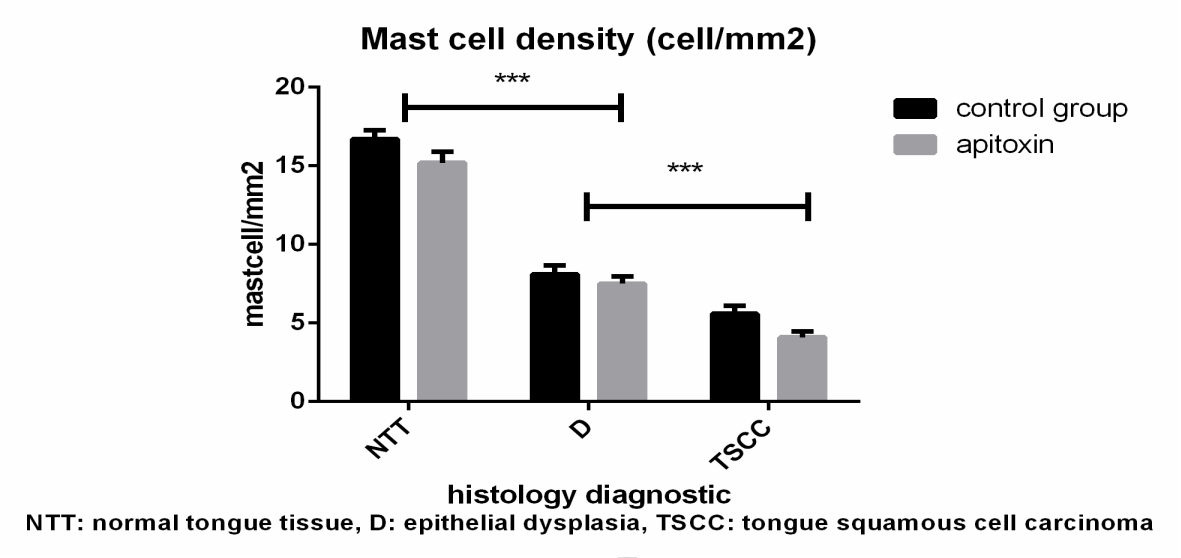

Figure 4: The graph shows a significant reduction (p> 0.001) of peritumoral mast cells in dysplastic lesions and squamous cell carcinoma.