Effects of propolis on lingual mucosa response of hamsters submitted to experimental carcinogenesis

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OBJECTIVE; To assess the tissue reaction of the lingual mucosa in hamsters submitted to daily, alternating, topical applications of 9,10-dimethyl-1,2-benzanthracene (DMBA) and a commercial brand of an ethanol propolis extract (EPE).

METHODS; A total of 60 hamsters were divided into three groups with two experimental periods (13 and 20 weeks). The lateral edge of the tongue was submitted to daily, alternating, topical applications of 0.5% DMBA and 8% EPE (EPE group, n=20), 0.5% of DMBA and aqueous propolis extract (APE group, n=20) and 0.5% of DMBA and saline solution (DMBA group, n=20). The occurrence of clinical and histological alterations was analyzed, along with the measurement of the area and volume of the clinical alterations, the determination of structural and cytological alterations of the squamous epithelial tissue with atypias and the measurement of the histological area of squamous cell carcinomas.

RESULTS; There were no significant differences among groups regarding any of the variables analyzed in the two evaluation periods. At week 13, a single squamous cell carcinoma occurred in the EPE group. At week 20, the greatest occurrence of squamous cell carcinoma was also in the EPE group.

CONCLUSION; The mechanism of EPE (30% alcohol content) affecting the onset of tissue reaction and the promotion of carcinogenesis has not been clarified yet.

KEYWORDS; propolis; carcinogenicity tests; 9,10-dimethyl-1,2-benzanthracene; chemoprevention; Mesocricetus

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It has been established that cancer can be induced by the carcinogen 9,10-dimethyl-1,2-benzanthracene (DMBA)\cite{1,2}. DMBA belongs to the class of aromatic polycyclic hydrocarbons, which are byproducts of the combustion of tobacco and other organic substances\cite{3,4}. In animal models, topical administration of DMBA\cite{5,6} promotes the emergence of dysplastic lesions and cancer after 10 and 13 weeks respectively\cite{7}. Such lesions in the retropharyngeal space in hamsters exhibit morphological alterations, which are similar to those that occur in the squamous epithelial tissue of the oral mucosa in humans\cite{8}.

The use of natural substances in the fight against cancer in research aims to search for chemoprevention with fewer side effects\cite{9}. The experimental DMBA-induced carcinogenesis model has been used to test the possible chemopreventive effects of certain substances, such as propolis\cite{10,11}. Propolis is a resinous substance produced by Apis mellifera bees from plant resins in order to seal and protect the hive against fungi and bacteria\cite{12,13} which has shown to have numerous pharmacological and biochemical properties\cite{14}.

The propolis extracts and isolated compounds employed in the majority of studies\cite{14,15,16,17} are prepared exclusively for the purposes of the respective studies and are not available on the market. However, ethanol propolis extracts (EPEs) have been approved for sale without a medical prescription\cite{18} and used by the general population for the relief of throat inflammation, halitosis and other conditions. The anti-inflammatory action has already been demonstrated\cite{19}.

Alcohol-based drugs administered topically or as a mouth wash\cite{20} and alcohol solutions\cite{21,22} have been associated with morphological alterations of the oral mucosa. Moreover, there has been an increase in the incidence of tongue cancer in humans\cite{23,24}. Thus, individuals exposed to risk factors for carcinogenesis such as smoking and the consumption of alcoholic beverages, may use an EPE to relieve halitosis or some symptoms in the oral cavity or oropharynx through self-medication, thereby increasing the risk of developing oral cancer.

The aim of the present study was to assess the tissue response of the lingual mucosa in hamsters submitted to daily, alternating, topical applications of DMBA and a commercial brand of an EPE with 30% alcohol content.

1 Materials and methods

1.1 Sample size calculation By admitting the confidence level of 95%, the standard deviation of 0.93\textsuperscript{25} and the difference between the groups of 0.6, a result of 9.2 animals per group was obtained. Add to the calculation 10% to compensate for losses resulting in a sample of 10 animals per group, thus totally 60 animals were needed for the study.

1.2 Ethical considerations This study was carried out in compliance with national and international guidelines for ethical animal experimentation and received approval from the Animal Research Ethics Committee of the Universidade Federal dos Vales do Jequitinhonha e Mucuri (Brazil) under process number 004/2010.

1.3 Animals The sample was made up of 60 male and female hamsters (Mesocricetus auratus) aged 90 d, with a mean body weight of (126.80±14.26) g. The animals were acquired from the animal housing facility of the Renê Rachou Research Center of the Oswaldo Cruz Foundation (Belo Horizonte, Minas Gerais, Brazil) and acclimatized in the laboratory for 14 d at room temperature under a 12 to 12 h light-dark cycle in plastic cages measuring 60 cm × 50 cm × 22 cm, with free access to a balanced ration (Nuvilab CR1, Nuvital\textsuperscript{R}, Colombo, Paraná, Brazil) and water. Each cage contained a maximum of four animals and was lined with wood shavings. The cages were cleaned twice a week. This is an in vivo, almost-experimental operated, randomized, quantitative study.

1.4 Chemicals The carcinogen DMBA (Sigma-Aldrich\textsuperscript{B}, St. Louis, MO, USA) was diluted in acetone to obtain a solution with a concentration of 0.5%. The EPE and aqueous propolis extract

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(APE) were acquired from the same manufacturer (Apaiário Mackillani® Ltda, Santa Bárbara, Minas Gerais, Brazil) in the local market and used without dilution. According to the information provided by manufacturer, the EPE and APE were produced with 11.56% of dried Brazilian propolis extract. The alcohol content in the EPE was 30%. The total content of polyphenols and flavonoids in the EPE and APE obtained from these commercial extracts was determined. The determination of total polyphenols in the EPE and APE was performed using the Folin-Ciocalteau colorimetric method[33]. Total flavonoid content was determined using the method described by Park et al[34]. The dry extracts of commercial preparation were determined by adding 5.0 mL of propolis extract to porcelain capsule and submission at 105 °C until constant weight. The analysis was made in triplicate.

1.5 Experimental protocol The hamsters were randomly divided into three groups with 20 in each, namely, 0.5% DMBA and 30% EPE (EPE group), 0.5% DMBA and APE (APE group) and 0.5% DMBA and saline solution (DMBA group).

Then the groups were subdivided based on the evaluation time of 13 and 20 weeks. The substances were toplically administered to the right lateral edge of the tongue using a No. 1 camel hair brush (Tigre®, São Paulo, Brazil). The application of DMBA was alternated every 24 h with EPE, APE or saline solution, depending on the grouping (there was no application of any substance once a week).

Each daily topical application consisted of four consecutive brushings. Excess substance was removed from the brush prior to the application to avoid accidental swallowing by the hamsters.

The animals were individually weighed in weeks 1, 3, 5, 7, 9, 11, 13, 15, 17 and 19. One day prior to euthanasia in each experimental period, an analysis was performed for the alterations of lingual mucosa. The types of clinical alterations examined were based on the method proposed by Lima and Taveira[31] with modifications, including the diffuse erythematous or diffuse whitish lesions (alterations of a reddish or whitish coloration); white plaque characterized by a flat, white, well-circumscribed elevation; diffuse of whitish berry-shaped lesions (alterations in color and textures, with small rounded irregularities on an opacified surface); verrucous plaque characterized by a flat, circumscribed elevation with a papillomatous surface; exophytic lesion; nodular or tumorous mass.

At each evaluation time, 10 hamsters from each group were anesthetized with 10 mg/kg of xylazine and 200 mg/kg of ketamine for euthanasia. After decapitation, the tongues were dissected and fixed in a 10% buffered formalin solution for 48 h. The specimens were embedded in paraffin, cut, dehydrated in alcohol, cleared in xylol and stained with hematoxylin and eosin (HE).

1.6 Histological analysis The histological analysis was performed by a single examiner using an optical microscope (Olympus® BX 41, Shinjuku-Ku, Tokyo, Japan) at different magnifications. The histological alterations encountered were classified based on the presence and number of structural alterations and cytological alterations[25]. The classification of the degree of squamous epithelial dysplasia (SED) with the presence of structural and cytological alterations was based on the criteria established by Katz et al[26] and Gale et al[27]. The degree of malignancy of squamous cell carcinomas was classified based on Johnson et al[28]. The mean occurrence of structural and cytological alterations in the epithelium was calculated for SED. The histological area of the squamous cell carcinomas was calculated using the Motic Images Plus program. Version 2.0 (Motic® China Group Co. Ltd., Copyright 2007, GUIYANG, China).

1.7 Statistical analysis The data were tabulated with the aid of the SPSS 17.0 program, (SPSS® Inc, Chicago, Illinois, USA). Normality tests were performed prior to the application of statistical analyses. Repeated-measure one-way analysis of variance was used to determine the variations in mean body weight of the animals. Chi-squared test was used to compare the occurrence of clinical and histological alterations among the groups and Fisher’s exact test for pair-by-pair comparison. Either the Kruskal-Wallis or Mann-Whitney test was used for comparison of mean histological areas among the groups. When \( P \leq 0.05 \), it was considered as statistical significance.

2 Results

2.1 Body weight The mean body weight of the animals increased throughout the course of the study. The increase followed a significant linear progression in the APE and EPE groups, whereas the variation followed a significant quadratic progression in the DMBA group, with a progressive loss beginning in week 15. See Figure 1.

![Figure 1](image)

**Figure 1** Mean body weight of different groups during the experimental periods

EPE: ethanol propolis extract; APE: aqueous propolis extract; DMBA: 9,10-dimethyl-1,2-benzanthracene.

2.2 Clinical alterations There was no significant difference found among the groups for the different
types of clinical alterations at the two evaluation periods (weeks 13 and 20). See Figure 2. Euthanasia was performed early in one animal in the DMBA group (week 18), which exhibited an extensive lesion on the tongue and cachexia. It was also performed in one animal in the EPE group as well (week 19). Both of these two hamsters were excluded from the clinical analysis of the lesions.

2.3 Histological aspects There was a greater occurrence of mild SED in the APE group at week 13 in comparison with the other groups using the chi-square test ($P<0.05$). However, this difference lost its significance in the pair-by-pair comparison using Fisher’s exact test (Table 1). There was no significant difference found among groups at weeks 13 and 20 regarding the occurrence of histological alterations and respective mean structural and cytological alterations.

At week 13, the following structural alterations were found in cases of mild SED in the EPE and APE groups including the irregular epithelial stratification, loss of polarity and drop-shaped epithelial projections. Premature cellular keratinization occurred in only one case in the APE group. In these same cases of mild SED, the following cytological alterations were found in the APE group, including the increase in the nucleus to cytoplasm ratio, number of nuclei and nucleus size and hyperchromasia (Figure 3B). The increases in the cell and nucleus size and the number of nuclei were found in the EPE group. In the only case of moderate SED in the DMBA group, the structural alterations were irregular epithelial stratification with the loss of polarity and the cytological alterations were changed in nucleus and cell size, pleomorphism and mitosis. In the cases of severe SED, the most common structural alterations in the APE and DMBA groups were irregular epithelial stratification, the loss of polarity in the basal layer and premature cellular keratinization (Figure 3C); moreover, the presence of keratin pearls and an increase in the number of atypical mitoses were observed in the EPE group (Figure 3D). In these cases of severe SED, the cytological alterations in the DMBA group were an increase in the nucleus to cytoplasm ratio, nucleus size and number and hyperchromasia. Atypical mitoses occurred in the cases of severe SED in the APE group.

At week 20, the structural alterations in the only case of mild SED in the DMBA group were irregular epithelial stratification and the loss of polarity and the cytological alterations were an increase in the number and size of the nucleoli. The only common structural alteration in all groups in cases of severe SED was irregular stratification; moreover, the APE and DMBA groups both exhibited premature cellular keratinization. Keratin pearls occurred in the DMBA group and there were an increased number of mitoses in the EPE group. All types of cytological alterations occurred in cases of severe SED in the EPE group.

No statistically significant differences were found among the groups at weeks 13 and 20 regarding the occurrence of squamous cell carcinomas and respective mean histological areas. At week 20, the greatest occurrence of squamous cell carcinomas was found in the EPE group, with exophytic growth and well-differentiated degrees of malignancy. The largest mean histological area of the squamous cell carcinomas was found in the APE group. The cases

![Figure 2 Clinical alterations in lingual mucosa of different groups](image)

A: White plaque (arrow) in aqueous propolis extract group at week 13; B: Exophytic lesion (arrow) in ethanol propolis extract group at week 13; C and D: Exophytic lesions (arrows) in ethanol propolis extract group at week 20.

<table>
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<tr>
<th>Table 1</th>
<th>Distribution of histological alterations in lingual mucosa in EPE, APE and DMBA groups at week 13 (n, %)</th>
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<td>Group</td>
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<td>EPE</td>
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* $P<0.05$, vs APE group, SED: squamous epithelial dysplasia; SCC: squamous cell carcinoma; EPE: ethanol propolis extract; APE: aqueous propolis extract; DMBA: 9,10-dimethyl-1,2-benzanthracene.
of squamous cell carcinoma occurred both in APE and DMBA groups, with endophytic growth involving nearly the entire tongue and moderately differentiated degrees of malignancy. See Figure 4.

![Figure 3](image1)

**Figure 3** Histological alterations at week 13 tested by light microscopy
A: Squamous epithelial tissue with hyperplasia (star) and hyperkeratosis (arrow) in DMBA group, with absence of structural and cytological alterations (HE, ×100); B: Mild squamous epithelial dysplasia in APE group (HE, ×200); C: Severe squamous epithelial dysplasia in EPE group, with keratin pearl (arrow) and premature cellular keratinization (HE, ×200); D: Severe squamous epithelial dysplasia in DMBA group, with premature cellular keratinization (stars) (HE, ×100).

DMBA: 3,10-dimethyl-1,2 benzanthracene; APE: aqueous propolis extract; EPE: ethanol propolis extract; HE: hematoxylin and eosin.

![Figure 4](image2)

**Figure 4** Histological alterations at week 20 tested by light microscopy
A: Well differentiated squamous cell carcinoma in EPE group, endophytic growth (HE, ×40 and ×200); B: Moderately differentiated squamous cell carcinoma in APE group, endophytic growth involving nearly entire tongue (HE, ×40 and ×200).

APE: aqueous propolis extract; EPE: ethanol propolis extract; HE: hematoxylin and eosin.

### 3 Discussion

Cancer cells emerge from a genomic transformation of a eukaryote due to gene mutation, which changes the normal phenotype into a cancerous phenotype. Mutations can occur due to the action of carcinogens, such as DMBA\(^{13,14}\). This substance was used in the present study due to the production of free radicals (byproducts of its metabolism that trigger mutations in the DNA) during the onset of DMBA-induced carcinogenesis\(^{15,16}\).

The topical application of 0.5% DMBA in acetone induces cancerous lesions on the lingual mucosa with or without previous scarification\(^{16,17}\). In the present study, DMBA and the propolis test substances were topically administered without previous scarification or sedation of the hamsters, as this has been demonstrated to be a valid model of lingual carcinogenesis\(^{16,18}\) that causes less stress to the animals.

Propolis extracts and their isolated chemical components with proven chemopreventive effects\(^{13,16,14,12}\) are not available to the general population. Moreover, commercially available propolis extracts sold at drugstores\(^{14}\) have not previously been tested in an induced carcinogenesis model. In the present study, the choice of the commercial brand of propolis extract was based on the fact that our research team had previously used these extracts in a study to investigate their anti-inflammatory effects\(^{17}\). Individuals at risk for the development of cancerous lesions, such as those who smoke or ingest alcoholic beverages, may self-medicate with an EPE to relieve halitosis or some symptom in the oral cavity or oropharynx. As oral structures come into direct contact with the EPE, which contains alcohol, it is important to assess the tissue reaction of the lingual mucosa submitted to both oxidative stress and treatment with an EPE.

As genomic alterations may vary from hamster to hamster or between different types of tissue in the same individual, the alternating applications of DMBA, EPE and APE were constant throughout the two evaluation periods in the present study. The 13-week and 20-week evaluations are similar to those employed in previous studies\(^{13,14}\). In the analysis of clinical and histological alterations, initiating or chemopreventive effects can be evaluated at week 13 and chemoprevention potential or cancer promotion and progression can be evaluated at week 20.

In recent years, propolis and its derivatives have been investigated due to their chemopreventive potential\(^{13,14,15}\) stemming from the presence of flavonoids and caffeic acid\(^{20,23}\), which neutralize free radicals, thereby reducing oxidative stress\(^{21}\).

In this study, the method of comparison between groups was performed by analysis of morphological features in the tissue of hamsters in different groups, and we consider it as a limitation of this study.

The chemopreventive potential of propolis extracts has been determined in different anatomic sites\(^{16,18,15,20}\). In the present study, the lingual mucosa was the site of choice for the application of the substances.
due to the increase in the incidence of tongue cancer in humans and the fact that few studies have been conducted on carcinogenesis on the tongue of hamsters.

The mean body weight of the hamsters increased throughout the experiment, meaning that cancer induction in the tongue did not have a negative effect on the diet. However, there was a reduction in mean body weight in the DMBA group beginning at week 15. This corroborates the findings reported by Lajolo et al. regarding weight loss related to DMBA toxicity and those reported by Li et al. regarding weight loss related to extensive lesions in the oral cavity. Such weight loss may account for the early euthanasia for ethical reasons of one animal in the DMBA group due to cachexia related to an extensive lesion on the tongue.

The premature occurrence of SCC in the EPE group (week 13) may have been a shorter latency period carcinogenesis in the former group, which could be attributed to the solvent. As ethyl alcohol is considered a co-carcinogen due to the fact that it increases tissue permeability and induces oxidation through acetaldehyde, the alcohol content in the EPE used in the present study may have enhanced the absorption of DMBA and potentiated its carcinogenic action. This is in agreement with findings described by Bazo et al., who treated colon cancer in rats induced by 1, 2 dimethylhydrazine and found a greater occurrence of aberrant intestinal crypt foci in the group treated with 32% of ethyl alcohol.

The equal occurrence of hyperplasia and hyperkeratosis with an absence of atypias at week 13 in the EPE and DMBA groups and the lesser occurrence of such conditions in the APE group suggest that the components of the APE do not protect the lingual mucosa from the tissue reactions triggered by carcinogenesis, as there was a greater occurrence of mild SED in the APE group in comparison to the other groups within this timeframe. The lower total content of polyphenols and flavonoids in the dry extract of the APE tested in the present study may be one of the reasons for the absence of chemoprevention in the group treated with this substance. However, further studies are needed to clarify the molecular aspects of the tissue reactions.

The larger mean histological area of the squamous cell carcinomas in the APE group suggests an association with the lower concentration of phenolic compounds, whereas the greater occurrence of squamous cell carcinomas in the EPE group suggests a tissue reaction promoted by carcinogenesis. The results of the present study are in disagreement with those reported by Cavalcante et al., who also induced cancer on the tongue of hamsters using 0.5% DMBA topical solution in acetone and treated the animals with an ethanol extract of Brazilian green propolis; the authors found a reduction in the number of structural alterations, cytological alterations and the degree of malignancy of the lesions in the group treated with the green propolis extract. These differences between studies may be explained by the fact that the authors used the propolis extract with greater dry extract content and administered the substance orally through gavage, thereby avoiding contact between the ethanol solvent and the oral mucosa.

Thus, based on the findings of the present study, the APE did not affect the tissue response of DMBA-induced carcinogenesis on the tongue of hamsters and the solvent in the EPE may have shortened the latency period and favored carcinogenesis. The mechanisms are not yet clarified. Further in vitro and in vivo studies are needed to test other commercial brands of propolis with different concentrations of dry extract and alcohol content.

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5 Competing interests

The authors declare that they have no competing interests.

REFERENCES

2 Darchun V, Hadler HI. Metabolic and carcinogenic studies with 9, 10-dimethyl-1, 2-benzanthracene. Cancer Res. 1953; 16(4): 316-323.
7 Eisenberg E. Neoplasia following cessation of DMBA application to hamster buccal pouch. J Dent Res. 1977;
56(11): 1430.
23 Park YK, Koo MH, Sato HH, Contado JL. Survey of some components of propolis which were collected by Apis mellifica in Brazil. Arqú Biol Tecnol. 1995; 38: 1253-1259.
31 Eveson JW, MacDonald DG. Hamster tongue carcino-