Propolis effect in vitro on canine Transmissible Venereal Tumor cells

Efeito in vitro da Própolis sobre células do Tumor Venéreo Transmissivel canino

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Summary: Transmissible venereal tumor (TVT) is a sexually transmissible neoplasm, attracting researchers’ interest because of its origin, manner of transmission, and possibility of spontaneous regression, which modify the behavior of this tumor in comparison to other neoplasias. Chemotherapy procedure is still the preferred treatment for patients with TVT in spite of its toxic side effects that lead to treatment interruption in some cases. Since antitumor property of propolis has been reported, this work attempted to verify its possible antitumor action on TVT malignancy. Five animals from the Veterinary Hospital, FMVZ, UNESP, Campus of Botucatu, Brazil, were used. Based on the cell morphology stained with Giemsa, a new nomenclature was proposed by the Pathology Veterinary Service of this University, and the TVT-cells were divided into three groups: "lymphocyte-like" transmissible venereal tumor, "plasmocyte-like" transmissible venereal tumor and "mixed" transmissible venereal tumor. In this study, propolis was active against TVT-cells. Propolis showed an effective antitumoral activity against TVT-cells, including the "plasmocyte-like" cells, considered the most malignant form, with a cytotoxic effect with the highest propolis concentration after 48h.

Introduction

Transmissible venereal tumor (TVT) is a contagious and sexually transmissible neoplasm with an unclear origin and affecting only canines. It has a world wide distribution but is detected mainly in tropical and subtropical zones (Varaschin et al., 2001).

Despite its ubiquitous distribution and studies on its origin and classification, the nature and behavior of these tumor cells remain controversial. Related studies support the hypothesis of a histiocytic origin (Tinucci-Costa, 1999; Albanese et al., 2002; Murgia et al., 2006).

Its diagnosis may be done through cytological or histological examinations. Cytological evaluation of fine needle-aspirations reveals abundant round or oval cells with diameter of 14 to 30 mm, cytoplasmatic border well-delineated. Cellular nuclei are round or oval, usually eccentric with variable size. They appear with clumped chromatin and contain one or more prominent nucleoli. The nuclear:cytoplasmic ratio is high (Boscos et al., 1999). The cytoplasm is discretely basophilic with small, empty space and multiple vacuoles mainly located close to the cytoplasmic border (Varaschin et al., 2001).

Studies on TVT of natural origin do not show any predisposition of gender or breed, and it is found mainly in adult animals during reproductive age. TVT may also have extra-genital location (Rodrigues et al., 2001) and metastasis to many other organs has also been reported (Boscos et al., 1999; Ferreira et al., 2000; Park et al., 2006).
The normal diploid number of chromosomes in the somatic cell of the dog (*Canis familiaris*) is 78, and 76 of these are acrocentric. TVT has a stable number of about 59:16 metacentric chromosomes and 43 acrocentric chromosomes. The constant and specific chromosomal aberrations, the identical rearrangement of the LINE/c-myc in all tumors, including the Brazilian ones (Portela et al., 2003), suggest that TVT cases developed from the same origin and subsequently were transmitted continually as allografts in many different geographical locations (Chu et al., 2001).

Although chemotherapy has been used for TVT treatment with further tumor regression, in many cases there are toxic side effects due to this therapeutic procedure, with eventual treatment interruption (Ogilvie, 1996). Besides, Tinucci-Costa (1999) and Brandão et al. (2002) reported that some TVTs are resistant to chemotherapy.

Propolis is a resinous beehive product with a complex composition, produced by bees from the secretions of trees, flowers, leaves and pollen. Bees use it to seal combs, cover irregular surfaces or other insects and eventual intruders that die inside the beehive, in order to avoid their decomposition (Banskota et al., 1997; Banskota et al., 2001).

Propolis presents several therapeutic properties, such as antibacterial, antifungal, anti-inflammatory, immunomodulatory, among others (Sforcin et al., 2002; Orsi et al., 2005; Sforcin et al., 2005; Sforcin, 2007). Many authors have reported its antitumor property (Matsuno et al., 1997; Banskota et al., 2001). Thus, the goal of this work was to verify the possible *in vitro* action of propolis on canine venereal transmissible tumor (TVT).

**Material and methods**

**Clinical data of the dogs**

This work conforms with Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation (n. 60/2002).

From January to July 2003, 30 dogs were subjected to physical examination, anamnesis and complete clinical history at the Veterinary Hospital, FMVZ, UNESP, Campus of Botucatu. From those, 25 dogs of either gender, any breed or age, with cytological TVT diagnosis and absence of previous antitumor treatments were used for culture standardization. Five dogs included in these conditions were selected for the propolis assays, and among these five tumors, 2 of them were plasma cells-like TVT, 2 were lymphocyte-like TVT and one was a lympho-plasma cell TVT form.

**Cytopathological analysis**

Cytopathological analyses were carried out on tumor smears obtained by puncture or exfoliation for the TVT diagnosis and, based on the cell morphology and using the new nomenclature proposed by the Patology Veterinary Service of this University, the tumors were classified in "lymphocyte-like" transmissible venereal tumor, "plasmocyte-like" transmissible venereal tumor and "mixed" transmissible venereal tumor. These smears were stained with Giemsa (Santos do Amaral, 2007).

**Propolis and TVT cell cultures**

Propolis was collected in the Beekeeping Section, FMVZ, UNESP, Campus of Botucatu, located on the Lageado Experimental Farm. Propolis samples were collected from colonies of Africanized honeybees (*Apis mellifera*) with plastic nets, which were later frozen to remove the propolis.

The propolis sample was analysed by gas-chromatography, gas chromatography-mass spectrometry and thin layer chromatograph, revealing that its main components are phenolic compounds (flavonoids, aromatic acids, benzopyranes), di- and triterpenes, essential oils, among others. Propolis was ground and mixed with 70% ethanol (30 g propolis to 100 mL of 70% ethanol), and protected from bright light, under moderate shaking, for 7 days. After this period, solutions were filtered and used at adequate concentrations in the biological assays (Sforcin et al., 2005).

TVT cells from biopsies of each animal were put in RPMI 1640 medium and processed, in order to carry out the cell cultures immediately. TVT cells were counted in a hematocytometer by Trypan blue exclusion in order to obtain a final concentration of 2x10^6 cells/mL, and were incubated at 37 °C under 5% CO2 tension into 96-well microplates with flat bottom in RPMI 1640 medium supplemented with 20 mM HEPES (Sigma Chemical Co., USA), 2x10^-5 M 2-mercaptoethanol (Sigma), 0.2% sodium bicarbonate, 1% glutamine 2 mM and 10% of heat-inactivated fetal calf serum. Then, propolis was added to the monolayers of each 5 animals in the following 4 concentrations: 10, 25, 50 and 100 µg/well (100 µl). The final volume in each well was 100 µl. All assays were carried out in duplicate (Orsi et al., 2005).

Propolis effect was analyzed after 6, 24 and 48 h, comparing propolis-treated cells with the control cells. Cell viability was analyzed by Trypan blue exclusion. In order to observe a possible effect of the propolis solvent, other cells were incubated only with 70% ethanol (20 µl), corresponding to the highest propolis concentration (100 µg).

**Statistical analysis**

Friedman’s test was used in order to analyze cell viability in the cultures, for each propolis concentration according to the time period. Kruskall-Wallis’s test was used to analyze the time period according to propolis concentrations.
Analysis of variation was used to analyze cellular concentration in the cultures, for each propolis concentration according to the time period and for every time period according to propolis concentrations. The value of each animal was considered to be the average of the two duplicates.

**Results and discussion**

Differences between cellular lineages were seen in morphological characteristics of TVT, influencing its biological behavior (Varaschin et al., 2001). According to cell characteristics, a new terminology for TVT has been suggested by our group and tumors were classified into lymphocyte-like TVT, plasma cell-like TVT and lympho-plasma cell-like TVT forms (Amaral et al., 2004; Bassani-Silva, 2005; Gaspar, 2005). This morphology classification shows TVT malignancy: plasma cell-like TVT shows a higher frequency of nuclear abnormalities associated with a larger expression of P-glycoprotein, an elevated rate of metastasis and cellular proliferation in comparison to lymphocyte-like TVT or lympho-plasma cell-like TVT forms. Plasma cell-like TVT is the most injurious and also the most malignant (Bassani-Silva, 2005; Gaspar, 2005).

To be sure that the culture did not contain any other cells than TVT, morphologic analysis of culture cells by Giemsa as well as chromosome analysis revealed that the cells were really from TVT. No antibiotic was added to the cultures within 48 h. Our preliminary 25 cultures showed that within this time period, antibiotics were not necessary. So, we chose this time period to carry out our cell cultures.

Comparing propolis-treated cells with control cells, one may verify that propolis showed a time-concentration effect on TVT.

After 6 h, propolis (100 µg) showed a significant antitumor activity (p<0.05). It was also effective at lower concentrations, but in a long-term way (after 24 h with 50 µg, and 48 h with 25 µg) (Tables 1 and 2). With regards to TVT morphology, plasma cell-like TVT was more resistant to propolis action (Table 3).

One may verify a similar number of 70% ethanol-treated cells and control, indicating the absence of propolis solvent effect and suggesting that the results were exclusively due to propolis components.

The chemical composition of propolis is very complex and is dependent upon the source plant. Bud exudates of different poplar species are the main sources of propolis in temperate zone, including Europe, Asia and North America. Samples originating from these regions are characterized by similar chemical composition; the most important constituents appeared to be phenolics: flavonoids, aromatic acids and their esters. The main vegetal source of propolis in Botucatu, São Paulo State, Brazil, is *Baccharis*

### Table 1 - TVT cell viability (average %) after 6, 24 and 48 h of incubation with different propolis concentration (P) (µg) and 70% ethanol (20 µl)

<table>
<thead>
<tr>
<th>Group</th>
<th>Time period</th>
<th>0 h</th>
<th>6 h</th>
<th>24 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>52.07±</td>
<td>81.41±</td>
<td>76.12±</td>
<td>60.83±</td>
</tr>
<tr>
<td>P (10 µg)</td>
<td></td>
<td>52.07±</td>
<td>44.38±</td>
<td>9.39±</td>
<td>1.47±</td>
</tr>
<tr>
<td>P (25 µg)</td>
<td></td>
<td>52.07±</td>
<td>31.56±</td>
<td>1.00±</td>
<td>0±</td>
</tr>
<tr>
<td>P (50 µg)</td>
<td></td>
<td>52.07±</td>
<td>0.41±</td>
<td>0±</td>
<td>0±</td>
</tr>
<tr>
<td>P (100 µg)</td>
<td></td>
<td>52.07±</td>
<td>0±</td>
<td>0±</td>
<td>0±</td>
</tr>
<tr>
<td>70% Ethanol</td>
<td></td>
<td>52.07±</td>
<td>80.79±</td>
<td>75.06±</td>
<td>60.02±</td>
</tr>
</tbody>
</table>

Different capital letters indicate significant differences between each time period according to propolis concentration (α=0.05).

### Table 2 - TVT cell concentration (x 10⁶ cells/mL) after 6, 24 and 48 h of incubation with different propolis concentration (P) (µg) and 70% ethanol (20 µl)

<table>
<thead>
<tr>
<th>Group</th>
<th>Time period</th>
<th>0 h</th>
<th>6 h</th>
<th>24 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>2.00a</td>
<td>2.52a±</td>
<td>1.70a±</td>
<td>1.15a±</td>
</tr>
<tr>
<td>P (10 µg)</td>
<td></td>
<td>2.00a</td>
<td>1.28a,±</td>
<td>0.28a,±</td>
<td>0.08a,±</td>
</tr>
<tr>
<td>P (25 µg)</td>
<td></td>
<td>2.00a</td>
<td>0.96a,±</td>
<td>0.06a,±</td>
<td>0.02a,±</td>
</tr>
<tr>
<td>P (50 µg)</td>
<td></td>
<td>2.00a</td>
<td>0.31a,±</td>
<td>0.03a,±</td>
<td>0.01a,±</td>
</tr>
<tr>
<td>P (100 µg)</td>
<td></td>
<td>2.00a</td>
<td>0.02a,±</td>
<td>0.01a,±</td>
<td>0.0a,±</td>
</tr>
<tr>
<td>70% Ethanol</td>
<td></td>
<td>2.00a</td>
<td>1.85a,±</td>
<td>1.56a,±</td>
<td>1.20a,±</td>
</tr>
</tbody>
</table>

Different letters indicate significant differences by the variation analysis for repeated measures on entirely random experiment, for every group between each circumstance and for every circumstance, between each group, with a significance of α=0.05.

n=5 averages of each animal.
The main constituents of our propolis sample were isolated and identified: flavonoids are present in small quantities in Brazilian propolis (kaempferid, 5,6,7-trihydroxy-3,4'-dimethoxyflavone, aromadendrine-4'-methyl ether); a prenylated p-coumaric acid and two benzopyranes: E and Z 2,2-dimethyl-6-carboxy-ethenyl-8-prenyl-2H-benzopyranes); essential oils (spathulenol, (2Z,6E)-farnesol, benzyl benzoate and prenylated acetophenones); aromatic acids (dihydrocinnamic acid, p-coumaric acid, ferulic acid, caffeic acid, which are common for poplar propolis, 3,5-diprenyl-p-coumaric acid, 2,2-dimethyl-6-carboxy-ethyl-8-prenyl-2H-1-benzo-pyran); di- and triterpenes, among others. Seasonal variations in propolis composition are not significant and are predominantly quantitative (Bankova et al., 1998).

Twenty-three components have been isolated from Brazilian propolis, with cell-toxicity against fibrosarcoma in men and murine colon carcinoma (Banskota et al., 1998). Artepilin-C is an isolated component of Brazilian propolis with cytotoxic activity against tumor cells in vitro. This cytotoxicity was related to DNA fragmentation, inducing apoptosis (Matsumo et al., 1997).

Orsolic et al. (2004) showed that according to the results obtained in their study on the growth and metastatic potential of a transplantable mammary carcinoma of CBA mouse, the antitumor activity of tested compounds can be related to the immunomodulatory properties of the compounds, their cytotoxicity to tumor cells, and their capacity to induce apoptosis and necrosis. They also showed that experimental data corroborate that polyphenolic compounds isolated from propolis and propolis itself could be potentially useful in the control of tumor growth in experimental models.

The literature reports immune suppression during TVT growth, allowing metastasis. In order to reduce the side effects of chemotherapy and considering that propolis possesses antitumor, anti-metastatic and immunomodulatory activities, its introduction as a therapeutic procedure in vivo could provide new contribution to TVT treatment, as well as to other neoplasia treatments.

There are no works dealing with propolis and TVT, demonstrating the originality of our research and its great contribution to the therapeutic procedures.

Bibliography


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Table 3 - Propolis (P) effect, according to its concentration and period of incubation, on different TVT cell morphology.*

<table>
<thead>
<tr>
<th></th>
<th>P (10 µg)</th>
<th>P (25 µg)</th>
<th>P (50 µg)</th>
<th>P (100 µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=2 averages of each animal</td>
<td>n=2 averages of each animal</td>
<td>n=1 average</td>
<td>n=1 average</td>
</tr>
<tr>
<td>Plasma cell-like TVT</td>
<td>0 h</td>
<td>6 h</td>
<td>24 h</td>
<td>48 h</td>
</tr>
<tr>
<td></td>
<td>2.00</td>
<td>1.95 ± 0.75</td>
<td>0.47 ± 0.28</td>
<td>0.19 ± 0.16</td>
</tr>
<tr>
<td></td>
<td>2.00</td>
<td>1.42 ± 0.61</td>
<td>0.20 ± 0.04</td>
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</tr>
<tr>
<td></td>
<td>2.00</td>
<td>0.77 ± 0.94</td>
<td>0.07 ± 0.01</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>2.00</td>
<td>0.06 ± 0.04</td>
<td>0.03 ± 0.01</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Lymphocyte-like TVT</td>
<td>0 h</td>
<td>6 h</td>
<td>24 h</td>
<td>48 h</td>
</tr>
<tr>
<td></td>
<td>2.00</td>
<td>1.25 ± 1.76</td>
<td>0.24 ± 0.34</td>
<td>0.02 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>2.00</td>
<td>0.98 ± 1.39</td>
<td>0.01 ± 0.02</td>
<td>0 ± 0</td>
</tr>
<tr>
<td></td>
<td>2.00</td>
<td>0.01 ± 0.02</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Lympho-plasma cell-like TVT</td>
<td>0 h</td>
<td>6 h</td>
<td>24 h</td>
<td>48 h</td>
</tr>
<tr>
<td></td>
<td>2.00</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2.00</td>
<td>0</td>
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<td>0</td>
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<td></td>
<td>2.00</td>
<td>0</td>
<td>0</td>
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</tr>
</tbody>
</table>

* 2 x 10^6 cells/mL.
Results are expressed in mean ± standard deviation.


