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Original article

Positive effects of antitumor drugs in combination with propolis on canine osteosarcoma cells (spOS-2) and mesenchymal stem cells

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ABSTRACT

The combination of lower concentrations of antitumor drugs (carboplatin – CARB, doxorubicin – DOX, and methotrexate – MET) with propolis was investigated against canine osteosarcoma (spOS-2) and mesenchymal stem cells (MSC) *in vitro*. The mechanism of action in the combinations was analyzed. spOS-2 cells were incubated up to 72 h with propolis (50 μg/ml) alone or in combination with CARB (10–400 μmol/l), DOX (0.5–2 μmol/l) or MET (50–200 μmol/l). Cell viability was assessed by MTT assay, apoptosis/necrosis by flow cytometry, and MSC was incubated with the optimum combination. Propolis alone exerted no cytotoxic action against spOS-2 cells, whereas CARB (400, 200 and 100 μmol/l) exhibited the highest cytotoxic effects comparing to DOX and MET. The combination of propolis with the lowest concentrations of CARB led to better results comparing to CARB alone, which was not observed using DOX and MET. Apoptosis was involved in the action of propolis + CARB in spOS-2 cells. MSC were not affected by CARB/propolis, indicating that the cytotoxic action of the combination was specific to tumor cells but not to normal ones. Propolis improved the action of CARB against spOS-2 cells using lower concentrations of this drug, without affecting MSC. These findings are relevant and indicate a possible application of propolis in OSA treatment.

1. Introduction

Propolis has been used in folk medicine for centuries showing uncountable biological activities, such as antitumor, immunomodulatory, anti-inflammatory, antimicrobial, antioxidant and others [1]. The ancient Greeks, Romans, and Egyptians were aware of the healing properties of propolis and made extensive use of its medicinal properties. In the middle ages, propolis was not very popular and its use in mainstream medicine declined. However, the knowledge of its medicinal properties survived in traditional folk medicine. Interest in propolis rearose in Europe during the renaissance [2]. Several works have been carried out *in vitro* and *in vivo* demonstrating propolis potential for the development of new antitumoral drugs [1,3].

Canine cancers may occur spontaneously showing a similar clinical pathophysiology to equivalent human cancers. Osteosarcoma (OSA) or osteogenic sarcoma is a primary bone neoplasm frequently diagnosed in dogs [4]. The biological behavior of OSA is aggressive and initially restricted to the bone microenvironment, with metastatic progression [5]. Although relatively rare in humans, the rate of canine OSA is 75× higher than that of humans. High risk breeds for developing canine osteosarcoma tend to be some of the larger and giant dogs, including the Rottweiler, Great Pyrenees, mastiff, Dobermann pinscher, Irish wolfhound, Scottish deerhound. Working across species, researchers may combine scientific findings to understand the origins of cancer in order to develop novel therapies to benefit both human and animals [6].

Chemotherapeutic agents such as carboplatin (CARB), cisplatin, and doxorubicin (DOX) have been used as a therapy in veterinary medicine to induce remission of the tumor [7]. However, the outcome of chemotherapy in dogs is unpredictable and may result in failure to respond to cytotoxic drugs [8]. Therapeutic studies involving *in vitro* models have used primary cultures rather than monoclonal neoplastic cell strains, because the primary culture is more similar to what occurs in patients, by presenting different neoplastic clones. For this reason, the primary culture became an excellent model for research in oncology [9]. In the search of new anticancer drugs, propolis has been pointed out because of its cytotoxic action *in vitro* towards different tumor cells. The main mechanisms of its antitumor effects *in vitro* involve apoptosis,

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cell cycle arrest and interference on metabolic pathways [10,11]. Besides, propolis exhibited an immunomodulatory action in tumor-bearing mice [12,13].

There are different types of propolis in Brazil such as green, red and brown propolis, and we used a sample collected in the Beekeeping Section of the University that was previously analyzed by chromatographic techniques. The main constituents of this green sample were phenolic compounds (flavonoids, aromatic acids and benzopyranes), diterpenes and triterpenes and essential oils, among others [1,3]. The effects of propolis were investigated in combination with lower concentrations of three different anticancer drugs (CARB, DOX and methotrexate – MET), in order to verify whether such combinations could reach the same efficiency of the drug alone. The mechanisms of action of propolis alone or in combination were also investigated. The effects using the best combination were assayed in mesenchymal stem cells (MSC).

2. Material and methods

2.1. Ethical aspects and spOS-2 primary cultures

Tumor fragments were taken from a dog in the Veterinary Hospital and FMVZ Veterinary Pathology Service – UNESP, *Campus* of Botucatu, Brazil. The dog's owner was informed about this study, permitting the publication of the data after signing a Free and Informed Consent Form.

Cells were transferred to 25 cm^2 flasks containing Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich Quimica, Madrid, Spain), supplemented with 10% fetal bovine serum (Gibco, Paisley, UK), 100 U/ml penicillin, 100 µg/ml streptomycin, and 2.5 µg/ml amphotericin B. Primary cultures of canine osteosarcoma were categorized according to a biochemical panel by alizarin red and by target proteins such as vimentin, cytokeratin, osteocalcin, osteopontin, osterix, and cyclo-oxygenase-2 (Cox2) by flow cytometry. Cox2 was also evaluated by immunohistochemistry and separated into Cox2-positive or Cox2-negative cultures. Thus, spOS-2 refers to a culture with upregulated Cox2 expression.

This study was approved by the FMVZ Ethics Committee – UNESP (protocol 98/2008).

2.2. Mesenchymal stem cells

MSC were kindly provided by the Biobank of Cells of the Laboratory of Investigative and Comparative Pathology, School of Veterinary Medicine and Animal Science, UNESP.

2.3. Propolis, antitumor drugs and their combinations

Propolis was collected in the Beekeeping Section, UNESP, Campus of Botucatu. After grinding, ethanolic extracts of propolis were prepared (30 g of propolis, completing the volume to 100 ml with 70% ethanol) and left for a week in the absence of bright light at room temperature, under moderate shaking. Extracts were filtered and their dry weight was calculated (130 mg/ml).

Propolis was used in different concentrations: 50, 25 and 10 µg/ml, according to previous standardization in our laboratory [10]. Carboplatin, doxorubicin, and methotrexate were purchased from Vancel®-Darrow Laboratórios S/A and their concentrations were defined according to literature: CARB = 400, 200, 100, 50, 25 and 10 µmol/l; DOX = 2, 1 and 0.5 µmol/l [4,14–16], and MET = 200, 100 and 50 µmol/l [17–20].

Propolis and the drugs were diluted in DMEM as described above.

In order to verify the effects of propolis in combination with the drugs, propolis was added to CARB, DOX and MET using the concentrations that affected spOS-2 cell growth without leading to cytotoxic effects near 0%, comparing cell viability to the effects of the drugs alone.

In vitro assays were carried out in triplicate.

2.4. Viability assay

After detachment from the flasks using trypsin, the cells were cultivated in a 96 wells U-bottomed plate (Corning) at a final concentration of 2×10^4 cells/ml, adding 100 µl/well. After 24 h incubation for cells adherence, the supernatant of cell culture was discarded and the researched solution was added. The same procedures were performed with 70% ethanol (propolis solvent), using 0.385, 0.1925 and 0.077 µl 70% ethanol/ml, corresponding respectively to the concentration present in 50, 25 and 10 µg/ml of the ethanolic extract of propolis.

To evaluate the effects of the combinations, propolis (50 µg/ml) was added to CARB (50, 25 and 10 µmol/l), to DOX (2 and 1 µmol/l), and to MET (50 and 25 µmol/l). The stimuli (100 µl) were added to the 96-well plates and control cells only contained the medium. Assays were carried out in triplicate and cell viability was assessed after 6, 24, 48 and 72 h.

Cell viability was performed using the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT – Sigma-Aldrich, USA) colorimetric assay. After each period of time, supernatants of cell cultures were discarded and 100 µl of MTT (1 mg/ml) was added to the culture cells for 3 h. Afterwards, MTT was taken out and 100 µl of dimethylsulfoxide (DMSO) was added to dissolve the formazan salt. Optical densities (O.D.) were read at 540 nm in an ELISA reader and the percentage of cell viability was calculated using the formula: [O.D. test / O.D. control] × 100. Assays were carried out in triplicate, comparing the effects to control in the respective time scale.

Cell viability was also used to evaluate cell growth after incubation with stimuli, comparing the effects over time to the control at 6 h, to verify a cytotoxic or cytostatic action.

Mesenchymal stem cells were cultivated similarly to OSA cells and MTT test was used to determine cell viability, in order to verify whether the cytotoxic action of the combination was specific to tumor cells but not to healthy ones.

2.5. Evaluation of necrosis/apoptosis by flow cytometry

In this assay, only the combinations that exhibited the highest activities were assessed. Thus, only propolis (50 µg/ml), CARB (10, 25 and 50 µmol/l) and their combinations were evaluated.

Cells (2×10^5 cells/ml) were cultured in 24 well plates adding 300 µl of DMEM in each well. After 24 h for cell adherence, supernatants were removed and stimuli were added in a final volume of 1 ml for 6, 24, 48 and 72 h. Afterwards, supernatants were collected and put in cytometry tubes without discarding dead cells, and trypsin (250 µl) was added to the wells for 1 min at 37 °C. Plates were shaken carefully, 250 µl of fetal bovine serum was added and the final volume transferred to the corresponding tubes.

Subsequently, tubes were centrifuged for 10 min at 200 × *g*, supernatants were discarded, and the pellets were resuspended in 200 µl PBS pH 7.4 containing Ca^{+2} and Cl–. This content was divided into two tubes: one tube was used as negative control and no substance was added after this procedure and, to another tube, 5 µl of annexin V (A13201 – combined with Alexa Fluor 488 – Invitrogen) was added. After 30 min, 1 µl of propidium iodide (PI) was added and the sample was homogenized. After 10 min, the tubes were transferred and read in the flow cytometer FACS Calibur BD TM.

The dot plot system identified living cells, apoptotic cells and necrotic cells and those with double reading. Data was based on a sample of 10,000 cells and expressed in percentages [21].

2.6. Statistic analysis

Profile analysis was employed to compare the groups and moments, and to evaluate a possible interaction moment x group. The significance level of 5% was adopted.