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Anti-genotoxicity and anti-mutagenicity of *Apis mellifera* venom

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ABSTRACT

The search for substances able to inhibit and/or diminish the effects of genotoxic and mutagenic substances has been the target of several investigations performed in recent times. *Hymenoptera* venoms constitute a considerable source of substances with pharmacological potential. The present study aimed to evaluate the cytotoxic, genotoxic and anti-genotoxic, mutagenic and anti-mutagenic potentials of *Apis mellifera* venom in HepG2 cells. In this evaluation, the MTT test was applied to determine the most appropriate concentrations for the genotoxicity and mutagenicity tests. It was verified that the concentrations of 0.1, 0.05 and 0.01 μg/mL were not cytotoxic, hence these concentrations were used in the experiments. For the evaluation of the genotoxic and mutagenic potential of the bee venom the comet assay and the micronucleus test were applied, respectively. The concentrations mentioned above presented both genotoxic and mutagenic potential for HepG2 cells and it was necessary to test lower concentrations of the venom (10 pg/mL, 1 pg/mL and 0.1 pg/mL) for the anti-genotoxicity and anti-mutagenicity tests, which were performed subjecting the cells to the action of MMS (methyl methanesulfonate) in order to verify the ability of the venom to inhibit or diminish the action of this compound, which has a recognized action on the genetic material. Pre-, post-treatment and simultaneous treatment with and without incubation with the venom were performed. It was observed that the lowest three concentrations tested did not present any anti-genotoxic and anti-mutagenic activity on the cells. The use of bee venom for pharmacological purposes in treatments such as cancer must be done with extreme caution, since it was observed that even at very low concentrations the venom can induce genotoxicity and mutagenicity in human cells, as was verified for the HepG2 cells.

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1. Introduction

DNA of living organisms is constantly exposed to agents that can cause damage to its structure [1]. One of the most effective ways to prevent damages in the genetic material and the possible diseases related to these, such as cancer, would be the use of bioactive substances, e.g., those with anti-genotoxic and anti-mutagenic activity [2]. Therefore, the search for substances that show these characteristics has increased considerably.

Several studies have focused on anti-mutagenic substances from plants [3–7], but little has been done with substances derived from animals, such as venoms. Considering that venoms contain various substances and many chemicals, a more detailed study on the action of these compounds on cells can also be promising in the search for substances with anti-mutagenic action. Among the animal venoms, those from wasps and bees, according to Habermann [8], are complex mixtures of pharmacologically and bio-chemically active agents.

Bee venom (BV) contains a variety of peptides such as melittin, apamin, adolapin and the mast-cell de-granulating peptide, besides enzymes such as phospholipase A_2, biologically active amines (histamine, epinephrine) and non-peptide compounds (lipids, carbohydrates and free amino acids) [9]. According to Terwilligert and Eisenbergg [10], melittin is a small protein with approximately 26 amino acid residues, which is the main toxic compound of the *Apis mellifera* bee venom. Although it is soluble in water when in the form of monomer or tetramer, this polypeptide is integrated rapidly into the membranes and disrupts the phospholipid bilayer. According to the authors, probably due to the action of melittin, there is an increase in the activity of phospholipase A_2, triggering countless effects on living cells. This venom is used in the treatment of several diseases such as arthritis [11], bursitis, tendonitis, herpes zoster, multiple sclerosis, wounds, gout, burns and infections [12]. Moreover, there are studies that indicate that BV inhibits the proliferation of cancer cells [13,14], and is involved in angiogenesis regulation, growth suppression and delay in the metastatic dissemination [15]. Thus, the venom of *A. mellifera* is a rich source

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of substances with several biological functions and with pharmacological potential. Since only a few studies have focused on the effects of BV on the genetic material of the exposed organisms, the aim of this study was to verify if the raw venom of this *Hymenoptera* presents anti-genotoxic and anti-mutagenic potential.

2. Material and methods

2.1. Biological material

2.1.1. Human cell culture

HepG2 human hepatoma cells were obtained from the American Type Culture Collection (ATCC No HB 8065, Rockville, MD) and were used in the MTT test, comet assay and micronucleus test.

2.1.2. Apis mellifera venom

The lyophilized BV was obtained from the Centre for the Study of Social Insects (Centro de Insetos Sociais—CEIS) of the Instituto de Biociências of the Universidade Estadual Paulista (UNESP), campus of Rio Claro, Brazil.

2.2. Methods

2.2.1. HepG2 cell culture

The cells were grown in 25-cm^2 culture flasks in 5 mL of MEM (Minimum Essential Medium–Cultilab), supplemented with 10% of foetal bovine serum (FBS) and 0.1% of antibiotic-antimycotic solution (penicillin 10.000 U.I./mL/streptomycin 10 mg/mL, Cultilab) in a CO_2 incubator (5%), until they reached confluence. In these conditions, the cell cycle of this cell line is of approximately 24 h.

2.2.2. MTT test

The MTT test (Thiazolyl Blue Tetrazolium Bromide—CAS nr 298-93-1, Sigma) was performed with HepG2 cells, according to the protocol of Mosmann [16], with some modifications. In each well of a 96-well plate, 2.34×10^4 cells were seeded. After a period of 24 h for cell stabilization, the medium was removed and 200 μL of culture medium (without serum) was added for the negative control (NC), culture medium without serum plus Triton X-100 at 1% for the positive control (PC) and culture medium without serum plus the test mixture (different concentrations of the bee venom). Incubation was for a period of 3 h, then the test substance was removed and 150 μL of MTT solution (5 mg/mL) was added. The plate was incubated for 4 h, in an incubator at 37 °C. After this period, the MTT solution was discarded and in each well 100 μL dimethyl sulfoxide (DMSO) was added.

The plates were then read in spectrophotometer with a microplate reader (Apparatus Multiskan FC—Thermo Scientific) with a 540-nm filter.

2.2.3. Comet assay with HepG2 cells

The comet assay was performed to evaluate the genotoxic and anti-genotoxic potential of the BV. The assay was carried out according to the protocol described by Singh et al. [17] and Tice et al. [18] with some modifications. The assays were conducted in triplicate per treatment.

Both for the genotoxicity and the anti-genotoxicity assay, 5×10^5 cells were seeded in 25-cm^2 culture flasks, which were pre-incubated for 24 h at 37 °C, in 5% CO_2 in a humid atmosphere, for stabilization. After this period, two evaluations were made, one to assess the genotoxicity, where the cells were exposed to different concentrations of the BV for 3 h, and the other to evaluate the anti-genotoxicity, where 4 types of treatment were performed:

- pre-treatment (PT): the cells were exposed to the different concentrations of the BV for 3 h. After this period, the medium was removed and the cells were exposed to new culture medium containing methyl methanesulfonate (MMS, CAS n. 66-27-3), at the concentration of 4×10^{-2} M, for another 3 h;
- post-treatment (PostT): in this test the cells were exposed to MMS (4×10^{-2} M) for 3 h, the medium was removed and the cells were exposed to new culture medium containing different concentrations of the BV for another 3 h;
- simultaneous treatment (ST): the cells were exposed simultaneously to MMS (4×10^{-2} M) and to the different concentrations of the BV for 3 h;
- simultaneous treatment with incubation (STI): MMS (4×10^{-2} M) was pre-incubated with the BV, for 1 h at 37 °C. After this incubation period, the cells were exposed, for 3 h, to this mixture.

Besides these treatments, the cells were also exposed to a negative control (NC, 50 μL of PBS) and a positive control (PC, MMS, 4×10^{-2} M).

After the treatment periods, both for the genotoxicity and antigenotoxicity evaluation, the cells were collected in a suspension and the cell viability was tested with Trypan Blue (Gibco), according to the method described by Salvadori et al. [19]. Five μL of the cell suspension was mixed with 5 μL of Trypan Blue, followed by a counting of 100 cells for the observation of the amount of cells stained white (live) and blue (dead).

After assessment of the cell viability, 20 μL of the cell suspension was mixed with 120 μL of low melting-point agarose at 37 °C. Then, this cell suspension was placed on slides previously coated with normal-melting agarose and covered with coverslips. After solidification at 4 °C (15 min), the coverslips were removed and the slides incubated in lysis solution (1 mL of Triton X-100, 10 mL of DMSO and 89 mL of lysis stock—NaCl 2.5 M, EDTA 100 mM, Tris 10 mM and ~8 g of NaOH, pH = 10), in the dark, at 4 °C, for at least 1 h. After lysis, the slides were transferred to an electrophoresis unit and covered with alkaline buffer (NaOH 300 mM + EDTA 1 mM, pH > 13), where the slides remained for 20 min for stabilization. After this period, the samples were subjected to electrophoresis at 39 V, 300 mA (~0.8 V/cm) for 20 min. The slides were removed and neutralized in Tris buffer (pH 7.5), fixed in absolute ethanol for 10 min and stored at 4 °C until the time of analysis. The slides were stained with 50 μL of GelRed® solution (15 μL of GelRed 10,000× in water, 5 mL of NaCl at 1 M and 45 mL of distilled water) and immediately analyzed with a Leica epi-fluorescence microscope, magnification of 400×, filter B3[4] (excitation: i = 420–490 nm, barrier: I = 520 nm). One hundred nucleoids per slide were analyzed, totalling 600 nucleoids per treatment. The nucleoids were visually classified and allocated in one of the four classes (0, 1, 2, 3) according to the migration of the fragments as follows: class 0, no tail; class 1, small tail with size smaller than the diameter of the head (nucleus); class 2, size of the tail equal to the diameter of the head or even twice the diameter of the head and class 3, tail larger than twice the diameter of the head [20].

The total score was obtained by multiplying the number of cells in each class by the class damage, according to the formula: Total score = $(0 \times n_1) + (1 \times n_2) + (2 \times n_3) + (3 \times n_3)$, where n = number of cells in each class analyzed. Thus, the total score can vary from 0 to 300.

2.2.4. Cytokinesis-block MN test (CBMN)

The cytokinesis-block micronucleus test (CBMN) was used to evaluate the mutagenicity and anti-mutagenicity of different concentrations of the BV and was performed according to the protocol described by Natarajan and Darroudi [21], with some modifications.

Both for the mutagenicity and anti-mutagenicity test, 5×10^5 cells were seeded in 25-cm^2 culture flasks, which were incubated for 24 h in an incubator at 37 °C, 5% CO_2 in humid atmosphere, for stabilization. After this period, two evaluations were made,

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