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IN VITRO RADIATION PROTECTION OF PERIPHERAL BLOOD MONONUCLEAR CELLS BY *TETRAGONA CLAVIPES* PROPOLIS

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ABSTRACT

Background: Research in breast cancer has shown notable relevance in recent years, bringing serious concern on the public health policies due to its high incidence and mortality, especially in Brazil. Limitations due to the toxic effects in the normal tissues and the depletion in the immune system have often been present in the breast cancer radiotherapy and chemotherapy. It is a promise a drug development of vegetal origin that induce immune system protection in patients submitted to radiotherapy and chemotherapy, if such compounds inhibit the depletion of the circulating cell number. **Objectives:** To evaluate the *in vitro* effect of the extract of Teragona *Clavipes* Propolis (TCP) in the viability of the human Peripheral Blood Mononuclear Cells (PBMC). **Methods:** The non-irradiated control, the irradiated control and the radiated and exposed to TCP (n=3) (GIE) groups were established. The cells were irradiated with predetermined doses of 2 and 5Gy, supplemented with TCP-extracts in aqueous solution at concentrations of 0.5, 1, 5 and 10 %. Post-irradiated sample was collected and viability assay was carried out at the time of 24, 48 and 72h. **Results:** A significant decrease of PBMC viability was observed after 2 and 5Gy. However, GIE showed an increase of cell viability, especially in the 5 and 10% concentrations of the extract incubated in culture, even after 5Gy. **Conclusions:** The findings showed that the aqueous extract of TCP is an exogenous protective agent to irradiated PBMC. This study opens a relevant perspective on the role of Propolis use as an adjuvant agent in protecting the immune system of patients undergoing breast radiotherapy.

INTRODUCTION

Breast cancer has often grown a hormone-dependent malignant tumor, responsible for most of the cancer-related mortality in women. While the advances in the treatment and the prevention of breast cancer have emerged over the last decade, the phenomenon of multiple drug resistance has been the main causes of the aggravation of its morbidity being a reflection of the radiotherapy and chemotherapy failures [1] .

Radiotherapy represents the main part of the primary conservative treatment of breast carcinoma, holding the goal of a better *in situ* tumor control [2, 3]. According to Perez et al. (2015), conformational protocols, assembled in the 3D planning system for the treatment of breast cancer, consider that the breast exposure should be carried out in two opposite tangential fields in multiple fractions of 1.8 to 2.0 Gy daily, five days a week, accumulated up to 45 to 50 Gy. [4] Deep fields, expanding the radiation portals, covering a large part of the chest, mediastinum and lung, can be indicated in patients with lymph node involvements. Such fields can also include the irradiation of the internal mammary chain, when taken in

addition from the medial border 1, 3 or 4 cm beyond the median line. Radiotherapy´s patients submitted to large fields and treated with cytotoxic chemotherapy have often compromised their immune system, thus cell blood monitoring is required and such data can limit the therapy.

Drug development of vegetal origin that induce radiation protection to the immune system of patients in radiotherapy is a promise, considering that such compounds may inhibit cell phenotype changes and reduce the depletion of the number of circulating cells. Therefore, it is important to identify non-toxic compounds, effective and of low cost that can serve as immune protection in patients submitted to cancer treatment to prevent opportunistic infections. [4]

Many synthetic compounds are being tested and have grown particular interest, but these compounds have shown a limited clinical success. These substances have antioxidant properties that occur naturally, as endogenous enzymes (glutathione, superoxide dismutase, catalase) or hormones (melatonin), vitamins (C and E vitamins), carotenoids and phytochemicals (flavonoids, Curcumin) [5]. The synthetic compounds are often toxic in the doses required for radiation protection, which limits the clinical use [6,7].

Propolis, a natural compound, is a resinous substance collected by bees from different plant parts and is used to seal any openings in the hive and to eliminate possible invaders [8]. It is known to contain a variety of chemical compounds as steroids phenolic acids, esters of phenolic acids, flavonoids and terpenoids, such as CAPE and Artepillin C [9].

The main chemical constituent present in Propolis is the Caffeic acid phenethyl ester (CAPE). It is proven that this compound possesses biological activities, including antibacterial, antiviral, antioxidant, and anticarcinogenic and antiinflammatory effects [10]. Propolis supplementation with radiotherapy treatment provides a very measurable protection against DNA damage caused by ionizing radiation in leukocytes of patients during radiotherapy treatment [11]. In addition, the immune activity provided by the use of Propolis and related compounds increase hematopoietic regeneration and survival after induction by radiation [12].

Research on the radioprotective action of Propolis has increased and the knowledge of its property has been studied extensively *in vitro* and *in vivo* over time [4]. Propolis prescription becomes an interesting approach as adjuvant therapy in the modulation of cellular chemotaxis associated with radiation, in the treatment of hypoxic tumors. The monitoring of the size of the tumor, as well as inhibition of nocireceptors, is essential to determine the prognosis of the treatment [13].

The present *in vitro* study proposes to investigate the role of the extract of *Tettragona clavipes* Propolis on the viability of the human peripheral blood cells (PBMC), exposed to two doses of ionizing radiation.

METHODS

Aqueous Propolis Extract (APE) – The Aqueous Extract of Propolis was obtained from the Fazenda Poranga located in the Municipality of Itacoatiara, State of Amazonas - Brazil, from the bee hive of the *Tetragona clavipes* species. The propolis sample was kept under refrigeration at 4 °C at Molecular Biology Laboratory of the Institute of Exact Sciences and Technology of the Federal University of Amazonas. Subsequently, the extract of propolis was prepared as described by Matsushige et al. (1996) with modifications. In a Becker was added 500 mL of deionized water and 100 g of crushed crude propolis in blender, and stored for 24 h. The mixture was heated in thermostated bath with water at 80 ° C for two hours. Subsequently this solution was turbolized in a blender and filtered through a funnel of kitassato. The filtrate was dried in a water bath for 48 h. Finally, the resulting powder was resuspended in RPMI. The APE concentrations used in the experiments were 0.5%, 1%, 5% and 10%, respectively.

Pre-established lines and in vitro culture maintenance. PBMCs obtained from healthy volunteers were set in the culture. Cell cultures were maintained in RPMI-1640 medium supplemented with 10 % fetal bovine serum and antibiotic Gentamicin (50 $\mu g.\mu L^{-1}$) and Streptomycin (500 $mg.mL^{-1}$), in T-25 culture bottles in humid atmosphere containing 5 % CO_2 at 37 °C.

Separation of PBMC. The PBMCs of patients were separated according to the procedure described by Gazzinelli et al., 1983 [14]. The heparinized blood was applied in 15 mL tubes, siliconized, containing a mixture of Ficoll diatrozato (Organon Teknika Corporation; Durham, NC), in the proportion of one part of Ficoll-diatrozato to two parts blood. The solution was subjected to centrifugation for 30 min, in 1,400 rpm at room temperature. At the end of the spin cycle, a ring of mononuclear cells at the interface between the Ficoll and the plasma was observed. It was carefully removed with the aid of a Pasteur pipette and transferred to sterile, taped 15 mL tubes (Falcon No. 2070). The 15 mL volume was completed by RPMI-1640 solution (Gibco®) not supplemented and was submitted to another centrifugation (10 min, 1,200 rpm). The cells were washed twice (10 min, 1,200 rpm). Finally, an aliquot of cell suspension was collected and diluted (1:20) at Eppendorf tube containing 90 μL of Turck solution and the cell number was determined by Neubauer Chamber count, in an optical microscope. The cell concentration was adjusted to a suspension containing 1.0×10^6 $cell.mL^{-1}$ with RPMI-1640 supplemented. All manipulation of the cells was performed under sterile conditions, in the laminar flow cabinet (Biological Cabinet BBL 60474 model).

Phitohemagglutinin (PHA) Assay - Phitohemagglutinin is obtained from a variety of *Phascolus vulgaris*, demonstrating intense mitogenic activity. It is used to investigate the proportion of T lymphocytes present in the individual associated with their ability in the immune response. Its mechanism of action was associated with mitogenic activity at a concentration of 0.1 - 0.2 / mL to stimulate the basic differentiation and consequent PHA mitosis to the lymphocyte suspension in 5 mL of culture medium added with Bovine Fetal Serum 20% for blast stimulation.

Group controls. The two control groups were prepared with PBMCs in a condition of non-irradiated and non-water Propolis extract presence, kept in culture as previously described.

Radiation dose kinetics. T25 bottles containing PBMC were sealed with parafilm and transported inside a rigid capped plastic bottle, previously decontaminated with 70 % alcohol, to the location of irradiation. A 4-cm tissue-equivalent slab plate was placed underneath the T25 bottle, as well as the culture medium was filled up to 5-mm high, in relation to the surface of cells attached. This procedure was necessary to achieve electronic balance and ensure the prescribed doses in the adherent cells. T25 bottles of confluent culture underwent irradiation with Co60, in the laboratory of gamma irradiation – LIG at the *Centro de Desenvolvimento da Tecnologia Nuclear* – CDTN. The amount of 2,0 and 5,0 Gy were applied. After exposure, the cells returned to CO_2 incubator.

Time kinetics. The *in vitro* cultures, subject to 2,0 and 5,0Gy, were kept in 5% CO_2, 37° C incubator after irradiation. Aliquots of irradiated and non-irradiated cells (control) were collected in the present times of 24, 48 and 72 h post-irradiation, respectively.

Viability assessment. In triplicate samples, 100 μL of cell aliquots of 24, 48 and 72 h after-irradiation were plated in each well of ELISA flat-plates (12 × 8) and kept in incubation for 8h. A value of 20 μL (5 mg. mL-1) of MTT was added to each well and the samples were returned to the incubator where viable cells metabolized the MTT for 4 h. After metabolism, 80 μL were disposed and added 80 μL of isopropanol (0.04 M) in each well. The plates were incubated for more 6 h. Cell viability was evaluated by measuring the optical density (OD) in ELISA reader at 595 nm wavelength. The first row corresponds to the white of the ELISA reader. The plates were read with 595 nm wavelength in ELISA Elx800 apparatus. The MTT assay quantified the cell viability and the cellular proliferation based on the cleavage of tetrazolium salts (MTT). After incubation of the cells, a dye solution was assembled which OD is measured by the ELISA reader, and then the absorbance was correlated with the number of cells.

Statistical analysis. Intragroup comparison was carried out, according to the two doses of 2,0 and 5,0 Gy and the different concentrations of Propolis extracts. The t-Student test was used to obtain the significant difference between the average values obtained in the readings. It was adopted a significance level of 5 %. The software used for the analysis was Origen 8 for Windows. The mean values followed by the same capital letters (in rows) do not differ statistically from one another. Means followed by the same lowercase letters (in column) do not differ statistically from one another.

RESULTS

The PBMC viability exposed to the extract of *Tetragona clavipes* Propolis in the 0.5, 1, 5 and 10 % concentrations were depicted in Tab.1. The data were presented in the collected time kinetics of 24, 48 and 72 h after extract incubation. It has been demonstrated that viable cells were capable of forming crystals of formazan product for the cleavage of tetrazolium salt.
The MTT cell viability test was based on viability and quantification of cell proliferation based on tetrazolium salt cleavage (MTT). After incubation of the cells subjected to this essay, a dye solution was formed, which is measured through the ELISA reader (Enzyme-linked Immunosorbent Assay), and it was possible to correlate the absorbance with the number of cells.

The qualitative evaluation of the cell viability of PBMC exposed to the Propolis extract of the bee species *Tetragona clavipes* was carried out, in 4 previously determined concentrations, whose samples were collected at a time kinetics of 24, 48 and 72 h after the incubation with the extract in the concentrations of 0.5, 1, 5 and 10 % compared to control (cells not receiving EPA) and control with PHA (cells that received immune stimulus) (Table 1). Viable cells have been shown to be able to form formazan crystals around them by cleavage of the tetrazolium salt.

Table 1 - Viability values in time kinetics of 24, 48 and 72 h, determined by the MTT test in *in vitro* culture of PBMCs before and after exposure to 0.5 % concentrations; 1 %; 5 % and 10 % Aqueous Propolis extract (APE). Means followed by the same capital letters (in rows) do not differ statistically from one another. Means followed by the same lowercase letters (in column) do not differ statistically from one another.

Time	**Treatments**						**Mean of time**
	Cont.	**PHA**	**Ext. 0,5%**	**Ext. 1%**	**Ext. 5%**	**Ext. 10%**	
24	1,38cD	2.92aA	0.99cE	1.73cC	2.62bB	3.10bA	0.48
48	1.91bD	2.95aB	1.30bE	2.48bC	2.92aB	3.25abA	0.71
72	2.42aC	2.98aB	2.52aC	2.87aB	2.95aB	3.30aA	1.39
Mean	1.90	2.94	1.60	2.36	2.83	3.21	

Considering the result of the interaction between time (line) and extract (column) (Table 1) it is possible to verify that at the time of 24 h (capital letters in the line) there was a significant difference (0.05 %) among the mean values of the control (non treated) between the mean values of cells receiving PHA (Phitohemaglutinin) treatment and extract concentrations. This same significant difference was observed in the average optical density for other times.
The PBMC's cells showed high viability in the time interval of 72 h. In all concentrations (Table 1), the mean optical density increased significantly ($p > 0.05$ %) at that time in relation to the other time intervals and to the groups of cell cultures that were not supplemented with Propolis or received less concentration of Propolis.

Table 2 - Viability values determined by the MTT test of *in vitro* culture of PBMCs, before and after radiation exposure at 2Gy dose and submitted to concentrations of 1% 10% of aqueous Propolis extract. Means followed by the same capital letters (in rows) do not differ statistically from one another. Means followed by the same lowercase letters (in columns) do not differ statistically from one another.

Treatment	**Time**			**Mean of Treatment**
	24	48	72	
Control	0.46 cAB	0.59 cA	0.39bB	0.48
Extract 1%	0.77bB	0.94bA	0.43bC	0.71
Extract 10%	1.43aA	1.39aA	1.35aA	1.39
Mean	0.88	0.97	0.72	

The table 2 shows that already in the 24 h there is a significant decrease ($P < 0.005$) in PBMCs survival before treatment with Propolis in relation to cell cultures that were

supplemented with Propolis extract. It is interesting to note that at the pre-established times there was no significant increase in cell proliferation in cell cultures that were subjected to the 1 % concentration of Propolis extract, in detriment of concentration increase (10 %).

As shown in Figure 1, the cell viability levels of PBMCs in the group that only received 5 Gy radiation, significantly reduced ($p < 0.005$) in relation to the two groups of cell cultures receiving PHA and Extract at 10 %. Regarding the level of survival in groups of cells that received 5 Gy radiation, and exposed to the extract, the survival curve was significantly higher than the control levels ($p < 0.005$). Considering the pre-established time intervals, a non-significant difference in cell growth was observed in cultures of cells receiving radiation treatment plus Propolis and those receiving PHA 1μ $.mL^{-1}$.

Table 3 – Viability values in time kinetics of 24, 48 and 72 h, determined by the MTT test in *in vitro* culture of PBMCs, before and after radiation exposure at 5Gy doses, and submitted to concentrations of 1 % and 10 % aqueous extract of Propolis. Means followed by the same capital letters (in rows) do not differ statistically from one another. Means followed by the same lowercase letters (in columns) do not differ statistically from one another.

	Time			**Mean of Treatment**
Treatment	24	48	72	
Control	0.48cB	0.36cB	0.86bA	0.57
Extract 1%	0.74bA	0.81bA	0.57cB	0.70
Extract 10%	1.03aB	1.23aA	1.32aA	1.19
Mean	0.75	0.80	0.91	

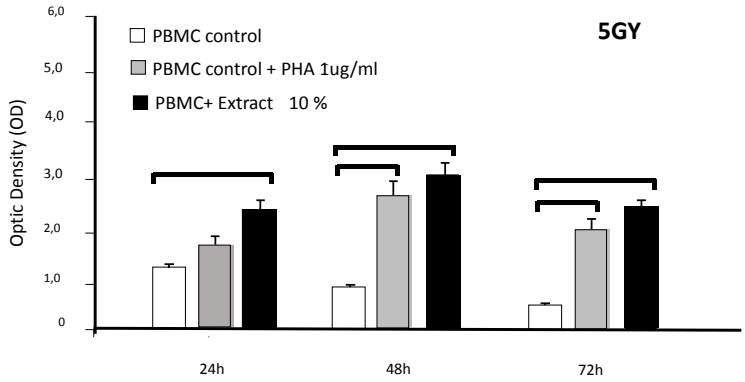

Figure 1 – Viability data in time kinetics of 24, 48 and 72 h, determined by MTT test in *in vitro* culture of PBMCs, before and after radiation exposure at 5Gy doses, submitted to concentrations of 10% of extract Aqueous solution of Propolis and 1 μg.mL^{-1} PHA.

DISCUSSION

The present study has investigated the influence of Brazilian Propolis, particularly from Itacoatiara, Amazonas State, on cytotoxic damage and the proliferative potential of PBMC cells irradiated with Co-60. Although the exact mechanism of action of Propolis on radiation protection is not fully elucidated, several studies have shown that the pharmacological properties of Propolis are attributed mainly to the presence of flavonoids due to their action

as scavengers of free radicals [15]. Such scavengers of free radicals are likely to have key role in radiation protection, since ionizing radiation induces toxicity which is measured primarily by free radicals and its action in DNA [15,16]. According to Ebeid et al., 2016, Propolis supplementation with radiotherapy treatment provides a very measurable operation against DNA damage by ionizing radiation in leukocytes of breast cancer patients and inhibits overexpression of RRM2 (Ribonucleotideo reductase 2). In addition, Propolis has beneficial effects on serum antioxidant capacity and improves digestive use of iron and hemoglobin regeneration efficiency [11].

Aqueous extract of Propolis stimulated PBMC cell growth at 5 Gy doses (Figure 1). The combined treatment with Propolis (10%) and radiation stimulated cell growth by inhibiting the cytotoxic effect of radiation, especially after incubation for 72 h (Figure 1). The results showed that the extract of Propolis starting at the 1% concentration until 10%, introduced radiation protective effect on PBMCs against the damage caused by the interaction of ionizing radiation with its biological components. Ionizing radiation is widely used for the treatment of breast cancer. However, one of the limitations of the radiation use is its toxic effects on normal tissues. Human blood cells showed radio sensitivity in all gamma-rays from Co60 doses [16, 17, 18, 19]. databased on this findings, s Propolis showed a stimulatory effect on cell proliferation in PBMCs.

CONCLUSION

The association of Propolis and radiation exposure on cell culture showed no significant reduction in the PBMC viability at 24, 48 and 72 h. Thus, the Propolis of *Teragona Clavipes* offered a protection against damage caused by ionizing radiation in PBMC. This finding supports the potential use of Propolis in combination with radiation therapy, in order to protect immune circulating cells from genotoxic damage caused by radiation. Studies on the chemical constituents responsible for its radio-protection effect is a required to better characterize suchsubstance.

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