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Interaction of bracken-fern extract with vitamin C in human submandibular gland and oral epithelium cell lines

Mariana Campos-da-Paz [a], Luciana O. Pereira [b], Leandro Santos Bicalho [c], José G. Dórea [c], Marcio J. Poças-Fonseca [b], Maria de Fátima M. Almeida Santos [b,*]

[a] *Departamento de Biologia Celular, Instituto de Ciências Biológicas, Universidade de Brasília, 70910-900 Brasília, DF, Brazil*
[b] *Departamento de Genética e Morfologia, Instituto de Ciências Biológicas, Universidade de Brasília, 70910-900 Brasília, DF, Brazil*
[c] *Departamento de Nutrição, Faculdade de Ciências da Saúde, Universidade de Brasília, 70910-900 Brasília, DF, Brazil*

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Abstract

he consumption of bracken-fern (*Pteridium aquilinum*) as food is associated with a high incidence of cancer in humans and animals. Thus far, the carcinogenic effects of bracken-fern consumption could be related to chromosome aberrations verified in animal and in human peripheral lymphocytes. We tested the *in vitro* effects of vitamin C (10 and 100 μg/ml) on the reversibility of DNA damage caused by bracken-fern on human submandibular gland (HSG) cells and on oral epithelium cells (OSCC-3) previously exposed to bracken-fern extract. DNA damage (i.e. nuclei with increased levels of DNA migration) was determined by comet assay, cell morphology was evaluated by light microscopy and cellular degeneration was assessed by the acridine orange/ethidium bromide fluorescent-dyeing test. Results showed that vitamin C alone did not reduce DNA damage caused by bracken-fern in HSG and OSSC-3 cells. However, at a higher concentration (100 μg/ml), vitamin C induced DNA damage in both cell lines. Moreover, vitamin C (10 and 100 μg/ml) together with bracken-fern extract showed synergistic effects on the frequency of DNA damage in HSG cells. In addition, cells treated with bracken-fern extract or vitamin C alone, or with their association, showed apoptosis morphological features, such as chromatin condensation, cytoplasmic volume loss, changes in membrane symmetry and the appearance of vacuoles; these alterations were observed in both cell lines. These results demonstrate that bracken-fern extract was cytotoxic to HSG and OSCC-3 cells, causing cell death by apoptosis, and that vitamin was not able to revert these effects.

Keywords: Bracken-fern; Vitamin C; DNA damage; Apoptosis; HSG cells; OSCC-3 cells

1. Introduction

Bracken-fern (*Pteridium aquilinum*) can induce acute and chronic toxicity in man and farm animals [1–3]. In cattle, chronic bracken-fern toxicity causes carcinomas in urinary bladder [4] in bovine presenting enzootic hematuria, which is a relatively common condition in bracken-infested pasturelands [5]. Epidemiological studies have related the occurrence of neoplasms in humans to the consumption of bracken crosiers [2,6] or milk from cows feeding on bracken [7,8]. It has been also suggested that the consumption of water contaminated with bracken-derived substances can be toxic [9].

Long-term exposure to bracken produces ionizing-radiation-like effects [10,11]. Radiomimetic compounds are associated to the generation of free radicals [12], which would be the primary effectors of DNA damage and chromosome aberrations induced by the plant. Moreover, a high incidence of structural chromosome aberrations was verified in human peripheral lymphocytes of bracken-fern consumers [13], in lymphocytes of animals grazing on bracken-fern [14], and in mice bone marrow and peritoneal cells [15].

Considering that bracken-fern-induced DNA damage could be related to free radicals generation, it is reasonable to assume that antioxidant dietary constituents – such as vitamin C – could revert such damage. The antioxidative effects of this vitamin are mediated by the scavenging of reactive oxygen and

* Corresponding author at: Universidade de Brasília, Campus Universitário Darcy Ribeiro, Instituto de Ciências Biológicas, ICC, Sul AT149, CEP 70910-970 Brasília, DF, Brazil. Tel.: +55 61 3307 2169; fax: +55 61 3273 4942.
E-mail address: lindocas@unb.br (M.d.F.M.A. Santos).

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nitrogen species [16], and by regeneration of other small molecule antioxidants, such as α-tocopherol and glutathione (GSH) [17]. Furthermore, vitamin C inhibits the mutagenic activity of several carcinogenic substances [18]. Notwithstanding the antioxidant activity, vitamin C is also known as a pro-oxidant substance *in vitro* [19]. In this case, its interaction with transitional metal ions is accompanied by H_2O_2 production [17,20]. There are reports that vitamin C heightens the cytotoxic effects of chemotherapeutic drugs, such as arsenic trioxide, through free radical generation [21].

A correlation between bracken-fern consumption and oral cancer has not yet been reported. Nonetheless, since the mouth is the first body compartment exposed to the plant, and head and neck cancers are the sixth leading cause of cancer mortality [22], it is important to study cell lines that can contribute to the understanding of the genetic damages possibly involved in the etiology of such neoplasies. Considering the association between genetic alterations and cancer, and the occurrence of neoplasies related to bracken intake, the aim of the current study was to evaluate the effects of vitamin C on oral cavity-derived cells (oral epithelium – OSCC-3 – and human submandibular gland, HSG) presenting DNA damage induced by bracken-fern extract. OSCC cells have been used in carcinogenesis mechanisms, antitumoral treatments and cytotoxicity studies [23–25]. HSG cells have been employed in tissue engineering, cell differentiation, apoptosis induction and cytotoxicity experiments [26–29].

2. Materials and methods

2.1. Plant extract and reagents

Bracken-fern was collected in the savanna (*cerrado*) of Brasilia and identified by botanists of the University of Brasilia, Brazil. The aqueous extract was obtained as described before [15]. Briefly, fronds were washed and oven dried at 37 °C and ground in a Willey mill. The resulting powder was extracted with distilled water—31 mg of extract for 1 ml. The extract was filtered and sterilized through a 0.22-μm nitrocellulose membrane and stored in cold and dark until use. Bracken-fern extracts and vitamin C (ascorbic acid, CEVITON®, ARISTON) were diluted in culture medium at the determined experimental concentrations. Hydrogen peroxide (Sigma Chemical Co., St Louis, MO, USA) was used as oxidizing agent. HSG cells and human oral epithelium cells (OSCC-3) were a generous gift of Prof. B. J. Baum (National Institute of Health Bethesda, USA).

2.2. Culture conditions

In order to test the effects of vitamin C on the reversibility of DNA damage caused by bracken-fern, HSG and OSCC-3 cells were grown at 37 °C, in an atmosphere of 5% of CO_2 in Dulbecco's modified Eagle's medium, DMEM, (GIBCO-BRL), pH 7.4, supplemented with 10% fetal calf serum, 100 U/ml penicillin and streptomycin (100 μg/ml).

For comet and acridine orange/ethidium bromide staining assays, HSG and OSCC-3 cells (5×10^4) were seeded in 25 cm^2 tissue culture flasks and stabilized for 24 h. For morphological analysis by light microscopy, 10^5 cells were grown over microscope coverslips placed in six-well culture plates. All experiments were run in duplicate. The bracken-fern concentration used in the tests was based on cell viability after the trypan blue exclusion test. Experimental groups consisted of cells treated with bracken-fern (670 μg/ml for HSG cells and 1340 μg/ml for OSCC cells), alone or in combination with vitamin C (10 and 100 μg/ml), cultured for 48 h. Negative controls consisted of untreated cultures. Hydrogen peroxide (1 mM), added to culture 4 h before cell processing, was used as positive control. After cultivation, cells were washed, harvested, re-suspended in phosphate buffer saline (PBS) and used for the tests.

2.3. Comet assay

We used the comet assay described by Singh et al. [30] with some modifications. Briefly, 30 μl of cell suspension (10^5–10^6 cells) were mixed with 120 μl of low melting point agarose (0.5% in PBS) and spread over microscopy slides that had been previously covered with a layer of agarose type II (1.5% in PBS). The slides were immersed in cold lysing solution (2.5 M NaCl, 0.1 M EDTA, 0.01 M Tris and 1X Triton X-100) for 1 h at 4 °C. The slides were then placed in a denaturating electrophoresis buffer (300 mM NaOH, pH 13, 1 mM EDTA,) and subject to 25 V (300 mA) for 20 min. After electrophoresis, the samples were neutralized in 0.4 M Tris buffer (pH 7.5) and stained with 50 μl ethidium bromide (20 μg/ml; Sigma).

Approximately 200 cells (100 per replicate slide) per experimental group were randomly analyzed using a fluorescence microscope (Axioplan-Carl Zeiss) at 400×, with a 515 to 560-nm exciting filter and a 590-nm barrier filter. The category of DNA damage was assigned to five classes (0–4) based on the visual aspect of the comets (nucleoids), considering the extent of DNA migration, according to the criteria established by Visvardis et al. [31]. Nucleoids with a bright head and no tail were classified as class 0 (undamaged nucleoids) and nucleoids with a small head and long diffuse tails, as class 4, i.e. as highly damaged nucleoids. Nucleoids with intermediate characteristics were classified as classes 1, 2 or 3. The index of DNA damage (IDD) was estimated quantitatively by the equation described by Jalonszynski et al. [32];

$$\mathrm{IDD} = \frac{(n_1 + 2n_2 + 3n_3 + 4n_4)}{(\Sigma/100)}$$

where IDD is the DNA damage in arbitrary units, n_1–n_4 the number of classes 1–4, and Σ is the total number of scored nucleoids, including class 0. IDD values ranged from 0 (all undamaged nucleoids) to 400 (all maximally damaged nucleoids). Arbitrary units were converted to the percentage of damaged DNA (frequency of DNA damage), with the score of 400 as 100% damaged DNA.

2.4. Detection of apoptosis and necrosis

In order to assess the frequency of cell death mechanism (apoptotic or necrotic), cells were collected, centrifuged and re-suspended in PBS. Twenty microliters of the cell suspension (10^5–10^6 cells) were stained with a fresh solution of acridine orange (100 μg/ml)/ethidium bromide (100 μg/ml)—1:1 for 5 min. Cells were then examined using a fluorescence microscope (Olympus; barrier filter O 530 NM). Apoptosis or necrosis and cell survival were evaluated by visual analysis according to previously established criteria [33,34]. Acridine orange penetrates into living and dead cells, emitting green fluorescence as a result of intercalation into double-stranded DNA, and red–orange fluorescence after binding with single-stranded RNA. Ethidium bromide emits red fluorescence after intercalation in DNA of cells with altered cell membrane (at necrosis or at a late stage of apoptosis). Thus, considering that apoptotic cells show fragmented nucleus and condensed chromatin, four cell stages can be identified by this assay (Fig. 1): (i) living cells, with uniform green nucleus (Fig. 1a), (ii) early apoptosis (cell membrane is still preserved but chromatin condensation and an irregular green nucleus are visible) (Fig. 1b), (iii) late apoptosis (ethidium bromide penetrates through altered cell membrane and stains the nuclei in red, while fragmentation or condensation of chromatin is still observed) (Fig. 1c and d), and (iv) necrosis (uniformly red-stained cell nuclei) (Fig. 1e).

2.5. Statistical analysis

The statistical analysis of the acridine orange/ethidium bromide double-staining data was based on cells/group after arcsine $\sqrt{x}$ transformation (x = number of death cells). The comet assay data was analyzed considering two parameters: (i) frequency of cells with damaged DNA; (ii) IDD. Statistical analysis of the data was based on cells/group after arcsine $\sqrt{x}$ transformation (x = number of cells with DNA damage) or log x transformation (x = IDD). Trans-