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Haematological and immunological effects of repeated dose exposure of rats to integerrimine *N*-oxide from *Senecio brasiliensis*

Fabiana Elias [a,b], Andreia O. Latorre [a], Fernando Pípole [a], Mitsue Haraguchi [c], Silvana L. Górniak [a], Isis M. Hueza [d,*]

[a] Research Centre for Veterinary Toxicology (CEPTOX) – Department of Pathology, School of Veterinary Medicine and Animal Sciences, University of São Paulo, Pirassununga 05508-270, SP, Brazil
[b] Veterinary School, UPIS-Faculdades Integradas, Brasília 70390-125, DF, Brazil
[c] Biological Institute of São Paulo, São Paulo 04014-002, SP, Brazil
[d] Instituto de Ciências Ambientais, Químicas e Farmacêuticas, Universidade Federal de São Paulo (UNIFESP), Diadema 09972-270, SP, Brazil

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ABSTRACT

This study is the first in the literature to focus attention on the possible immunotoxic effect of integerrimine *N*-oxide content in the butanolic residue (BR) of *Senecio brasiliensis*, a poisonous hepatotoxic plant that contains pyrrolizidine alkaloids (PAs). PAs have been reported as a pasture and food contaminant and as herbal medicine used worldwide and are responsible for poisoning events in livestock and human beings. After the plant extraction, BR extracted from *Senecio brasiliensis* was found to contain approximately 70% integerrimine *N*-oxide by elemental and spectral analyses (^{1}H and ^{13}C NMR), which was administered to adult male Wistar Hannover rats at doses of 3, 6 and 9 mg/kg for 28 days. Body weight gain, food consumption, lymphoid organs, neutrophil analysis, humoural immune response, cellular immune response and lymphocyte analysis were evaluated. Our study showed that integerrimine *N*-oxide could promote an impairment in the body weight gain, interference with blood cell counts and a reducing T cell proliferative activity in rats; however, no differences in the neutrophil activities, lymphocytes phenotyping and humoural and cellular immune responses were observed. It is concluded that doses of integerrimine *N*-oxide here employed did not produce marked immunotoxic effects.

1. Introduction

Pyrrolizidine alkaloids (PAs) are natural phytotoxins found in thousands of plant species around the world. It is estimated that PAs are present in about 3% of all flowering plants (Roeder, 1995; Smith and Culvenor, 1981). Currently, about 660 PAs and their *N*-oxide derivatives are known, and half of them present toxic properties (Molyneux and Panter, 2009; Roeder, 1995, 2000; Stegelmeier et al., 1999; Trigo, 2010).

Plants with PAs are common causes of natural poisonings that affect livestock, wild animals (Lucena et al., 2010) and humans, as people consume traditional herb prescriptions, such as bush-tea in Jamaica (Bras et al., 1961; Brooks et al., 1970) or herbal medicines. These herbal products, such as those that contain *Symphytum officinalis* (commonly called comfrey), *Heliotropium indicum* and *Eupatorium cannabinum*, are known to contain PAs and have been reported to cause intoxication events in industrialised countries such as USA and UK (Fu et al., 2002; Mei et al., 2010; Stickel and Seitz, 2000; Roeder and Wiedenfeld, 2009). Indirect intoxication can also occur by the ingestion of contaminated foodstuff, such as the milk from poisoned dairy cattle (Dickinson, 1980; Molyneux and James, 1990), eggs from poultry that has consumed PA-contaminated grain (Edgar and Smith, 1999) or even honey produced by bees who pollinate contaminated fields (Boppré et al., 2005; Deinzer et al., 1977; Kakar et al., 2010). Thus, PA exposure is a problem for both veterinary and public health.

Among plants that contain PAs as a constituent, some genera are especially important, such as *Senecio*, which contains many species of worldwide concern. For example, common groundsel (*S. vulgaris*, L.) has been found to contaminate salads (e.g., ready-packed rucola and salad mixtures) in Middle Europe. Additionally, tansy ragwort (*Jacobaea vulgaris* syn. *Senecio jacobaea*, L.) is a common Western European plant inadvertently introduced into Eastern Europe, Australia, New Zealand and the north-western regions of North America. *S. jacobaea* from North America and *S. brasiliensis* from Brazil have been discussed recently due to their extensive expansion into pastures, which has led to a great number of horse and cattle intoxications (Lucena et al., 2010; Stegelmeier et al., 2009; Wiedenfeld and Edgar, 2010).

PAs produced by *Senecio* spp. are ester alkaloids derived from necines, retronecine and otonecine. However, these agents must be transformed into a pyrrole compound by microsomal liver enzymes to become the active toxic molecule, which possesses a

* Corresponding author. Tel.: +55 11 3319 3308; fax: +55 11 4033 6428.
E-mail address: imhueza@unifesp.br (I.M. Hueza).

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strong bonding affinity for nucleophilic groups. After oral uptake and absorption, PAs are first hydroxylated by hepatic monoxygenases of cytochrome P-450 into an unstable compound that rapidly dehydrates to didehydropyrrolizidine alkaloids, resulting in a spontaneous rearrangement to an aromatic pyrrole system. These pyrrole compounds generate stabilised carbonium ions by the loss of hydroxyl groups that react rapidly with nucleophilic mercapto, hydroxyl and amino groups on proteins and the amino groups of purine and pyrimidine bases in nucleosides like DNA and RNA. In addition, the hydrolysis of didehydropyrrolizidine alkaloids results in pyrrole compounds with alkylating activity towards DNA, proteins and cysteine residues (Culvenor et al., 1969, 1971). Based on these reports, it is not surprising that pyrrole compounds can damage proteins, RNA and DNA, resulting in protein synthesis impairment, cellular function loss, tumour development and an anti-mitotic effect. Indeed, it is well established that PAs can promote megalocytosis, carcinogenesis and teratological effects (Fu et al., 2004; Mattocks and Cabral, 1984; Petry et al., 1984).

While the toxic effects of PAs on different organ systems, namely the liver, are amply covered, very little is known about the possible toxic effects of PAs on the immune system, a system highly dependent on protein synthesis and lymphocyte proliferation to establish a vigorous functional immune response. Thus, the purpose of the present study was to investigate the potential immunotoxic effects of PAs from *S. brasiliensis* on different branches of the immune response in rats.

2. Materials and methods

2.1. Plant material

Leaf samples of *S. brasiliensis* were collected in Pelotas, Rio Grande do Sul, Brazil, in February 2008. A voucher specimen was deposited in the Federal University of Pelotas Herbarium, Brazil, under the number 24592 and was identified as *Senecio brasiliensis* (Spreng.) Less. var. *brasiliensis*. Dry leaf samples of *S. brasiliensis* (900 g) were macerated in 92% ethanol for 3 days. After total solvent evaporation under reduced pressure at 40 °C, an ethanol residue was obtained that was acidified with 10% tartaric acid and filtered to obtain a waxy residue and an acidic solution. This solution was consecutively fractionated with ethyl acetate and chloroform to obtain an ethyl acetate and chloroform solution. Total solvent evaporation was performed under reduced pressure at 40 °C to achieve both residues: the ethyl acetate residue and the chloroform acid residue. Subsequently, the remaining acidic aqueous solution was alkalised with 0.5 M ammonium hydroxide (NH_4OH) to pH 9–10 and was extracted consecutively with chloroform and *n*-butanol saturated with water. The chloroform and butanol solutions were evaporated completely for their waste products, chloroform alkaline and butanolic residue (BR).

These residues were submitted to elemental and spectral analyses (^1H and ^{13}C NMR), which were performed at the Analytical Laboratory of the Institute of Chemistry, University of São Paulo and revealed the presence of integerrimine *N*-oxide only in the BR. The ^1H and ^{13}C spectral data of the BR were consistent with previously reported findings by Reina et al. (2001). From these results, the purity of the extracted product was determined to be 69%. Thus, the doses employed in the present study of 3, 6 and 9 mg BR per kilogram of body weight contained 2.07, 4.14 and 6.21 mg, respectively, of integerrimine *N*-oxide.

2.2. Animals

Male adult Wistar Hannover rats (10 weeks of age) were obtained from our colony in the Department of Pathology, School of Veterinary Medicine and Animal Sciences, University of São Paulo. All rats were housed separately and received food (Nuvilab CR1® – balanced rodent feed produced as recommended by the National Research Council and National Institute of Health – USA) and water *ad libitum* and were maintained under controlled conditions of temperature (22–25 °C), relative humidity (50–65%) and lighting (12 h/12 h light/dark cycle). Cages were checked daily until the end of the experimental period for rats' clinical signs, soft faeces, food waste and mortality. Food consumption and body weight gain were measured every 3 days. Experiments were performed in accordance with the ethical principles for animal research adopted by the Bioethics Committee of the School of Veterinary Medicine and Animal Sciences (process 1461/2008).

2.3. Reagents

Dichlorofluorescein diacetate (DCFH-DA) was obtained from Molecular Probes. Concanavalina a (ConA), freund's complete adjuvant (FCA), lipopolysaccharide (LPS 0127:B8), peroxidase, phorbol myristate acetate (PMA), propidium iodide (PI), RNAse A and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. Foetal bovine serum (FBS), HEPES, RPMI culture medium-1640 and trypan blue were obtained from Gibco. Keyhole limpet hemocyanin (KLH) was obtained from Calbiochem. FITC-labelled anti-CD3 (clone G4.18), PE-labelled anti-CD4 (clone OX-35), PerCP-labelled anti-CD8a (clone OX-8), PE-labelled anti-IgM (clone G53-238), FITC-labelled anti-CD45R (clone HIS24) and purified anti-CD32 (clone D34-485) Rat BD Fc Block™ antibodies were purchased from BD Pharmingen.

2.4. Lymphoid organs, serum biochemistry and neutrophil granulocyte evaluation

Twenty-eight rats were randomly divided into one control and three experimental groups. Rats from experimental groups were treated once daily with 3, 6 or 9 mg BR/kg body weight by gavage for 28 days. On day 29, all rats were anaesthetised for blood collection from the caudal cava vein, and after euthanasia by cervical dislocation, lymphoid organs (thymus and spleen) were harvested for lymphoid organ analysis.

Blood collection was performed with or without heparin to evaluate haematological and neutrophil parameters and serum biochemical analyses, respectively. The haematological parameters were assessed by automatic Horiba® ABX equipment. Serum biochemistry was analysed using a Roche Hitachi 917 analyser with corresponding reagents. The measured markers were serum albumin, total protein, alkaline phosphatase (AP), alanine transaminase (ALT), aspartate transaminase (AST), and γ-glutamyl transpeptidase (GGT).

To perform neutrophil granulocyte analysis, blood samples (100 μL) were directly used to analyse the phagocytic activity and the oxidative burst of neutrophil. The method of analysis was adopted from Hasui et al. (1989). To analyse the oxidative burst, cells obtained from each rat (2×10^5 cells/tube) were incubated for 30 min at 37 °C with either dichlorofluorescein diacetate (DCFH-DA) or DCFH-DA + phorbol myristate acetate (PMA) or DCFH-DA + *Staphylococcus aureus* conjugated with propidium iodide (SAPI). To analyse the phagocytic activity, cells were incubated for 30 min at 37 °C with either SAPI or DCFH-DA and SAPI. Ten thousand neutrophils were then acquired by flow cytometry (FACS Calibur FACSCalibur™ flow cytometer equipped with Cell Quest Pro® software (Becton Dickinson [BD] Immunocytometry System), and the data were analysed using FlowJo 7.2.2® software (Tree Star Inc, Ashland, KY).

For lymphoid organ analysis, the thymus and spleen were removed from euthanised rats and weighed. The spleen was disrupted with two pieces of ground glass, and the red blood cells were removed by lysing with a 0.4% ammonium chloride esterile solution, resulting in a single splenocyte suspension in cold RPMI-1640 culture medium. Bone marrow cell suspensions were achieved by flushing the marrow cavity of the left femur of each rat with ice-cold RPMI-1640 medium using a sterile syringe with a 26-gauge needle. Viability was assessed via a trypan blue dye exclusion test, and cell numbers were determined by a haemocytometer. Moreover, fragments of liver, spleen, thymus, lungs, and kidney were collected and fixed in 10% formalin, routinely embedded in paraffin, cut into 5-μm thick sections and stained with haematoxylin and eosin (HE) for histopathology.

2.5. Humoural immune response

Forty rats were randomly divided into one control and three experimental groups. Rats from experimental groups were treated once a day with 3, 6 or 9 mg BR/kg body weight by gavage for 28 days. On Day 21 of their regimens, subsets of rats ($n = 10$/treatment group) were immunised by intraperitoneal (IP) injection of 2.0×10^9 sheep red blood cell (SRBC) in 0.9% saline (normal saline). Seven days later, blood samples were harvested from the euthanised rats, allowed to clot in a vertical tube at room temperature for 45 min and then were separated by centrifugation at 3200*g* for 10 min. The serum was collected, and the anti-SRBC antibody titre was estimated as follows: 25 μL of twofold diluted serum in normal saline was challenged with 25 μL 1% (v/v) SRBC suspension in a microtitre plate. The plate was incubated at 37 °C for 1 h and then checked for haemagglutination, and the highest dilution causing haemagglutination was taken as the antibody titre. Antibody titres were expressed in a graded manner, with the minimum dilution (i.e., 1/2) being ranked as "1" against which the mean ranks of the different groups were compared.

The Plaque-Forming Cell assay (PFC) was also used to assess the status of the humoural immune response of each host. Briefly, rat spleens were used to generate a corresponding single-cell suspension (10^7 cells/mL) in RPMI-1640 medium at 4 °C. The splenocyte suspension, SRBC, and guinea pig complement were added to a 1.0-cm^2 PFC well to achieve a final concentration of 2.0×10^6 splenocytes/mL, 7% SRBC, and 10% complement in a final volume of 50 μL; duplicate wells were prepared for each rat sample. The wells were then covered with glass slides (22 × 22 mm) and sealed with varnish. Each PFC well was incubated at 37 °C for 3 h before the number of lysate plaques produced by the 10^5 splenocytes in the well was counted under a light microscope (40× magnification). Data are expressed as the total PFC/10^6 splenocytes.

2.6. Cellular immune response (delayed type hypersensitivity; DTH)

Forty rats were randomly divided into one control and three experimental groups. Rats from experimental groups were treated once a day with 3, 6 or 9 mg BR/kg body weight by gavage for 28 days. Sensitisation with KLH was performed as described by Exon et al. (1990). Briefly, KLH (5 mg/mL) was injected into