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Original Article

The effect of the carotenoid bixin and annatto seeds on hematological markers and nephrotoxicity in rats subjected to chronic treatment with cisplatin

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ABSTRACT

This study assessed the protective effect of the carotenoid bixin and annatto seeds against possible nephrotoxicity induced with a single peritoneal administration of pharmacological cisplatin in male Wistar rats. After 48 h, the blood cell differential count showed a significant reduction in neutrophil counts in rats that received a diet rich in bixin when compared to the group that received only cisplatin. The use of cisplatin led to an increase in kidney weight. The carotenoid bixin attenuated renal injury, characterized by increased polymorphonuclear infiltration. No protective effect was observed with respect to Annatto. These results demonstrate the role of toxic cisplatin and suggest that bixin affords a protective effect against cisplatin-induced nephrotoxicity in adult Wistar rats.

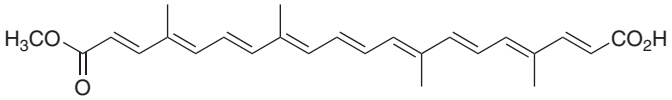
Introduction

Cisplatin [*cis*-diamminedichloroplatinum(II) – CDDP] is a chemotherapeutic agent used alone or in combination with other antineoplastic agents in the treatment of lung, brain, throat, esophagus, stomach, colon, bladder, testicular, ovarian and uterine and other cancers. However, cisplatin nephrotoxicity is the principle dose-limiting factor for the use of cisplatin (Santos et al., 2012). It has been suggested that the generation of reactive oxygen species and lipid peroxidation is responsible for the cisplatin-induced renal tubular impairment (Chirino and Pedraza-Chaverri, 2009).

The physiological functions of patients undergoing chemotherapy should be monitored using periodic laboratory tests to analyze parameters of global renal function, such as creatinine and urea, and obtain the blood cell count to determine general immune status (Sodré et al., 2007).

Carotenoids are compounds that have antioxidant properties capable of sequestering free radicals. They are found in various foods and may have a protective effect against oxidative damage caused, for example, by chemotherapy (Antunes and Bianchi, 2004; Rios et al., 2009; Bautista et al., 2004).

Annatto is a natural color extracted from the outer coating of the seeds of the Annatto tree (*Bixa orellana* L., Bixaceae), which is native to the Amazon region in South America (Mercadante, 2001; Gomes, 2007). Its color varies from yellow to red, and the most abundant carotenoid is *trans*-bixin (**1**), which accounts for around 80% of the total pigment content of the seeds (Mercadante, 2001; Giuliano et al., 2003; Bautista et al., 2004). In addition to its coloring action, annatto also has functional properties that form the basis of a variety of roles and actions in living organisms due to the presence of antioxidant compounds (Krinsky, 1994). Studies have shown that bixin has beneficial effects, including the reduction of oxidative stress and its ability to act as a chemo-preventative agent and to reduce the nephrotoxic effects of antitumor agents (Bertram and Bortkewicz, 1995; Agner et al., 2005; Rios et al., 2009).



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In an attempt to minimize the side effects caused by cisplatin, this study evaluates the antioxidant activity of the carotenoid bixin

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and annatto seeds on cisplatin-induced nephrotoxicity in adult male Wistar rats by examining biochemical and histological aspects to assess renal function and morphology and performing complete blood counts.

Materials and methods

Plant material

This study examined annatto (*Bixa orellana* L., Bixaceae) from the Eldorado access, ICN (189644) 10.VII.2009, collected and identified by J.M. Wiest.

Assays

Animals

The biological assay was performed in the Vivarium of the Food Science Department of the Institute of Food Science and Technology of the Federal University of Rio Grande de Sul in Brazil. The animals were housed in ventilated rooms at a temperature of $24 \pm 2\,^{\circ}C$ with a 12–12 h light–dark cycle and $60 \pm 5\%$ humidity and given *ad libitum* access to food and water.

The animals were divided at random into four groups: CG (control group), CPG (cisplatin group), CPBG (cisplatin/bixin group), and CPAG (cisplatin/annatto group). The biological trial lasted 28 days. The CG group received commercial rodent feed, the CPG group received commercial feed plus cisplatin ($5\,mg\,kg^{-1}$ body weight), the CPBG group received commercial feed with bixin ($0.065\,mg\,kg^{-1}$ body weight) and cisplatin ($5\,mg\,kg^{-1}$ body weight), and the CPAG group received commercial feed with cisplatin ($5\,mg\,kg^{-1}$ body weight) and annatto ($0.500\,mg\,kg^{-1}$ body weight). The doses of bixin and annatto used in pretreatment were based on data found in the literature on nephroprotective effects (FAO/WHO, 2007; Antunes and Bianchi, 2004). A single dose of cisplatin ($5\,mg\,kg^{-1}$) was administered 48 h before the end of the experiment to induce oxidative stress.

Obtaining annatto and bixin crystals

Bixin crystals were obtained using a methodology developed by Rios and Mercadante (2004). The seeds were first washed in hexane and methanol to remove hydrophilic and hydrophobic impurities and the bixin was then extracted using ethyl acetate. The extract obtained through this process was dried in a rotary evaporator ($T < 30\,^{\circ}C$) and rediluted in dichloromethane. For the crystallization process, the extract was heated on a hot plate ($T < 50\,^{\circ}C$). After being chilled, absolute ethanol was added and the extract was then cooled in an ice bath and placed in a freezer at $14 \pm 2\,^{\circ}C$ for 24 h. The bixin crystals were filtered and washed with chilled absolute ethanol, dried in a vacuum oven for 24 h, and then stored at $-18\,^{\circ}C$ until use.

Purification of bixin

The purity of bixin was determined using a method described by Tocchini and Mercadante (2001), in which the bixin is extracted from annatto seeds previously washed with petroleum ether and a mixture of methanol and dichloromethane (1:1). After concentration, the extract was successively purified by silica gel thin layer chromatography using both ethyl acetate and petroleum ether (3:2) as mobile phases. In the first chromatography, silica gel layers (Merck) were prepared in the laboratory and the two separated bands and everything remaining at the origin were scraped off and eluted with the methanol/dichloromethane mixture (1:1). After concentration, the band was applied again to ready silica plates and split into two bands. The major one at R_f 0.61 was scraped off and eluted with the methanol/dichloromethane mixture. The pattern obtained shows 99% purity, checked by High Performance Liquid Chromatography (HPLC) (Agilent 1100 series).

Cisplatin

Cisplatin [*cis*-diamminedichloroplatinum (II), CDDP; CAS Nº. 15663-27-1] was kindly donated in its commercial form by Quiral Química do Brasil S.A. (Platinil®).

Blood collection and doses of urea and creatinine

The animals were sedated for blood collection using benzoadiazepine ($0.25\,mg\,100\,g^{-1}$ body weight) and sodium pentobarbital ($4.6\,mg\,100\,g^{-1}$ body weight). A median incision was made in the ventral part of the abdomen and blood was collected from the ascending aorta. The blood was centrifuged for 10 min at $5081.31 \times g$ force to separate serum for urea and creatinine analyses.

Urea and creatinine levels were determined using commercial kits (Bio Liquid). Urea was quantified using a spectrophotometer (Micronal – B342II digital model) adopting the UV kinetic method, while creatinine was quantified using the colorimetric kinetic method. The results were expressed in mg/dl.

Blood cell differential count

The blood collected from the aorta was placed into flasks containing EDTA solution. The animals were subsequently sacrificed. The blood smears were stained (Instant Prov – New Prov) and manually analyzed to determine the blood cell differential count using an optical microscope. The blood cell differential count was expressed as a percentage (%).

Determination of kidney weight and preparation for histological analysis

The kidneys were washed free of blood (by perfusion) with a saline solution. A fixing solution was then added and the kidneys were weighed using a Micronal B 600 balance and fixed using Bouin's solution for 24 h at room temperature.

The material was prepared for histological analysis using routine techniques (Prophet et al., 1994). After fixing for 24 h, the sample tissues were cryoprotected using sucrose solutions at increasing concentrations (from 15% to 30%) and then frozen in nitrogen. Next, using a cryostat (Leitz, Digital 1702, Germany) at $-20\,^{\circ}C$, each piece was cut at 15 μm thickness intervals and the slice were placed on glass slides and stained using hematoxylin and eosin (HE) to assess tissue morphology. Histological parameters were obtained using an Olympus® Bx50 optical microscope (Olympus, Tokyo, Japan).

Ethical aspects

This study was approved by the Animal Research Ethics Committee of the Federal University of Rio Grande do Sul (reference number 17809), being considered ethically and methodologically adequate according to Resolution 196/1996 and complementary items of the Brazilian National Health Council.

Statistical analysis

Data was submitted to an analysis of variance (ANOVA) to detect significant differences between study groups. Tukey's test was applied to identify any difference between means using a significance level of $p \leq 0.05$.