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Genotoxicity, mutagenicity and cytotoxicity of carotenoids extracted from ionic liquid in multiples organs of Wistar rats

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ABSTRACT

The ionic liquid or melted salt 1-Butyl-3-methylimidazolium is an alternative process to extract natural pigments, such as carotenoids. Lycopene represents 80–90% of total of carotenoids presents in tomatoes and it has been widely studied due its potent antioxidant action. The aim of this study was to evaluate genotoxicity, mutagenicity and cytotoxicity of carotenoids extracted from ionic liquid using experimental model *in vivo*. For this purpose, a total of 20 male Wistar rats were distributed into four groups ($n = 5$), as follows: **control group**; received a corresponding amount of corn oil for 7 days by intragastric gavage (*i.g.*), **ionic liquid group**, received 10 mg kg^{-1} body weight for 7 days by gavage; **10 mg carotenoids group**, received 10 mg kg^{-1} bw dissolved in corn oil for 7 days by gavage and **500 mg carotenoids group**, received 500 mg kg^{-1} bw dissolved in corn oil for 7 days by gavage. Rat liver treated with ionic liquid exhibited moderate histopathological changes randomly distributed in the parenchyma, such as cytoplasmic eosinophilia, apoptotic bodies, inflammatory infiltrate and focal necrosis. DNA damage was found in peripheral blood and liver cells of rats treated with ionic liquid or carotenoids at 500 mg. An increase of micronucleated cells and 8-OhDG immunopositive cells were also detected in rats treated with carotenoids at 500 mg. In summary, our results demonstrate that recommended dose for human daily intake of carotenoids extracted by ionic liquid did not induce genotoxicity, mutagenicity and cytotoxicity in multiple organs of rats.

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1. Introduction

Ionic liquid is molten salts formed by an organic cation and different chemical class of anion. Its ionic properties have been used as extractor solvent under different applications and paradigms (Bi et al., 2010). Therefore, the use of ionic liquid is environmentally acceptable and it is recommended as sustainable processes of extraction, generating profitability and recognition among several companies dedicated to this purpose. An important advantage of ionic liquid resides on the fact that its ionic characteristics are high density and low vapor pressure facilitating the isolation process of organic compounds, which are usually slightly soluble (Dupont et al., 2002).

Carotenoids are good examples of compounds obtained from chemical synthesis and they are employed in the food industry. The conventional method for extracting carotenoids is quite complicated from natural sources due to low solubility. To overcome the problem, it is used volatile organic solvents, such as acetone and hexane. These solvents cannot be used in food products since they are toxic to humans. In this context, the development of new extraction techniques employing ionic liquids becomes relevant and promising so far. Among carotenoids, lycopene has high coloring strength and chemical/biological activities against reactive oxygen species (ROS). Numerous studies have proved that lycopene exerts beneficial effect on human health (Boyacioglu et al., 2016). Lycopene contains approximately 80–90% of total carotenoids present in tomatoes being widely distributed throughout fruit (Del Valle et al., 2006). Genotoxicity, mutagenicity and cytotoxicity studies may be used as biomarkers for understanding the adverse effects of ionic liquid and carotenoids after ingestion (Jodynis-Liebert et al., 2009; Aydin et al., 2013). Histopathological analysis, DNA damage, DNA repair capacity, and micronucleus are potential biomarkers traditionally used for this purpose. As a result and because of the lack of scientific evidence, the aim of the present study was to evaluate

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genotoxicity, mutagenicity and cytotoxicity induced by carotenoids extracted from liquid ionic in multiple organs of Wistar rats.

2. Material and methods

2.1. Tomato samples

Tomato samples were donated by a local market in the Santos city, SP, Brazil. A total of 10 kg of tomatoes (with no selection criteria) were homogenized and reduced. Sterilization process (121 °C for 15 min) was performed before initiating experimental design, according to Nunes and Mercadante (2004). Excess water from tomato samples was washed out with ethanol. After that, they were refrigerated at −40 °C before use.

2.2. Carotenoids preparation and chemical characterization

A total of five grams of tomato samples was submitted to exhaustive extraction, which was assisted by ultrasound agitation. The solvent used was ionic liquid imidazole derivate 1-buthyl-3-methyl-imidazolium chloride Fluka® (Darmstadt, Germany). The extraction process is described in the Brazilian patent application by De Rosso and Martins (2015).

The carotenoids were separated chromatographically *via* HPLC-PDA Shimadzu® (Kyoto, Japan) model LC-20a. It was used a C_{30} YMC column with the isocratic mobile phase elution mode (50% TBME and 50% methanol) at 0.9 mL/min with a 29 °C temperature column. The UV–vis spectra were acquired between 250 and 600 nm, and the chromatograms at 470 nm were processed. Standards Sigma-Aldrich® (Darmstadt, Germany) high purity (99%) of the all-*trans*-lycopene and all-*trans*-β-carotene (0.5–50.0 $\mu g\,mL^{-1}$) were used to create calibration curves with five concentration levels to quantitatively determining the total carotenoids samples using HPLC-PDA. The limit of detection (LOD) was calculated using the parameters of standard curve, as LOD = 3.3 × SD/S, where SD is the standard deviation of the response and S is the slope of the curve. For the all-*trans*-lycopene and all-*trans*-β-carotene analytical curves, the $R^2 = 0.999$, and the limit of detection was 0.1 $\mu g\,mL^{-1}$, whereas the limit of quantification was 0.5 $\mu g\,mL^{-1}$. The concentration was expressed in $\mu g\,g^{-1}$ of the initial sample. For the qualitative analysis, the ESI–MS/MS (mass spectra) was obtained using a LTQXL Thermo® Ion Trap (Waltham, MA, USA). The mass spectrometer was in the negative mode, and 5 μL of sample in TBME was injected *via* direct infusion with N^2 at 10 psi, flow rate of 10 $\mu L\,min^{-1}$, 4.52 kV of spray voltage, capillary voltage of 17 V and temperature of 250 °C. The ESI–MS/MS was used with collision energy of 40% (2 eV).

The ionic liquid residual in the carotenoids was determinate by HPLC-PDA Shimadzu® (Kyoto, Japan) model LC-20a. We used a C_{18} (5 μm, 4.6 × 250 mm) column Atlantis Waters® (San Antonio, TX, USA) with the isocratic mobile phase elution mode (10% acetonitrile and 90% water) at 0.9 mL/min. The temperature of column was set at 26 °C. The UV–vis spectra was ranged from 200 to 600 nm, and the chromatograms were processed at 230 nm. Standard 1-buthyl-3-methyl-imidazolium chloride Fluka® high purity (99%) was used on analytical curve acquisition with six concentrations (1–500 $\mu g\,mL^{-1}$). The analytical curve corresponded $R^2 = 0.999$. The limit of detection was 10 $ng\,mL^{-1}$, whereas the limit of quantification was 10 $\mu g\,mL^{-1}$.

2.3. Animals and experimental design

All experimental protocols involving animals are conformed to the procedures described in the Principles for the Use of Laboratory Animals Guidelines. The study was approved by the Animal Ethics Committee of Federal University of Sao Paulo, UNIFESP, SP, Brazil (Protocol CEUA no. 9987160315).

A total of twenty Wistar rats weighing about 250 g, 8 weeks old were distributed into four groups (n = 5), as follows: control group (non-treated group, CTRL); carotenoids obtained with ionic liquid groups at 10 mg/kg/day and at 500 mg/kg/day; and pure ionic liquid at 500 mg/kg/day. All animals were provided from Development Center of Experimental Models for Medicine and Biology (CEDEME) of Federal University of Sao Paulo, SP, Brazil. The rats were maintained under controlled conditions of temperature (23 ± 1 °C), light–dark periods of 12 h and free access to water and diet.

For seven days, all groups received 0.5 mL by gavage once a day: control group (sunflower oil); carotenoids at 10 mg obtained with ionic liquid group (10 mg/kg body weight of carotenoids diluted in sunflower oil); carotenoids at 500 mg obtained with ionic liquid group (500 mg/kg body weight of carotenoids diluted in sunflower oil) and pure ionic liquid group (500 mg/kg body weight of ionic liquid diluted in sunflower oil). The doses were chosen based on recommended values for human daily intake of carotenoids (Selvan et al., 2002). The daily dose was calculated in order to provide the equivalent principle for humans taking into consideration the rat metabolism (twice faster than humans). The carotenoids at 500 mg was the overdose corresponding to 100 times greater than the recommended daily dose. At the end of the experimental period, all animals were anesthetized with inhalational anesthetic halothane (Tanohalo™, Cristália™, SP, Brazil) and euthanized for tissue collection.

2.4. Histopathological evaluation

After completion the experimental design, liver and left kidney from all animals was removed, fixed in 10% neutral buffered formalin and embedded in paraffin blocks. Slices from 3 to 4 μm were gradually dehydrated in alcohol, cleared in xylene and then microscopically evaluated. Histopathological changes from liver and kidney were evaluated by semi-quantitative method according to da Silva et al. (2014) as follows: no injury (0); 1 – Watering degeneration (<30% of tissue degeneration); 2 – Increased eosinophilic staining cytoplasm, presence of congested vessels, vacuolization (30–50%% of tissue degeneration); 3 – Severe degeneration, necrosis and loss of structure (>50% of tissue degeneration).

2.5. Single cell comet assay

The protocol used for peripheral blood and liver cells followed the guideline outlined by Tice et al. (2000). Peripheral blood was transferred to plastic tubes, containing 1 mL of cold phosphate buffer solution (PBS, Ca^{+2}, Mg^{+2} free), and centrifuged for 5 min, 1000 rpm, at room temperature. A central fragment from liver was minced to PBS buffer. The supernatant was removed and the cell suspensions (~10 μL) were used for single cell gel (comet) assay. A volume of 10 μL of each tissue suspension was added to 120 μL of 0.5% low-melting point agarose at 37 °C, layered onto a pre-coated slide with 1.5% regular agarose, and covered with a coverslip. After brief agarose solidification in refrigerator, the coverslip was removed and the slides immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris–HCl buffer, pH 10, 1% sodium sarcosinate with 1% Triton X-100 and 10% DMSO) for about 1 h. Afterwards, the slides were washed in ice-cold PBS for 5 min, left in electrophoresis buffer (0.3 mM NaOH and 1 mM EDT A, pH > 13) for DNA unwinding during 20 min, and electrophoresed in the same buffer for 20 min at 25 V (0.86 V/cm) and 300 mA. Following electrophoresis, slides were neutralized in 0.4 M Tris–HCl (pH 7.5), fixed in absolute ethanol and stained with Syber-Green.

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