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The effect of d-galactose induced oxidative stress on *in vitro* redox homeostasis in rat plasma and erythrocytes

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ABSTRACT

We, herein, investigated the *in vitro* effects of galactose on thiobarbituric acid-reactive substances (TBA-RS), total sulfhydryl content, and on the activities of antioxidant enzymes, including catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), and butyrylcholinesterase (BuChE) in the blood of 30- and 60-day-old rats. We also determined the influence of the antioxidants, trolox, ascorbic acid and glutathione, on the effects elicited by galactose on the parameters tested. Galactose was added to the assay at final concentrations of 0.1, 3.0, 5.0 and 10.0 mM. Control experiments were performed without the addition of galactose. Rats were sacrificed by decapitation without anesthesia and a blood sample was removed for analysis. Galactose, at 3.0 mM, 5.0 mM and 10.0 mM, enhanced TBA-RS in the plasma of 60-day-old rats, while 10.0 mM galactose reduced total sulfhydryl content in the plasma of 30-day-old rats; 5.0 mM and 10.0 mM galactose enhanced CAT activity in the erythrocytes of 30- and 60-day-old rats and 10.0 mM galactose reduced SOD activity in the erythrocytes of 60-day-old rats. Galactose did not alter BuChE activity. Data showed that at the pathologically high concentration (greater than 5.0 mM), galactose induces lipid peroxidation, reduces total sulfhydryl content and alters antioxidant defenses in the blood of rats. Trolox, ascorbic acid and glutathione addition prevented most alterations in oxidative stress parameters that were caused by galactose. Our findings lend support to a potential therapeutic strategy for this disease, which may include the use of antioxidants for ameliorating the damage caused by galactose.

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1. Introduction

The enzyme, galactose-1-phosphate uridyltransferase (GALT), converts galactose-1-phosphate into glucose-1-phosphate, which can subsequently enter the glycolytic pathway. However, in galactosemic patients, the impairment of GALT enzyme does not allow these reactions to proceed and, galactose-1-phosphate accumulates in the cells [1].

Human GALT deficiency is detected through newborn screening in many developed countries with the objective of minimizing its acute pathology, which can include jaundice, cataracts, vomiting, diarrhea, hepatomegaly, sepsis and neonatal death [2]. Galactose restriction in the diet can immediately mitigate or prevent these acute manifestations, but does not appear to prevent longer-term complications that include ovarian failure, mental retardation, cognitive difficulties, psychiatric symptoms, speech and motor problems, among other complications [3–5].

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It was demonstrated that D-galactose treatment caused oxidative stress in mouse brain, resulting in neurodegeneration and cognitive dysfunction [6–9]. Furthermore, D-galactose seems accelerated aging in rodents [10,11] and caused oxidative stress and mitochondrial dysfunction in the livers of mice and rats [11–13].

Researchers have also demonstrated that different experimental models of galactose cause oxidative stress in Drosophila melanogaster, Musca domestica and rats [14,15]. Also, high level galactose exposure of genetically normal mice and/or dogs has also been associated with negative long-term outcomes that include neurodegeneration, and cognitive disability [16,17], diminished immune response [16] and retinal degeneration [18]. The literature shows that different substances like purple sweet potato color, melatonin and proanthocyanidins, due antioxidant capacity, protect against oxidative stress caused by galactose in animal models [19,20].

As such, in order to corroborate with previous studies and considering that there are few studies of galactose in the peripheral system of galactosemic patients and animal models, the purpose of this study was to investigate the *in vitro* effects of different concentrations of galactose (0.1- 10.0 mM) on plasma TBA-RS and total sulfhydryl content, on erythrocyte antioxidant enzyme activities (CAT, GSH-Px, and SOD), as well as on serum BuChE activity in rats. Furthermore, we also tested the influence of trolox, ascorbic acid and glutathione on the effects elicited by galactose on these same parameters.

2. Materials and methods

2.1. Animals and reagents

Thirty and sixty-day-old male Wistar rats (120–150 g and 200–250, respectively), obtained from the Univali University, Itajaí, Brazil, were used in the experiments. The animals from our own breeding stock were maintained on a 12 h light/12 h dark cycle at a constant temperature (22 ± 1 °C), with free access to water and commercial protein chow. The "Principles of Laboratory Animal Care" (NIH publication 85–23, revised 1985) were followed in all the experiments and the experimental protocol was approved by the Ethics Committee for Animal Research of the University of Region Itajaí, Itajaí Brazil, under the protocol number CEUA 01/14-14/03/2014. All chemicals were purchased from Sigma Chemical Co., St Louis, MO, USA.

2.2. In vitro *studies*

For *in vitro* experiments, erythrocytes, plasma and serum were pre-incubated for 1 h at 37 °C, without buffer, in the presence of galactose at final concentrations of 0.1, 3.0, 5.0 and 10.0 mM. Control experiments were performed without galactose addition and with addition of water, since water was used as vehicle for the preparation of galactose. After incubation, aliquots were taken to measure TBA-RS, total sulfhydryl content, the activity of antioxidant enzymes and BuChE activity.

2.3. Trolox (α-tocopherol), ascorbic acid and glutathione administration

The assays were divided into eight groups: Group 1 (control-saline), group 2 (galactose), group 3 (control – 1.0 mM trolox), group 4 (galactose + 1.0 mM trolox), group 5 (control – 1.0 mM ascorbic acid), group 6 (galactose + 1.0 mM ascorbic acid), group 7 (control – 1.0 mM glutathione), and group 8 (galactose + 1.0 mM glutathione). The concentrations of trolox, ascorbic acid and glutathione utilized in the present study were chosen according to previous studies [21,22].

2.4. Erythrocyte and plasma preparation

Erythrocytes and plasma were prepared from whole blood samples obtained from rats. Whole blood was collected and transferred to heparinized tubes for erythrocyte separation. Blood samples were centrifuged at 1,000 × *g*, plasma was then removed by aspiration and frozen at −80 °C until use in assays. Erythrocytes were washed three times with cold saline solution (0.153 mol/L sodium chloride). Saline solution was added to the erythrocytes, after homogenization, centrifuged at 13,500 x *g* for 10 min and then saline was removed. This procedure was performed 3 times to wash the erythrocytes. Lysates were prepared by the addition of 1 mL of distilled water to 100 μL of washed erythrocytes and frozen at −80 °C until determination of the antioxidant enzyme activities. For antioxidant enzyme activity determination, erythrocytes were frozen and thaw three times, and centrifuged at 13,500 × *g* for 10 min. The supernatant was diluted in order to achieve an approximate concentration of 0.5 mg/mL of protein.

2.5. Serum preparation

The blood was rapidly collected, centrifuged at 1,000 × *g* for 10 min and the serum was separated.

2.6. Thiobarbituric acid reactive substances (TBA-RS)

TBA-RS were determined according to the method described by Esterbauer and Cheeseman [23]. TBA-RS methodology measures malondialdehyde (MDA), a product of lipoperoxidation, caused mainly by hydroxyl free radicals. For the *in vitro* measurements, tissues were mixed with 10% trichloroacetic acid and 0.67% thiobarbituric acid and heated in a boiling water bath for 25 min. TBA-RS were determined by the absorbance at 535 nm. A calibration curve was obtained using 1,1,3,3-tetramethoxypropane as the MDA precursor and each curve point was subjected to the same treatment as that of the supernatants. TBA-RS content was calculated as nanomoles of MDA formed per milligram of protein.

2.7. Total sulfhydryl content

The total thiol group concentration was determined by the method of Aksenov and Markesbery [24]. Briefly, 50 μL of homogenate was added to 1 mL of phosphate-buffered saline (PBS), pH 7.4, containing 1 mM ethylenediamine tetraacetic acid (EDTA). The reaction was started by the addition of 30 μL ssay solutions with 1of 10 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) and incubated for 30 min at room temperature in a dark room. Total sulfhydryl content was determined by measuring the absorbance at 412 nm. Analyses of a blank (DTNB absorbance) was also performed. Results are reported as nmol 3-thio-2-nitro-benzoic acid (TNB)/mg protein.

2.8. Catalase assay (CAT)

CAT activity was assayed by the method of Aebi [25] using a UV–visible Shimadzu spectrophotometer. The method used is based on the disappearance of H_2O_2 at 240 nm in a reaction medium containing 20 mM H_2O_2, 0.1% Triton X-100, 10 mM potassium phosphate buffer, pH 7.0, and 0.1–0.3 mg protein/mL. One CAT unit is defined as 1 μmol of H_2O_2 consumed per minute and the specific activity is calculated as CAT units/mg protein.

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