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Effects of a moderate iron overload and its interaction with yacon flour, and/or phytate, in the diet on liver antioxidant enzymes and hepatocyte apoptosis in rats

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ABSTRACT

The effect of moderate Fe overload in the diet and its interaction with phytate, and/or yacon flour (YF), recognized as an inhibitor, and facilitator, of Fe absorption, respectively, was evaluated in healthy rats. For this purpose the following parameters were analyzed: (1) apparent iron (Fe), copper (Cu) and zinc (Zn) absorption; (2) blood Fe; (3) blood lipids (cholesterol, tryacylglicerol); (4) blood AST and ALT; (5) liver histology (histopathology, hemosiderin depots, apoptosis index; (6) liver fatty acid incorporation; (7) liver antioxidant enzyme activity. Moderate Fe overload may cause change in some liver markers (hemosiderin depots, apoptosis index and GPx) and blood lipids (total cholesterol and VLDL) and the interaction with yacon flour, and phytate, in the Fe overloaded diets may exert a protective effect on these alterations.

1. Introduction

The same properties which enable transition metals such as Fe to act as an efficient cofactor in several oxi-reduction reactions also enable it to mediate the oxidation of biomolecules when it is present in excessive amounts (Eid, Arab, & Greenwood, 2017). The liver is particularly prone to this toxic effect, since it is the largest storage site of Fe in the body.

In disorders inherently related to Fe metabolism such as hereditary hemochromatosis (HH), the excess of Fe can cause portal fibrosis, cirrhosis and hepatocellular carcinoma (Asare et al., 2006). The participation of Fe in such processes is due to the reversibility of its valence states, which is an important source of reactive oxygen species (ROS). Nevertheless, sophisticated antioxidant systems, both enzymatic and non-enzymatic, can protect the organism against the oxidative damage caused by these ROS (Bernotti et al., 2003).

However, large amounts or inadequate removal of such species may result in oxidative stress that can cause many metabolic dysfunctions and irreversible damage to several tissues and organs (Matés, Pérez-Gómez, & Castro, 1999). In this context, it is known that, in rats, even a moderate Fe overload induces the generation of ROS, which increases DNA damage and/or lipid peroxidation in cell membranes (Fischer et al., 2002). On the other hand, in these animals, the excess of Fe is usually obtained through parenteral injections of solutions containing Fe salts or through a modification of the Fe concentration in the diet mineral mixture, without evaluating factors which might interfere with the Fe bioavailability (Brunet et al., 1997; Fischer et al., 2002). Thus, the present work included in the diet of rats fed with moderate Fe overload, two components, whose interactions have not yet been fully evaluated concomitant with Fe overload: phytate and yacon flour rich in fructooligosaccharides (FOS), recognized by, respectively, decreasing and increasing the bioavailability of minerals (Lobo et al., 2011; Lopez et al., 2000).

Phytate (myo-inositol hexaphosphate – IP6) chelates minerals such as Fe, Zn and Cu decreasing their bioavailability (Lopez et al., 2000) being, for this aspect, often considered as an antinutritional factor.

Phytate is also considered a natural antioxidant reducing carcinogenesis and cell damage mediated by ROS (Vucenik & Shansuddin,

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2006) and also the concentration of free fatty acids in plasma, and triacylglycerols and cholesterol in the liver (Szkudelski, 2005; Yuangklang, Wensing, Lemmens, Jittakhot, & Beynen, 2005)

In turn, the conversion of fermentable carbohydrates, particularly fructans [inulin and fructooligosaccharides (FOS)], to short-chain fatty acids (SCFA) by the resident microbiota in the large intestine increases the mineral bioavailability by lowering the pH in this organ (Lobo et al., 2011). Neyrinck, Alexiou, and Delzenne (2004) demonstrated that FOS protected the liver of rats experiencing endotoxic shock caused by bacterial lipopolisaccharides, since more intense phagocytic activity of Kupffer cells was observed. The increased fermentation in the cecum apparently also promotes a more effective hydrolysis of phytate, thus counteracting its effects on mineral absorption (Lopez et al., 2000).

Yacon (*Smallanthus sonchifolis* [Poepp & Endl.] H. Robinson, Asteracea) is an Andean crop used in our study. Yacon FOS accumulate large amounts of fructans with a low degree of polymerization (DP < 10, FOS) (Itaya, Carvalho, & Figueiredo-Ribeiro, 2002). Therefore, the present study hypothesizes that yacon flour rich in FOS and phytate might counteract the possible deleterious effects of a moderate Fe overload on the liver, on the biochemical parameters related to Fe, and on the profile of serum lipids.

2. Material and methods

2.1. Animals and diets

The experimental protocol was approved by the Commission on Ethics in Animal Experiments of the Faculty of Pharmaceutical Sciences of the University of São Paulo. Thirty-four male Wistar rats, weighing 49.3 ± 3.9 g, were obtained from the colony of the Faculty of Pharmaceutical Sciences and the Chemistry Institute of the University of São Paulo. The rats were housed in individual stainless-steel wire-mesh cages under a controlled temperature (22 ± 2 °C), relative humidity of 55 ± 10%, with a 12-h light/dark cycle (lights on from 8 a.m. to 8p. m.). A modified AIN-93G diet (Reeves, Nielsen, & Fahey, 1993) was used for the study (Table 1). The dietary mineral mix (Sigma-Aldrich p.a. St. Louis, MO, USA.) was prepared in our facilities and then the amount of microencapsulated Fe was added in each diet according to our experimental design. Five diets were prepared (a control AIN93G diet and four diets with an moderate Fe overload) and supplemented with microencapsulated $FeSO_4{\cdot}7H_2O$ obtained from Fermavi Eletroquímica Ltda. (São Paulo, Brazil). Three out of the four diets with moderate Fe overload were supplemented with YF rich in FOS and/or phytate (myo-inositol hexaphosphate – Sigma-Aldrich St. Louis, MO, USA). Yacon tuberous roots, donated by São Sebastião Farm (Ibiúna, São Paulo, Brazil) were properly processed as described in a previous study (Lobo et al., 2011). They were autoclaved (121 °C, 20 min), freeze-dried (Liotécnica Ind. Com. Ltda, Embu, São Paulo, Brazil) and ground for obtaining the flour. The amounts of FOS and soluble sugars present in the YF were subtracted from sucrose and starch quantities of the standard diets. After preparation, diet samples were collected for chemical-composition analysis and stored (around 4 °C) until use. Phytate (myo-inositol hexaphosphate; Sigma-Aldrich, St Louis, MO, USA) was kindly donated by Dr Ralf Greiner (Centre for Molecular Biology, Federal Research Centre for Nutrition and Food, Karlsruhe, Germany). Soybean oil was provided by Cargill Agrícola S/A (Mairinque, São Paulo, Brazil) and did not contain antioxidants. Therefore, the antioxidant *tert*-butyl hydroquinone (0.03%) was added to the diets to prevent oil oxidation.

2.2. Experimental design

The animals were fed the AIN-93G basal diet for five days and then randomly assigned to one of the five diet groups (n = 7 per group) so that the average weights of each group were equal (Table 3). The rats were provided free access to deionized water and diets. The diets were prepared to contain 35 mg Fe/kg diet (control group) and 500 mg Fe kg/diet (moderate Fe overload groups), for 92 days. Additional moderate Fe-overload groups were supplemented with 18% yacon flour (3.6% FOS/kg diet) and/or phytate (0.6% phytate/kg diet). The groups were named: Control, moderate Fe Overload (IO), moderate Fe overload + Yacon Flour (IO-YF), moderate Fe overload + Phytate (IO-Phy), moderate Fe overload + Yacon Flour + Phytate (IO-YF-Phy). Food and demineralized water were offered *ad libitum*. The diet intake was recorded daily and the body weights were recorded every three days. Feces were collected during the last five days before euthanasia (from days 82 to 87), pooled and stored at −20 °C until analysis. Humidity in feces was determined through the weight loss of the samples heated in oven at 105 °C. At the end of the experiment, the rats were anaesthetized through an intraperitoneal route with a 1:1:0.4:1.6 (v/v/v/v) mixture of ketamine (100 mg/kg body weight; Vetaset, Fort Dodge, Iowa, USA), xylazine (25 mg/kg body weight; Virbaxil 2%, Virbac, São Paulo, Brazil), acepromazine (2 mg/ml; Acepran 0.2%, Univet S/A Indústria Veterinária, São Paulo, Brazil) and demineralized H_2O. After deep anesthesia (verified by lack of response to tactile tail stimuli) the animals were euthanized by exsanguination and their organs were removed and frozen for further analysis.

Table 1
Formulation of the experimental diets.[1]

Ingredients (%)	Experimental groups[2]				
	Control	IO	IO-YF	IO-Phy	IO-YF-Phy
Casein (78% protein)[3]	21.79	21.79	21.79	21.79	21.79
Fiber	5.0	5.0	5.0	5.0	5.0
Soybean oil[4]	7.0	7.0	7.0	7.0	7.0
L-cystine	0.3	0.3	0.3	0.3	0.3
Choline Bitartarate	0.25	0.25	0.25	0.25	0.25
Vitamin Mixture[1]	1.0	1.0	1.0	1.0	1.0
Modified mineral mix[1,*]	3.5	3.5	3.5	3.5	3.5
Sucrose	10.0	10.0	–	10.0	–
Corn starch	51.15	51.15	45.15	51.15	45.15
Yacon flour (18% FOS)[5]	–	–	20.0	–	20.0
Phytic acid[6]	–			0.6	0.6

[1] According to the recommendation of the *American Institute of Nutrition* (Reeves et al., 1993) with modifications: $FeSO_4$ was substituted by microencapsulated $FeSO_4$ in all diets, Control Group: 48.8 mg Fe/kg diet, IO group: 575.7 mg Fe/kg diet, IO-YF group: 585.5 mg Fe/kg diet, IO-Phy group: 556.1 mg Fe/kg diet, IO-YF-Phy group: 604.2 mg Fe/kg diet.

[*] Copper: Control group: 6.1 μg/g diet, IO group: 6.0 μg/g diet , IO-YF group: 6.9 μg/g diet, IO-Phy group: 5.5 μg/g diet, IO-YF-Phy group: 7.1 μg/g diet. [*]Zinc: Control group: 38.2 μg/g diet , IO group: 41.2 μg/g diet, IO-YF group: 43.3 μg/g diet, IO-Phy group: 42.1 μg/g diet, IO-YF-Phy group: 38.1 μg/g diet . Sucrose and part of the starch were substituted by yacon flour (*Smallanthus sonchiflolius*) in the formulation of the diets containing fructans.

[2] IO: Iron Overload, IO-YF: Iron Overload + Yacón Flour, IO-Phy: Iron Overload + Phytate, IO-YF-Phy: Iron overload + Yacón Flour + Phytate.

[3] Protein content in casein was determined by the micro-Kjeldahl method, using a conversion factor of 6.25 (AOAC, 2005).

[4] Cargill Agrícola S/A (Mairinque, São Paulo, Brazil).

[5] São Sebastião Farm (Ibiúna, São Paulo, Brazil); 18% ITF, 28.3% sucrose, 15.7% fructose, 6% IDF, 4% SDF, 4 μg Fe/g, 19 μg Cu/g and 16 μg Zn/g (Lobo et al., 2011).

[6] Myo-inositol hexaphosphate (Sigma-Aldrich, St Louis, MO, USA).

2.3. Chemical composition of experimental diets

The following components were determined: humidity, ash, total lipids and protein by the micro-Kjeldahl method (AOAC, 2005) (conversion factor of 6.25). Dietary Fe, Zn and Cu concentrations were determined by atomic absorption spectroscopy (AAS; AAnalyst 100, Perkin Elmer, USA) according to AOAC methods (2005) employing a hollow cathode lamp at 283.4, 213.9 and 324.8 nm, respectively, and slits of 0.2, 1.3 and 1.3 nm, respectively, after wet digestion with

HNO_3:H_2O_2 (5:1). The working standard solutions were prepared by diluting $FeCl_3$, $ZnCl_2$ and $CuCl_2$ standards (Tritisol, Merck, Darmstadt, Germany). Total fructans, glucose, fructose and sucrose in the YF were analysed by spectrophotometry (Steegmans, Iliaens, & Hoebregs, 2004). Insoluble (IDF) and soluble (SDF) dietary fibres were determined by the enzymatic–gravimetric method (Prosky, Asp, Schweizer, DeVries, & Furda, 1988).

2.4. Blood collection

Blood was collected from the inferior vena cava with 10% K_3EDTA for hemogram (hematocrit, hemoglobin, total red blood cells, total leukocytes and reticulocytes) and without EDTA for the other analyses. Blood samples obtained without anticoagulant were collected, and serum Fe concentrations and the unsaturated Febinding capacity (UIBC) were determined (Labtest Diagnóstica S/A, Lagoa Santa, Minas Gerais, Brazil). The total Fe-binding capacity (TIBC) and transferrin saturation were calculated from the values of serum Fe and UIBC. Blood swabs were prepared immediately after collection for differential count of leukocytes and morphological analyses (Rosenfeld, 1947). For detecting possible lesions caused by Fe overload, serum activity of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were evaluated by using commercial kits (Labtest Diagnóstica S/A, Lagoa Santa, Minas Gerais, Brazil). Triacylglycerols, total cholesterol and HDL-cholesterol were determined by an enzyme-colorimetric method (Labtest Diagnóstica S/A, Lagoa Santa, Minas Gerais, Brazil).

2.5. Hepatic parameters

After deep anaesthesia and exsanguination, the liver was perfused through the subhepatic vein with a NaCl solution (9 g/l) to drain blood out of the organ. The liver was then removed, rinsed with saline and weighed. To analyze the activity of the antioxidant enzymes, the left lateral lobe was cut and immediately frozen with liquid nitrogen and subsequently kept at −80° C for a maximum period of one week until analysis. The remaining liver was stored at −20° C until analysis.

2.5.1. Concentration of minerals

The livers were thawed at room temperature to determine Fe, Zn and Cu concentrations. Approximately 2 g of each liver, removed from the median lobe, were digested according to the AOAC standards (2005). The concentration of these minerals in the samples was determined by atomic absorption spectroscopy (AAS; AAnalyst 100, Perkin Elmer, USA), as previously described.

2.5.2. Incorporation of fatty acids

The lipid fraction was extracted according to the method of Folch, Lees, and Stanley (1957). Fatty-acid methyl esters were analyzed by gas chromatography according to the method described by Gressler et al. (2010).

2.5.3. Determinations of antioxidant enzyme activities in the liver

In order to determine the activity of the main antoxidant enzymes, liver samples were homogenized in 10 mM sodium phosphate buffer, pH 7.5, at the proportion of 1:10. After homogenization, the extracts were centrifuged at 10,000*g* for 10 min (SPIN 1, Incibras, SP, Brazil) (Azevedo-Martins & Curi, 2008). Supernatants of the samples were used for protein quantification and enzyme activity determinations. Catalase (E.C.1.11.1.6) activity was determined by measuring the decomposition of hydrogen peroxide at 230 nm and 30 °C (Azevedo-Martins & Curi, 2008). GPx (E.C.1.11.1.9) activity was determined as described by Azevedo-Martins and Curi (2008), following the rate of NADPH oxidation at 340 nm, and 37 °C, in an assay medium containing 50 mM phosphate buffer (pH 7.0), 3.6 mM sodium azide, 0.3 mM NADPH, glutathione reductase (0.25 U ml^{-1}) and 5 mM reduced glutathione. The reaction was initiated by the addition of *t*-butyl hydroperoxide (1.5 mM). One unit of GPx is defined as the amount of protein that oxidizes 1 mmol of NADPH per minute.

CuZn-SOD (E.C. 1.15.1.1) and Mn-SOD activities were determined according to Azevedo-Martins and Curi (2008) by measuring, at 25 °C, the decrease in the rate of cytochrome *c* reduction in a xanthine–xanthine oxidase superoxide generating system consisting of 10 mM cytochrome *c*, 100 mM xanthine, 50 mM sodium phosphate buffer (pH 10) and the necessary quantity of xanthine oxidase to yield a variation of absorbance of 0.025 per min at 550 nm. One unit of SOD activity is defined as the amount of protein necessary to inhibit 50% of the rate of cytochrome *c* reduction. CuZn-SOD was differentiated from Mn-SOD by the addition of 1 mM KCN (final concentration) to suppress, around 100%, the activity of the CuZn isoform. The enzyme activities were determined in a Pharmacia Biotech (Ultrospec 3000 model, Little Chalfont, UK) spectrophotometer and are expressed as U per mg of protein.

2.5.4. Histological analyses

In order to evaluate Fe overload, liver fragments were fixed in Metacarn (70% MetOH, 20% $CHCl_3$, 10% AcOH), processed, included in paraffin, cut at 5 µm and stained with hematoxylin and Perls (Behmer, Tolosa, & Freitas Neto, 1976). The histological sections were examined under an optical microscope with magnifications of 40, 100 and 400x. Hepatic hemosiderin deposition was evaluated by Perls stain and it was semi-quantitatively analyzed within a scale from 0 to 3, where 0 = parameter considered normal, meaning absence of hemosiderin pigment, 1 = minimum alterations featured by the presence of up to 2 Kupffer cells containing hemosiderin per field at 400x magnification and absence of portal macrophages and hepatocytes with pigments, 2 = moderate-degree alterations with 3 to 6 Kupffer cells with pigments, rare hepatocytes with pigments, 3 = marked alteration with more than 6 Kupffer cells containing pigments per field at 400× magnification and presence of portal macrophages and hepatocytes with pigments.

Apoptosis was evaluated using the method of fluorescence microscopy described by Ong, Heidor, Conti, Dagli, and Moreno (2006). Briefly, representative fragments of each hepatic lobe were fixed in Metacarn solution (60% methanol, 30% chloroform and 10% glacial acetic acid) for 24 h and included in paraffin. Five micrometre sections were stained with hematoxylin-eosin (H & E) for histopathological examination. Apoptotic bodies (AB) in normal or lesioned tissue were quantified by using an epifluorescence microscope (Nikon E-800, Japan) at a magnification of 400×. All AB were counted and the area of the histological section was measured. This analysis was carried out with the aid of Image Pro-Plus version 4.5 image-analyzing system.

2.5.5. Vitamin E

Approximately 200 mg of liver were homogenized with ethanol and *n*-hexane, and then centrifuged at 3500*g* for 10 min. An aliquot of 500 µl supernatant was collected; the sample was dried in gaseous nitrogen and then re-suspended with 500 µl mobile phase (70% acetonitrile, 20% dichloromethane, 10% methanol). For the analysis of vitamin E, 20 µl were injected into the chromatograph [(Shimadzu (Japan), model LC9, column type C18 (OD5) (25 cm × 0.46 cm)], using a 1.0 ml/min flow and a wavelength of 292 nm. The results concerning α-tocopherol were expressed as µM/g tissue, using the calibration curve drawn by the chromatograph.

2.6. Feces

Moisture in feces, quantitatively collected in a 5-day period, was determined through the weight loss of the samples heated in oven at 105 °C. The feces were pooled, ground in a mill, and stored at −20 °C for Fe, Cu and Zn determinations by AAS (AAnalyst 100, Perkin Elmer, USA), according to the standards of the AOAC (2005), as previously described. The absorption of minerals was determined by the following

equation (Lobo et al., 2011):

$$Apparent\ absorption\ (\%) = (total\ mineral\ intake - fecal\ mineral\ excretion)\ x\ total\ mineral\ intake^{-1} \times 100$$

2.7. Statistical analysis

The data were analyzed by SPSS (Statistical Package for Social Sciences) for Windows version 11.5. All tests were carried out adopting a significance level of 5%. Initially, descriptive statistics were used for evaluating mean and standard deviation of the analyzed variables. Pearsońs correlation coefficient was calculated to measure the degree of association among variables. The comparisons of the means of the variables among the five groups (Control, IO, IO-YF, IO-Phy and IO-YF-Phy) were carried out through ANOVA. A *Post-Hoc* Tukeýs test was applied to identify significant differences. The normality of the observations was verified through the non-parametric Komogorov-Smirnov test. The observed power was 85–95% for most tests.

3. Results

3.1. Chemical composition of experimental diets

Chemical analyses of the diets showed Fe concentrations of 48.8 in the control group, and 575.7, 585.5, 556.1 and 604.2 mg Fe/kg in the Fe overload groups (IO, IO-YF, IO-Phy and IO-Y-Phy groups, respectively), without significant differences between Fe overload groups. No significant differences were verified in the Zn and Cu concentrations of the diets (38.2, 41.2, 43.3, 42.1, 38.1 mg Zn/kg and 6.1, 6.0, 6.9, 5.5, 7.1 mg Cu/kg, in the control, IO, IO-YF, IO-Phy and IO-Y-Phy groups, respectively) or in the protein and lipid concentrations (18% and 6%, respectively, for all groups). YF analysis showed 18% total fructans, 28.3% sucrose, 15.7% fructose, 6% IDF, 4% SDF and 4 μg Fe/g.

3.2. Body weight and feed intake

Feed intake and body weight gain were not altered ($p > 0.05$) by Fe concentration in the diet or by the presence of YF and/or phytate (Table 2).

3.3. Fecal parameters

3.3.1. Humidity

There was not any significant difference in the amount of feces excreted by all groups over a period of five days (between the 81st and the 86th days of the experiment) in terms of dry weight. YF significantly increased the humidity of feces ($p = 0.000$) when compared to the groups without this component. This effect was even more intense in the interaction between YF and phytate (IO-YF-Phy group) (Table 2).

3.3.2. Fecal Fe, Cu and Zn

The increase in Fe intake by all IO groups increased the fecal excretion of this mineral when compared to the control group ($p = 0.000$). However, in the IO groups which received YF (IO-YF and IO-YF-Phy groups) there was a significant reduction in the excretion of this mineral when compared to the IO group ($p < 0.05$). There was no difference in the fecal excretion of Cu and Zn among the groups ($p > 0.05$) (Table 2).

3.3.3. Apparent absorption (%)

The increase in Fe fecal excretion observed in the IO groups was not reflected in a reduction in the apparent absorption of this mineral. On the contrary, all groups with moderate IO presented an increase in the apparent absorption of this mineral when compared to the control group ($p < 0.05$). Concerning Zn, a moderate Fe overload, even in the presence of phytate (IO and IO-Phy groups), did not significantly alter the apparent absorption of this mineral when compared to the control group. The moderate Fe overload did not alter Cu apparent absorption, but the presence of YF (IO-YF group) increased the apparent absorption of this mineral (+75%) when compared to the control group ($p < 0.05$) (Table 2).

3.4. Blood analysis

3.4.1. Fe status indices

There was no significant difference ($p > 0.05$) in serum Fe, transferrin saturation (TS, as %) or total Fe binding capacity (TIBC) (Table 3). The same was observed in the hemogram [hematocrit, hemoglobin, red blood cell count, total leukocytes and reticulocytes (data

Table 2
Feed intake, body weight gain, mineral intake and fecal mineral excretion of rats fed control and iron-overload diets supplemented or not with yacon flour and/or phytate.

Parameters	Experimental Groups[1]					
	Control	IO	IO-YF	IO-Phy	IO-YF-Phy	p*
Total Feed intake (g)	1372.1 ± 150.3	1421.0 ± 93.9	1336.3 ± 155.4	1437.8 ± 136.1	1443.6 ± 119.5	ns**
Body weight gain (g)	293.9 ± 33.0	288.9 ± 23.4	270.3 ± 37.0	290.1 ± 12.0	289.7 ± 31.8	ns
Feed Efficiency	0.21 ± 0.02	0.20 ± 0.02	0.20 ± 0.01	0.20 ± 0.02	0.20 ± 0.01	ns
Total Fe intake (mg/day)	0.8 ± 0.1^{a}	10.5 ± 1.4^{b}	9.0 ± 1.0$^{b.c}$	8.6 ± 0.5^{c}	10.0 ± 1.4$^{b.c}$	0.000
Total Cu intake (mg/day)	0.09 ± 0.01^{a}	0.09 ± 0.01^{a}	0.10 ± 0.01$^{a.b}$	0.09 ± 0.01^{a}	0.11 ± 0.01^{b}	0.000
Total Zn intake (mg/day)	0.55 ± 0.05^{a}	0.62 ± 0.03$^{a.b}$	0.61 ± 0.06$^{a.b}$	0.64 ± 0.05^{b}	0.58 ± 0.04$^{a.b}$	0.015
Fecal humidity (%)	28.3 ± 3.9^{a}	33.1 ± 2.3^{a}	39.7 ± 4.7^{b}	32.2 ± 4.0^{a}	46.5 ± 1.7^{c}	0.000
Total Fecal Fe (mg/day)	0.7 ± 0.3^{a}	4.3 ± 0.3^{b}	3.3 ± 0.2^{c}	4.1 ± 0.2^{b}	3.3 ± 0.3^{c}	0.000
Total Fecal Cu (mg/day)	0.08 ± 0.01	0.07 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	ns
Total Fecal Zn (mg/day)	0.4 ± 0.1	0.4 ± 0.1	0.3 ± 0.0	0.4 ± 0.2	0.3 ± 0.1	ns
Apparent absorption (%)***						
Fe	13.4 ± 1.2 ^{a}	58.87 ± 4.9$^{b.c}$	62.7 ± 5.5 ^{b}	52.3 ± 2.0 ^{c}	66.0 ± 4.8 ^{b}	0.050
Cu	21.2 ± 6.3 ^{a}	31.1 ± 10.4 $^{a.b}$	37.1 ± 6.8 ^{b}	20.8 ± 9.0 $^{a.b}$	35.6 ± 10.1 $^{a.b}$	0.050
Zn	34.0 ± 9.0 ^{a}	43.6 ± 18.0 ^{a}	47.7 ± 9.3 ^{a}	43.1 ± 4.3 ^{a}	48.3 ± 10.5 ^{a}	ns

Mean ± S.D. (n = 7: Control, IO, IO-FOS, IO-FOS-Phy, n = 6: IO-Phy).
^{a,b,c}Different superscripts letters on the same line indicate statistically different values ($p < 0.05$).
[1] Groups: Control diet, Iron Overload (IO), Iron Overload + Yacón Flour (IO-YF), ron Overload + Phytate (IO-Phy), Iron Overload + Yacón Flour + Phytate (IO-YF-Phy).
* Versus Control group.
** Non significant difference.
*** The feces were quantitatively collected in a five day period.

Table 3
Biochemical parameters of rats fed control and iron-overload diets supplemented or not with yacon flour and/or phytate for 92 days.

Parameters	Experimental Groups[1]					
	Control	IO	IO-YF	IO-Phy	IO-YF-Phy	p*
Fe (mg/dL)	161.67 ± 29.6	206.0 ± 80.0	195.9 ± 33.7	195.8 ± 42.8	216.6 ± 109.8	ns**
TIBC[2] (mg/dL)	405.0 ± 79.7	512.9 ± 171.7	484.0 ± 81.4	488.6 ± 62.3	506.4 ± 80.8	ns
Transferrin saturation (%)	41.0 ± 9.0	41.0 ± 10.2	41.3 ± 9.11	40.1 ± 7.2	41.0 ± 14.6	ns
Cholesterol (mg/dL)	67.4 ± 7.2^{a}	48.1 ± 1.7^{b}	51.6 ± 12.7^{b}	62.2 ± 8.7$^{a.b}$	54.9 ± 9.5$^{a.b}$	0.002
Triacylglycerol (mg/dL)	119.9 ± 33.9^{a}	87.1 ± 48.6$^{a.b}$	71.6 ± 26.7$^{a.b}$	96.0 ± 55.1$^{a.b}$	58.7 ± 12.1^{b}	0.047
HDL[3] (mg/dL)	44.29 ± 8.2	29.7 ± 11.5	37.57 ± 11.4	43.4 ± 10.4	41.29 ± 7.4	ns
VLDL[4] (mg/dL)	23.97 ± 6.8^{a}	17.4 ± 9.7$^{a.b}$	14.3 ± 5.4$^{a.b}$	19.2 ± 11.0$^{a.b}$	11.7 ± 2.4^{b}	0.047
AST[5] (UI/L)	58.7 ± 26.7	82.1 ± 32.3	59.3 ± 15.2	43.4 ± 26.1	44.9 ± 19.3	0.055
ALT[6] (UI/L)	36.7 ± 14.7	36.0 ± 10.7	29.9 ± 8.3	26.4 ± 4.2	26.1 ± 6.3	ns

Mean ± S.D. (n = 7: Control, IO, IO-YF, IO-YF-Phy, n = 6: IO-Phy).
[a,b,c]Different superscripts letters on the same line indicate statistically different values ($p < 0.05$).
[1] Iron Overload (IO), Iron Overload + Yacón Flour (IO-YF), Iron Overload + Phytate (IO-Phy), Iron Overload + Yacón Flour + Phytate (IO-YF-Phy).
[2] Total iron binding capacity.
[3] High density lipoprotein.
[4] Very low density lipoprotein.
[5] Alanine aminotransferase.
[6] Aspartate aminotransferase.
* Versus Control group
** Non significant difference.

not shown)].

3.4.2. Blood lipids moderate

Fe overload reduced serum cholesterol (−29%) in comparison to the control group ($p = 0.002$), which persisted in the presence of yacon flour (IO-YF group). In the interaction between IO-Phy and IO-YF-Phy, this parameter was the same as the level in the control group. The interaction IO-YF-Phy reduced triacylglycerol serum concentration (-51% in comparison to the control group, $p = 0.047$). The interaction IO-YF-Phy significantly reduced (-51%) the VLDL serum concentration ($p = 0.047$) (Table 3).

3.4.3. AST and ALT

There was a +40% increase in the AST serum concentration in the IO group when compared to the control group ($p = 0.055$), returning to the levels observed in the control group in the interactions IO-YF, IO-Phy and IO-YF-Phy. No difference was observed in the ALT serum concentration in all groups (Table 3).

3.5. Liver parameters

3.5.1. Fe, Cu and Zn concentrations

The increased Fe intake resulted in an increase in the hepatic concentration of this mineral in the IO and IO-Phy groups when compared to the control group ($p = 0.002$). However, in the IO-YF and IO-YF-Phy groups, there was no difference in Fe concentrations when compared to the control group. There was no difference among all groups in Cu and Zn hepatic concentrations (Table 4).

3.5.2. Fatty-acid incorporation

There was no statistically significant difference ($p > 0.05$) in the incorporation of fatty acids into the hepatic tissue among all experimental groups (data not shown).

3.5.3. Vitamin E and antioxidant enzymes activity

No difference was observed in the hepatic concentrations of vitamin E or in the activity of total SOD, total MnSOD and total CuZnSOD. The interaction IO-YF-Phy reduced (−25%) the catalase activity ($p = 0.036$). Moderate Fe overload increased GPx activity in all groups IO when compared to the control group ($p = 0.000$). Nevertheless, in the IO-Phy and IO-YF-Phy interactions there was a reduction in the activity of this enzyme ($p = 0.000$) when compared to the IO group (Table 4).

3.5.4. Histological analyses

3.5.4.1. Macroscopy. No gross alterations in the liver (size, color, consistence and section surface) were observed in any group.

3.5.4.2. Light microscopy. In the control group, liver histopathology was within normal parameters. In the Fe-overload groups, discrete tubular lymphocyte infiltrates and some cases of diffuse microvesicular vacuolar degeneration were observed in some animals.

3.5.4.3. Apoptosis. There was a considerable increase ($p = 0.000$) in the number of AB observed through fluorescence microscopy in all IO groups when compared to the control group (> 200%) (Table 5, Fig. 1).

3.5.4.4. Semi-quantitative evaluation of hemosiderin deposition. All animals in the control group did not present hemosiderin depositions (score 0). With the exception of the IO-YF-PHY group that presented zero index for two animals in a total of 7 (2/7), in the other IO groups all animals presented index 1 of hemosiderin deposition with a significant difference from the control group ($p = 0.000$) (Table 5, Fig. 1).

4. Discussion

In HH models, Fe overload leads to alterations in the serum Fe status indices, such as the increase in serum Fe, the degree of TS and the TIBC (Turbino-Ribeiro et al., 2003). Here we demonstrated that a moderate Fe overload did not alter the Fe status indices (Table 3), despite the increase in the daily Fe intake in the moderate Fe-overload (IO) groups (10 mg Fe/day versus 0.8 mg Fe/day in the control group), thus demonstrating that under a moderate Fe overload and in the absence of a congenital metabolic disease altering the homeostasis of the mineral, the mechanisms which regulate it are sufficient to maintain such parameters within normality.

In healthy humans and animals, Fe homeostasis is controlled by alterations in the efficiency of the mineral absorption. Higher Fe concentration in the diet leads to lower absorption (Hallberg, Hultén, & Gramatkovsky, 1997), and this regulation is mediated by the hepatic synthesis of hepcidin (Gulec, Anderson, & Collins, 2014). In contrast, we found an unexpected increase in the apparent absorption of this mineral (Table 3). We have previously shown that the increase in

Table 4
Mineral concentration and antioxidant activities in the liver tissue of rats fed control and iron-overload diets supplemented or not with yacon flour and/or phytate for 92 days.

Parameters	Experimental Groups[1]					
	Control	IO	IO-YF	IO-Phy	IO-YF-Phy	p*
Liver weight (g)	16.1 ± 4.0	15.1 ± 2.3	15.1 ± 3.2	15.8 ± 2.0	16.6 ± 3.1	ns**
Liver weight/body weight (%)	4.3 ± 1.0	4.1 ± 0.4	4.3 ± 0.9	4.3 ± 0.7	4.5 ± 1.0	ns
Fe (mg/g liver)	0.38 ± 0.07^{a}	0.69 ± 0.22^{b,c}	0.49 ± 0.11^{a,b}	0.82 ± 0.16^{c}	0.63 ± 0.25^{a,b,c}	0.002
Cu (μg/g liver)	15.2 ± 2.6	12.7 ± 2.0	13.2 ± 2.3	13.6 ± 1.4	13.0 ± 3.2	ns
Zn (μg/g liver)	90.1 ± 25.9	73.6 ± 13.3	83.6 ± 18.7	79.0 ± 20.8	66.4 ± 11.8	ns
GPx[2] (mM/min/mg protein)	121.2 ± 38.9^{a}	803.6 ± 178.4^{b}	596.0 ± 195.9^{b,c}	383.3 ± 101.0^{c}	380.6 ± 122.2^{c}	0.000
CAT[3] (μM/min/mg proteína)	6.1 ± 0.8^{a}	4.7 ± 0.8^{a,b}	4.8 ± 1.0^{a,b}	5.2 ± 1.1^{a,b}	4.5 ± 0.8^{b}	0.036
MnSOD[4] (U/mg protein)	9.7 ± 3.2	12.8 ± 2.4	11.2 ± 3.1	11.8 ± 2.9	11.1 ± 4.7	ns
CuZnSOD[5] (U/mg protein)	6.3 ± 1.7	10.5 ± 4.2	13.1 ± 7.7	6.9 ± 2.4	11.1 ± 3.5	ns
Vitamin E (μM/g)	594.4 ± 158.1	624.3 ± 101.9	607.2 ± 129.9	616.7 ± 104.1	659.1 ± 171.3	ns

Mean ± S.D. (n = 7: Control, IO, IO-YF, IO-YF-Phy, n = 6: IO-Phy).
[a,b,c]Different superscripts letters on the same line indicate statistically different values ($p < 0,05$).
[1] Iron Overload (IO), Iron Overload + Yacón Flour (IO-YF), Iron Overload + Phytate (IO-Phy), Iron Overload + Yacón Flour + Phytate (IO-YF-Phy).
[2] Glutathione peroxidase.
[3] Catalase.
[4] Mn superoxide dismutase.
[5] Cu-Zn superoxide dismutase.
* Versus Control group.
** Non significant difference.

hepatic Fe in response to dietary Fe overload after 12 weeks may occur due to changes in cellular dynamics in the gut (Lobo, Cocato, Sá, & Colli, 2014). Thus, since there were no changes in blood Fe, excess absorbed Fe would accumulate as ferritin (or hemosiderin) in the liver.

On the other hand, Fe overload did not alter Zn and Cu apparent absorption, except for group IO-YF where there was an increase in Cu apparent absorption in comparison to the control group ($p < 0.05$) (Table 2). However, this increase did not result in a difference in the hepatic concentration of these minerals. The same was observed by Vayenas, Repanti, Vassilopoulos, and Papanastasiou (1998), who demonstrated that the excess Fe does not interfere with Cu and Zn concentrations in this organ.

On its turn, the association of Fe overload and phytate (IO-Phy) (concentration of 0.6% in the diet), did not interfere with the apparent absorption of Fe, Zn and Cu, either. This result is in line with those found by Yuangklang et al. (2005), who demonstrated that, in the concentration of 0.4%, phytates did not reduce the apparent absorption of Ca and Mg or the hepatic concentrations of Fe, Zn and Cu. According to Szkudelski (2005), after ingestion with the diet, phytate undergoes dephosphorylation in the digestive tract, a process catalyzed by phytases, and the less phosphorylated and non-phosphorylated forms of inositol are absorbed, thus reducing its capacity to chelate minerals.

Despite the magnitude of the increase in Fe liver concentration in all IO groups, this was not so large as those found in rodents experiencing the HH model (Bacon & Britton, 1990), it was enough to increase the hepatic activity of glutathione peroxidase (GPx) in the same groups when compared to the control group (up to 200%), whilst a reduction in the activity of CAT was observed only in the IO-YF-Phy group (Table 4).

The enzyme GPx is present in all cell compartments (Schrader & Fahimi, 2004) and is one of the main factors responsible for the reduction of hydrogen peroxide (H_2O_2) to H_2O (a role also played by CAT), thus avoiding the formation of the hydroxyl radical ($OH^{\bullet}$), the most reactive oxygen species. In this way, the increase in GPx activity, without significant alteration in the activity of MnSOD, may involve two possible mechanisms.

In the first one, the amount of free Fe generated by a moderate Fe overload might have been enough to reduce cytoplasmic molecular O_2, via xantine oxidase, increasing the production of superoxide radicals ($O_2^{\bullet-}$), which in turn would have increased the production of H_2O_2 through Fentońs Reaction (Galaris & Pantopoulos, 2008). This process would involve the action of Cu-Zn-SOD (localized mainly in the cell cytoplasm) in the dismutation of $O_2^{\bullet-}$. In fact, a trend to an increase in the hepatic activity of this enzyme in all groups ($p = 0.09$) was observed in this work, except for the IO-Phy group. A second mechanism would involve the action of several enzymes inside the peroxisomes.

Table 5
Number of apoptotic bodies and Kupffer cells with hemosiderin deposition in the liver of rats fed control and iron-overload diets supplemented or not with yacon flour and/or phytate for 92 days.

Parameters	Experimental Groups[1]					
	Control	IO	IO-YF	IO-Phy	IO-YF-Phy	p*
Apoptotic bodies (No. of bodies/mm^2)	0.1 ± 0.06^{a}	0.29 ± 0.05^{b}	0.48 ± 0.14^{b}	0.27 ± 0.07^{b}	0.42 ± 0.18^{b}	0.000
Kupffer cells with hemosiderin deposition						
No. of animals per group	7	7	7	6	7	
Index 0**	7/7 (100%)	0/7 (0%)	0/7 (0%)	0/6 (0%)	2/7 (29%)	0.000
Index 1***	0/7 (0%)	7/7 (100%)	7/7 (100%)	6/6 (100%)	5/7 (71%)	0.000

Mean ± S.D. (n = 7: Control, IO, IO-YF, IO-YF-Phy, n = 6: IO-Phy).
[a,b,c]Different superscripts letters on the same line indicate statistically different values ($p < 0.05$).
[1] Iron Overload (IO), Iron Overload + Yacón Flour (IO-YF), Iron Overload + Phytate (IO-Phy), Iron Overload + Yacón Flour + Phytate (IO-YF-Phy).
* Versus Control group.
** Absence of hemosiderin pigment.
*** Presence of up to 2 Kupffer cells with hemosiderin per field in a magnification of 400×.

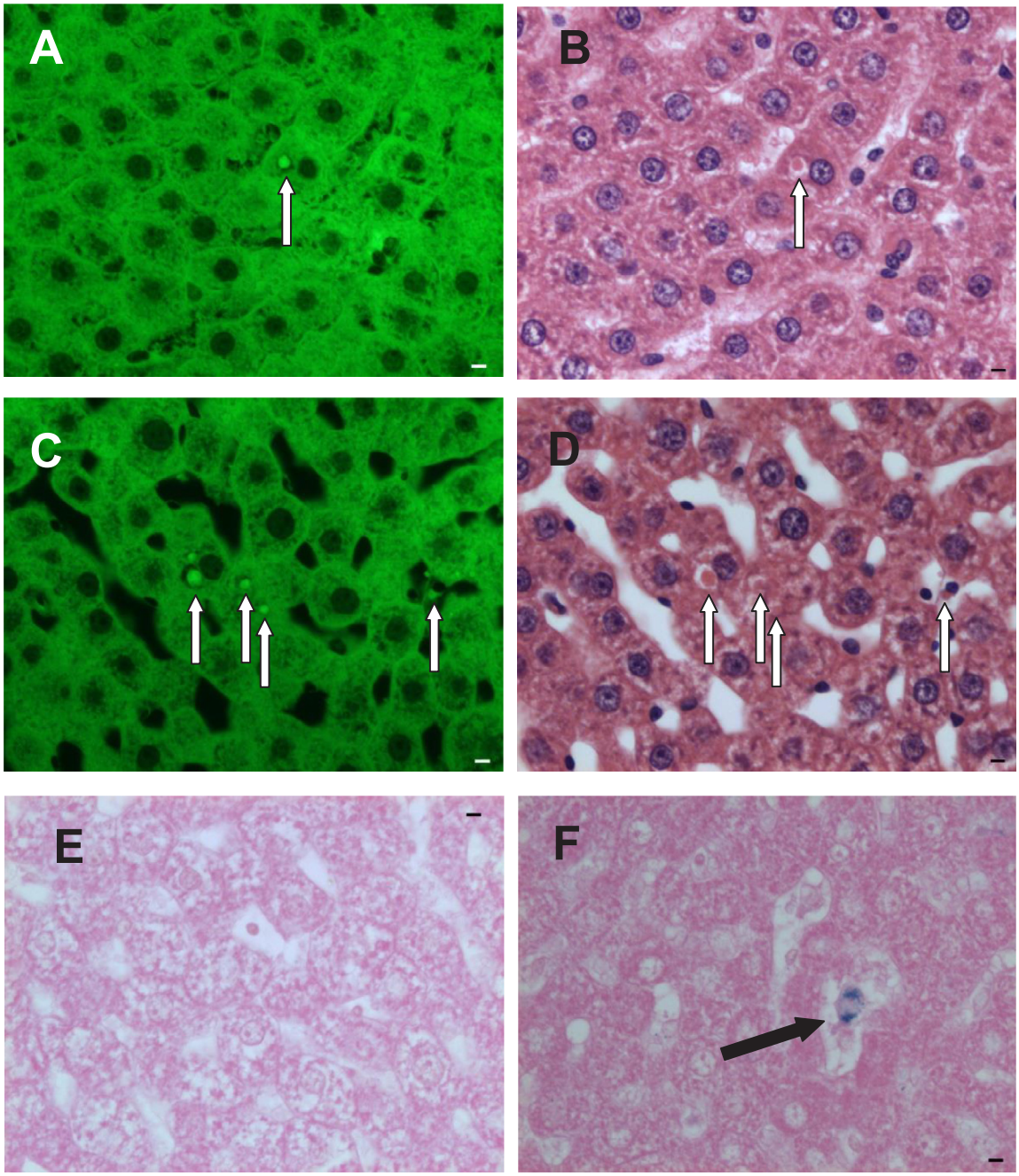

Fig. 1. Histological liver sections: (A) from a control-group animal, showing an apoptotic body (arrow) (fluorescence microscopy). (B) The same histological section of Fig. 1A (H & E). (C) from an animal of the iron overload groups, showing apoptotic bodies (arrows) (fluorescence microscopy). (D) The same histological section of Fig. 1C (H&E). (E) Histological section of a liver from a control-group animal showing no hemosiderin deposition (H&E). (F) Hemosiderin deposition around a Kupffer cell from an animal of the iron overload groups (H&E). Scale bar = 5 μm.

Such enzymes are able to generate H_2O_2 without producing the $O_2^{\cdot-}$ radical, which leads to the need for a higher GPx activity without a higher superoxide dismutase (SOD) activity.

The presence of phytate, both in the IO-Phy and in the IO-YF-Phy groups, despite having increased the GPx activity by 216% in comparison to the control group, reduced the activity of this enzyme by 52% in comparison to the IO group ($p < 0.05$) (Table 4), thus confirming the antioxidant properties of this compound. According to Muraoka and Miura (2004), phytate inhibits xantine oxidase and the scavenging radical $O_2^{\cdot-}$, both *in vitro* and *in vivo*, and this aspect, once more, confirms that a probable pathway related to the increase in the GPx activity in the IO group is through xantine oxidase.

On the other hand, to evaluate possible damage caused by moderate Fe overload on the hepatic tissue, the serum activities of alanine aminotransferase and aspartate aminotransferase (ALT and AST) were determined. No alteration in the activity of ALT was found, but there was an increase (+40% in comparison to the control group, $p = 0.055$) in the AST activity for the IO group (Table 3). In the presence of YF and/or phytate, the activity reversed to the one observed in the control group. The pattern of AST activity in the IO groups followed the same pattern observed in the GPx activity for the several groups, whereas the IO group showed the highest enzyme activity and the phytate groups showed a tendency to lower activities, when compared to the control. This, again, denotes the hepatic protective effect of phytate in the presence of a moderate Fe overload. This result was also found by Bhowmik et al. (2017) who, working with Fe overload in *in vitro* and *in*

vivo models, found that phytic acid attenuated oxidative stress and hepatic injury induced by excess Fe.

In this way, for a better understanding about the effects of a moderate Fe overload on the hepatic tissue, a histopathologic evaluation was carried out to measure the magnitude and distribution of Fe deposition in the liver, besides evaluating the formation of AB in this tissue. Moderate Fe overload caused a discrete increase in hemosiderin deposition within Kupffer cells in all IO groups (Table 5, Fig. 1). This distribution pattern is expected when the increase in body Fe stores is caused by chronic exposure to Fe supply in the absence of HH (Deugnier, Brissot, & Loréal, 2008).

Kupffer cells represent the largest population of resident macrophages in the organism, and are located in an anatomical and histological position which enables them to control the presence of foreign substances brought by portal blood (Vanderkerken et al., 1995). Nevertheless, the activation of Kupffer cells may trigger necrotic-inflammatory secondary processes through the activation of prostaglandins (PGE), leukotrienes, tumor necrosis factor-α (TNF-α), among others, leading to alterations in the hepatic tissue such as inflammation, fibrosis and apoptosis (Kolios, Valatas, & Kouroumalis, 2006).

In fact, it was demonstrated that a moderate Fe overload triggered a significant increase in the number of AB per area in the liver (a 3- to 5-fold increase when compared to the control group) (Table 5, Fig. 1). The presence of a great number of AB in the groups fed diets with the Fe overload and yacon flour (IO-YF and IO-YF-Phy) may be related to the systemic effects of the short chain fatty acid generated by the YF. Neyrinck et al. (2004) demonstrated in wistar rats subjected to an endotoxic shock induced by administration of lipopolysaccharides (LPS) and fed with inulin FOS (Raftilose P_{95}, Orafti), there was an increase in serum TNF-α and PGE-2 in FOS treated rats than in control rats without an increase in ALT activity. *In vitro*, liver cuts released more TNF and PGE-2 in the FOS group than in the control group, suggesting increased phagocytic activity of Kupffer cells. As already mentioned, both are involved in apoptotic processes in Kupffer cells (Kolios et al., 2006).

In the present study, there was no change in ALT activity among all groups, but the AST activity increased in the IO group ($p = 0.055$) compared to the control group. However, in the groups that received YF, the levels were numerically similar to those in the control group. So, the increase in apoptosis may be related to mechanisms of liver protection which are triggered by the YF.

Here, we also searched to know if the moderate Fe overload would alter the profile of fatty acids in the liver, the concentration of vitamin E and lipids in serum, known to be altered by the Fe overload (Brunet et al., 1997; Turbino-Ribeiro et al., 2003), and if the interactions of this Fe overload with the yacon flour, and/or phytate, could reverse this process. There was no difference in the profile of fatty acids (data not shown) and in the concentration of vitamin E in the liver among all groups (Table 4). The results obtained demonstrated that, similarly to what happens in high Fe overload, the moderate Fe overload decreased cholesterol ($p = 0.002$), which was also observed in the interaction IO-YF. Only for the IO-YF-Phy group, a decrease in serum triacylglycerols and VLDL ($p = 0.047$) was observed. The mechanisms for the hypocholesterolemic effects induced by an Fe overload are not well understood yet. Research involving the excretion of bile salts and the enzymes lecithin-cholesterol acyltransferase (LCAT) and acylcoenzyme A-cholesterol acyltransferase (ACAT) did not elucidate these issues (Brunet et al., 1997; Yuangklang et al. (2005); ; ; , .

The decrease in cholesterol, however, was not observed in the case of interaction with phytate (IO-Phy and IO-YF-Phy groups) (Table 3). The effect of phytate on serum cholesterol is controversial and the action mechanism has not been elucidated yet. In rats fed diets with excess cholesterol (10 g/kg), 0.4% phytate increased the serum concentration of cholesterol and had no effect on the concentration of triacylglycerols (Yuangklang et al., 2005). Nevertheless, in another work, the decrease in cholesterol was demonstrated with the addition of 1% phytate to the diet. No effect was observed on the serum concentration of triacylglycerols (Lee et al., 2007). On the other hand, Szkudelski (2005), showed in his research that increasing additions of phytic acid to diets free from cholesterol offered to rats, demonstrated that additions of 0.1% and 0.2% phytic acid to the diets did not alter serum cholesterol, but decreased serum triacylglycerols, whilst concentrations of 0.3% and 1% had no effect on this parameter.

Yacon flour, on the other hand, did not alter the effects of Fe overload on serum lipids (Table 4). It is known that FOS above 10% decreases serum triacylglycerols in rats fed diets with high lipid concentrations (Delzenne, Daubioul, Neyrinck, Lasa, & Taper, 2002) which, for hypercholesterolemic patients, would be a positive effect. For normocholesterolemic patients, this decrease would be undesirable because it would imply problems in the synthesis of hormones, the fluidity of cell membranes, among other aspects. Thus, the use of smaller amounts of FOS (as used in our experiment) in diets with moderate iron overload may protect the body from some of its deleterious effects without, however, further reducing serum lipids.

5. Conclusion

The results of the present study demonstrated that moderate Fe overload did not alter serum Fe levels, but resulted in increased AB in hepatic tissue, increased GPx activity and serum lipid profile, with a reduction in cholesterol. Its interaction with YF, and/or phytate, led to the reduction in the activity of GPx - caused by phytate - and increase (numerical, but not statistical) in apoptosis - in the groups with YF. Serum cholesterol returned to the levels of the control group in the interaction between Fe overload/yacon flour/phytate. These data indicate the protective effect of yacon flour and phytate for groups exposed to diets with moderate Fe overload.

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