



Neurotoxic effects of fructose administration in rat brain: implications for fructosemia

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ABSTRACT

Fructose accumulates in tissue and body fluids of patients affected by hereditary fructose intolerance (HFI), a disorder caused by the deficiency of aldolase B. We investigated the effect of acute fructose administration on the biochemical profile and on the activities of the Krebs cycle enzymes in the cerebral cortex of young rats. Rats received a subcutaneous injection of NaCl (0.9 %; control group) or fructose solution (5 μ mol/g; treated group). Twelve or 24 h after the administration, the animals were euthanized and the cerebral cortices were isolated. Peripheral blood (to obtain the serum) and cerebral spinal fluid (CSF) from the animals were also collected. It was observed that albumin levels were decreased and cholesterol levels were increased in CSF of animals 12 h after the administration of fructose. In addition, serum lactate levels were increased 12 h after the administration, as compared to control group. Furthermore, malate dehydrogenase activity was increased in cerebral cortex from treated group 24 h after the administration of this carbohydrate. Herein we demonstrate that fructose administration alters biochemical parameters in CSF and serum and bioenergetics parameters in the cerebral cortex. These findings indicate a possible role of fructose on brain alterations found in HFI patients.

Key words: brain damage, fructose, fructosemia, hereditary fructose intolerance, Krebs cycle, metabolism.

INTRODUCTION

Fructose (β -D-fructofuranose) is a glucose epimer widely found in food, especially fruits and honey (Hardinge et al. 1965, Somogyi and Trautner 1974).

There are several studies showing toxic effect of fructose *per se* (Yokozawa et al. 2008, Fan et al. 2014, Yeh et al. 2014) or by inducing glyoxal and methylglyoxal formation (Maruf et al. 2015). Recent studies have specifically described neurotoxicity elicited by this carbohydrate, inducing oxidative

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stress, inhibiting acetylcholinesterase activity and affecting brain mitochondrial function (Guimarães et al. 2014, Lopes et al. 2014, Mortensen et al. 2014).

Increased levels of fructose are found in hereditary fructose intolerance (HFI) (Steinmann et al. 2001). HFI (OMIM 229600) is an autosomal recessive inborn error of fructose metabolism caused by the deficiency of the enzyme fructose-1-fosfato aldolase (EC 4.1.2.7.), also known as aldolase B. This enzyme is present in liver, kidney, and small intestine (Van den Berghe 1986). The deficiency leads to the accumulation of fructose in biological fluids and tissues of patients (Steinmann et al. 2001). The onset of signs and symptoms coincides with the introduction of fruits in the diet. Continuous exposure of patients affected by HFI to fructose during infancy may result in liver damage, mental retardation, and even death (Gopher et al. 1990, Steinmann et al. 2001). Metabolic sequelae includes hypoglycemia, hyperuricemia, hypophosphatemia, hyperlactatemia, and metabolic acidosis (Cornblath et al. 1963, Perheetupa et al. 1972, Froesch 1976, 1978).

Considering that the identification of pathophysiological mechanisms of cerebral manifestations has not emerged yet, studies evaluating the effect of high levels of fructose in the brain are necessary. Previous studies from our group described an animal model of fructosemia induced chemically by administering fructose acutely to rats (Monteiro et al. 2012, Lopes et al. 2014). By using this experimental model, we evaluated the biochemical profile in cerebral spinal fluid (CSF) and serum and the activities of Krebs cycle enzymes in the cerebral cortex of young rats.

MATERIALS AND METHODS

FRUCTOSE SOLUTION

Fructose (Sigma-Aldrich; St. Louis, MO, USA) was dissolved in saline solution (NaCl 0.9 %) always on the day of the experiment and its pH was adjusted to 7.4.

ANIMALS

Twenty-four 30-day-old male Wistar rats obtained from the Central Animal House of Universidade do Extremo Sul Catarinense were used. Rats were kept with dams until weaning at 21 days of age. The animals had free access to water and to a standard commercial chow and were maintained on a 12:12 h light/dark cycle in an air-conditioned constant temperature (22 ± 1 °C) colony room. The Guide for the Care and Use of Laboratory Animals (National Research Council, 2011) and the “EC Directive 86/609/EEC” were followed in all experiments. All efforts were made to minimize the number of animals used and their suffering. This study was approved by the Local Ethical Committee on Animal Use for Research under the protocol 076/2013-2.

FRUCTOSE ADMINISTRATION AND TISSUES PREPARATION

The animals were divided into two groups: control group, which received a single subcutaneous injection of vehicle (0.9 % sodium chloride), and fructose group, which received a single subcutaneous injection of fructose (5 μ mol/g body weight or 0.9 mg/g of body weight), according to Monteiro et al. (2012) and Lopes et al. (2014).

Twelve or 24 h after the administration, the animals were euthanized by decapitation without anesthesia, and the brains, skeletal muscle and liver were rapidly excised on a Petri dish placed on ice and cerebral cortices were isolated. Peripheral blood was also collected in order to obtain the serum by centrifuging at 10,000 rpm for 10 min. CSF was collected from cisterna magna with a needle number 23. After collected, CSF was centrifuged in order to precipitate any possible blood contamination. Cerebral cortices were weighed and homogenized with SETH buffer, pH 7.4 (250 mM sucrose, 2 mM EDTA, 10 mM Trizma base, 50 IU/ml heparin). The homogenates were centrifuged at $800 \times g$ for 10 min at 4 °C, and the supernatants were kept at -80 °C until the determination of the enzyme activities. Skeletal muscle and liver were homogenized with

33 % KOH and the homogenates were used for glycogen content.

DETERMINATION OF GLUCOSE, CHOLESTEROL, ALBUMIN, LACTATE DEHYDROGENASE, AND LACTATE LEVELS

Glucose, cholesterol, and albumin were measured in CSF of the animals and lactate dehydrogenase (LDH; EC 1.1.1.27) and lactate levels were measured in serum samples from rats submitted to the animal model by using commercial kits (Labtest, Lagoa Santa, MG, Brazil), according to the instructions of the manufacturer.

DETERMINATION OF GLYCOGEN CONTENT

Glycogen content was measured in skeletal muscle and liver according to Krisman (1962), with slight modifications. The homogenates were boiled at 100 °C for 20 min, with stirring. After cooling, 96 % ethanol was added to the samples, and they were heated again to boiling and further cooling in an ice bath to precipitate the glycogen content. The homogenates were centrifuged at 1,300 x g for 15 min. The supernatant was then discarded and the pellets were neutralized with saturated NH₄Cl. The pellet was heated to 100 °C for 5 min and solubilized in water. Glycogen content was determined by treatment with iodine reagent and the absorbance was measured at 460 nm. The results are expressed as mg of glycogen/g of tissue.

KREBS CYCLE ENZYME ACTIVITIES

Citrate synthase activity

Citrate synthase (CS; EC 2.3.3.1) activity was measured according to Srere (1969). CS was assayed in a medium containing 0.1 mM 5',5''-dithiobis-(2-nitrobenzoate) (DTNB), 0.2 mM oxaloacetic acid, 0.1 % triton X-100, 0.1 mM acetyl-CoA, and 100 mM Tris-HCl, pH 8.0, and aliquots of cerebral cortex homogenates (3 mg protein). The activity was determined as DTNB reduction at $\lambda=412$ nm. The results are expressed as nmol TNB . min⁻¹ . mg protein⁻¹.

Isocitrate dehydrogenase activity

Isocitrate dehydrogenase (IDH; EC 1.1.1.41) activity was determined in 33 mM Tris buffer, pH 7.4, containing 33 mM Tris-HCl, 10 μ M rotenone, 1.2 mM MnCl₂, 0.67 mM ADP, 0.1 % Triton X-100, 0.3 mM NAD, cortical homogenates (75 mg protein), and 5 mM isocitrate (Plaut 1969). NAD reduction at $\lambda=340-400$ nm was followed and the results are expressed as nmol NADH . min⁻¹ . mg protein⁻¹.

Succinate dehydrogenase activity

Succinate dehydrogenase (SDH; EC 1.3.99.1) activity was determined according to the method of Fischer et al. (1985), by following the decrease in absorbance, due to the reduction of 2,6-dichloro-indophenol at 600 nm. The results are expressed as nmol . min⁻¹ . mg protein⁻¹.

Fumarase activity

Fumarase (EC 4.2.1.2) activity was assayed in 100 mM sodium phosphate buffer, pH 7.3, containing 50 mM L-malate. The activity was determined by measuring the increase of absorbance at $\lambda=250$ nm (O'Hare and Doonan 1985). The results are expressed as nmol fumarate . min⁻¹ . mg protein⁻¹.

Malate dehydrogenase activity

Malate dehydrogenase (MDH; EC 1.1.1.37) activity was measured according to Kitto (1969), in a buffer containing 10 μ M rotenone, 0.3 % Tween 20, 0.14 mM NADH, 0.30 mM oxalacetate, 50 mM potassium phosphate, pH 7.4, and 25 mg protein from cerebral cortex preparations. MDH activity was determined by following the reduction of NADH absorbance at $\lambda=340-400$ nm. The results are expressed as nmol NADH . min⁻¹ . mg protein⁻¹.

PROTEIN QUANTIFICATION

Protein concentrations were measured by the method of Lowry et al. (1951), using bovine serum albumin as standard.

STATISTICAL ANALYSES

Results are presented as mean \pm standard error of mean. Assays were performed in duplicate or triplicate and the mean or median was used for statistical analysis. Results were analyzed by Student's *t* test for independent samples. Differences between groups were rated significant at $p < 0.05$. All analyses were carried out in an IBM-compatible PC computer using the Statistical Package for the Social Sciences (SPSS) software 16.0.

RESULTS

We first investigated biochemical parameters in CSF of rats submitted to acute fructose administration. It was observed that total cholesterol levels were markedly increased 12 h after fructose administration [$t(9) = -4.64$; $p = 0.001$]. In contrast, albumin levels were decreased 12 h after the administration of this carbohydrate [$t(6) = 2.14$; $p < 0.05$]. It was not identified any difference of both total cholesterol and albumin levels in CSF between groups 24 h after fructose administration. Glucose levels were not altered either 12 h or 24 h in CSF after fructose administration (Table I).

The influence of acute fructose administration on LDH and lactate levels in serum of rats was also investigated and it was observed that LDH was not altered either 12 h or 24 h after administration. On the other hand, lactate serum levels were increased 12 h after fructose administration, as compared to control group [$t(8) = -2.32$; $p < 0.05$]. In contrast, lactate levels were decreased 24 h after fructose administration, although not significantly [$t(8) = 2.31$; $p = 0.066$] (Table II).

Furthermore, we determined glycogen content in skeletal muscle and liver of rats euthanized 12

or 24 h after fructose administrations. Fructose administration did not alter this parameter after 12 hours or 24 h in skeletal muscle and in liver (Table III).

Finally, we investigated the *in vivo* effect of fructose on Krebs cycle enzymes activities 24 h after the administration of this carbohydrate. It was demonstrated that MDH activity was increased in cerebral cortices of animals submitted to the animal model of fructosemia, as compared to controls [$t(10) = -2.49$; $p < 0.05$]. On the other hand, CS, IDH, SDH, and fumarase activities were not altered by fructose administration (Fig. 1).

DISCUSSION

Some patients affected by HFI may present neurological impairment (Labrune et al. 1990, Steinmann et al. 2001), but the mechanisms underlying these symptoms are not well understood. In the present study, we demonstrated the effects of the acute administration of fructose, the main metabolite accumulated in HIF, on cerebral metabolism of rats. The animal model used reflects the characteristic findings of fructosemic patients during metabolic crises. This model allowed us

TABLE I
Glucose, cholesterol, and albumin levels in cerebral spinal fluid of animals submitted to an animal model of fructosemia.

	Control	Fructose
	12 h	
Glucose	69.5 \pm 4.96	59.42 \pm 6.84
Cholesterol	6.88 \pm 0.98	13.5 \pm 1.00***
Albumin	0.31 \pm 0.06	0.18 \pm 0.01*
	24 h	
Glucose	34.9 \pm 7.05	36.3 \pm 4.32
Cholesterol	26.4 \pm 0.92	26.88 \pm 1.09
Albumin	0.07 \pm 0.006	0.09 \pm 0.03

Values are mean \pm standard error of mean for six independent experiments (animals) per group performed in duplicate. Data were expressed as mg/dL. * $p < 0.05$; *** $p < 0.001$ compared to control (Student's *t* Test).

to investigate the biochemical alterations that occur after a single ingestion of high fructose concentrations, when fructose levels in plasma and tissues rise drastically shortly after the increased ingestion of fructose. Previous works using animal models of fructosemia also used fructose injections (Klein et al. 1946, Phillips and Yu 1974), but at

different doses. Klein et al. (1946) described that brain fructose concentrations were 7 times lower than the plasma levels in cats. It was also considered that fructosemic patients with a fructose-free diet present plasma concentrations of 0.14 mg/mL, but the levels of this sugar can reach up to 10 times more after its ingestion (Levin et al. 1963).

TABLE II
Lactate dehydrogenase (LDH) and lactate levels in serum of animals submitted to an animal model of fructosemia.

	Control	Fructose
12 h		
LDH	494 ± 40.1	448 ± 73.2
Lactate	12.1 ± 0.97	21.7 ± 4.00*
24 h		
LDH	113 ± 2.86	119 ± 2.94
Lactate	18.8 ± 2.78	12.3 ± 0.44

Values are mean ± standard error of mean for six independent experiments (animals) per group performed in duplicate. Data were expressed as UI/dL for LDH and mg/dL for lactate levels. * $p < 0.05$ compared to control group (Student's t Test).

TABLE III
Glycogen content in skeletal muscle and liver of animals 24 hours after fructose administration.

	Control	Fructose
Skeletal Muscle	0.39 ± 0.05	0.41 ± 0.6
Liver	0.35 ± 0.04	0.33 ± 0.06

Values are mean ± standard error of mean for six independent experiments (animals) per group performed in duplicate. Data were expressed as mg of glycogen/g of tissue. No difference between groups were detected (Student's t Test).

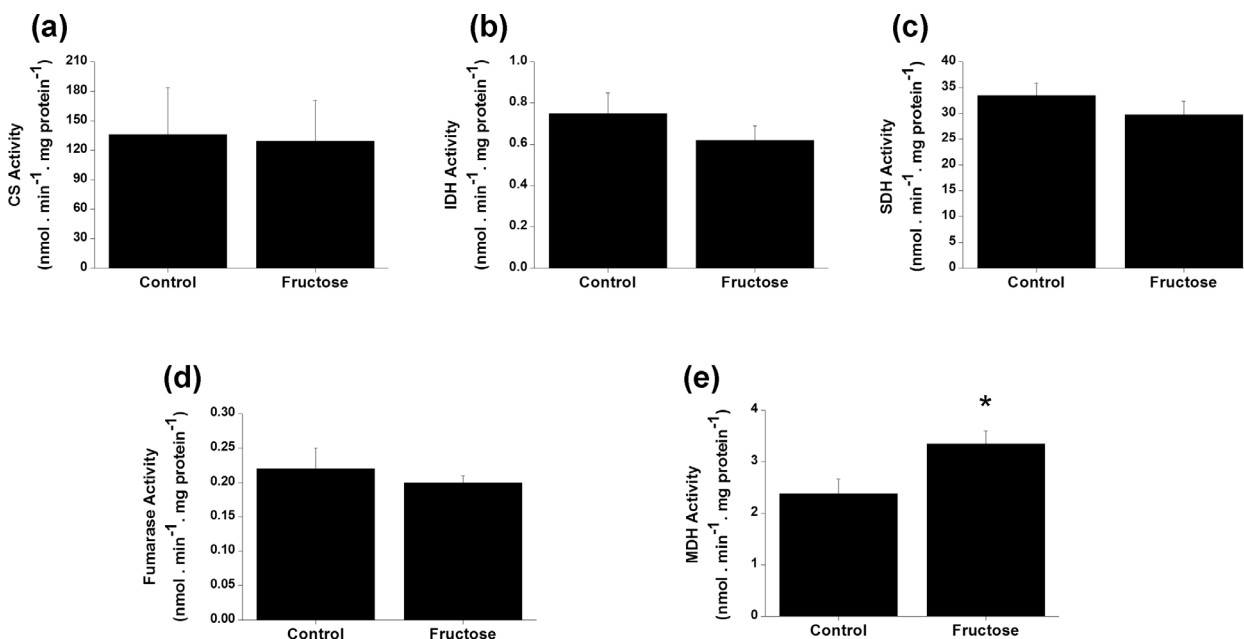


Figure 1 - Effects of acute fructose administration on the activities of Krebs cycle enzymes citrate synthase (CS) (a), isocitrate dehydrogenase (IDH) (b), succinate dehydrogenase (SDH) (c), fumarase (d), and malate dehydrogenase (MDH) (e) in rat cerebral cortex of rats 24 h after the administration. Values are means ± standard deviation for five to six independent experiments per group performed in duplicate and are expressed as $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$. * $p < 0.05$ compared to controls (Student's t test).

Initially, we evaluated the biochemical profile of CSF from animals submitted to an experimental model of fructosemia 12 and 24 h after acute fructose administration. It was observed that glucose levels were not altered in CSF of fructosemic animals 12 h nor 24 h after the administration of this carbohydrate. On the other hand, CSF albumin levels were decreased 12 h after the administration of fructose in animals as compared to control group, and these levels were restored 24 h after the administration. A decrease of this protein levels cannot be attributed to malnutrition, since CSF albumin levels, different from serum albumin, are not related to nutritional status of the organism. In this context, Wade and colleagues described that in fasting conditions, rat CSF albumin levels remain unaltered, while serum levels are decreased (Wade et al. 1988). Furthermore, Monteiro et al. (2012) showed that serum albumin levels were not altered 12 h or 24 h after acute fructose administration. In this scenario, decreased prealbumin levels in CSF were also observed in patients affected by multiple sclerosis and Alzheimer's disease (Hybelová et al. 2009, Ribeiro et al. 2012). Diminished albumin levels could also be attributed to proteolysis, which may occur due to the lack of energy substrates such as glucose, in order to provide carbons to oxidative reactions to further synthesize ATP. However, this possibility is unlikely, since glucose levels in CSF were not altered in our animal model. More studies should be conducted in order to clarify this effect.

In addition, cholesterol levels were increased in CSF from animals receiving fructose 12 h after the administration. It is widely reported that fructose stimulates lipid synthesis including cholesterol in liver (Hwang et al. 1987, Hulman and Falkner 1994, Laville and Nazare 2009, Tran et al. 2009, Chou et al. 2011), but it is still to be unraveled in the brain. Recently, cholesterol levels in CSF have been suggested as a marker of brain health since this parameter was found increased in cognitive impairment and Alzheimer's disease and the levels of cholesterol are associated with the progression of cognitive dysfunction (Leoni et al.

2013, Trushina et al. 2013). Increased cholesterol levels in CSF were also observed in patients affected by cerebrotendinous xanthomatosis (Salen et al. 1987) and in multiple sclerosis (van de Kraats et al. 2014). It is possible that increased cholesterol levels provoked by fructose administration could be related to the impaired cognitive function found in rats with high fructose intake (Cao et al. 2007, Stranahan et al. 2008, Ross et al. 2009, Hsu et al. 2015).

Our next step was to evaluate the effects of acute fructose administration on energy status in serum from animals and it was observed that LDH activity in animals receiving fructose did not differ from control groups. On the other hand, acute fructose administration increased serum lactate levels of fructosemic animals 12 h after the injection. Interestingly, Monteiro et al. (2012) showed that serum glucose levels were unaltered in the same animal model and time frames used in the present study. Increased lactate levels without glucose levels alterations suggest that glycolysis was not affected by fructose administration, but possibly reflect mitochondrial dysfunction. In line with previous data showing no alteration in serum glucose levels (Monteiro et al. 2012), no alteration of glycogen content in liver and skeletal muscle (tissues rich in this polysaccharide) of rats submitted to animal model of fructosemia in the present study was observed, corroborating the idea that glucose metabolism is not impaired in this experimental model.

Considering that increased serum lactate levels suggest mitochondrial dysfunction, we then evaluated the effect of acute fructose administration on Krebs cycle enzyme activities in homogenates from cerebral cortices of rats 24 h after the administration of this monosaccharide. It was observed that MDH activity was increased in this tissue, while CS, IDH, SDH, and fumarase activities were not altered in cerebral cortices of animals receiving fructose administration in comparison to control group.

It has been shown that MDH activity is increased in the brain of patients affected by schizophrenia (Bubber et al. 2011) and Alzheimer disease *post mortem* in regions affected by oxidative stress (Op den Velde and Stam 1976, Bubber et al. 2005). Shi and Gibson (2011) demonstrated that H_2O_2 increased MDH activity in hippocampal cell lines by increasing mRNA levels of the gene that expresses this enzyme. In this context, recently Lopes et al. (2014) demonstrated that acute fructose administration induced oxidative damage to lipids and proteins and altered enzyme antioxidant defenses in cerebral cortex. Thus, it is tempting to speculate that increased MDH activity caused by acute fructose administration might be due to oxidative stress.

The implication of increased MDH activity is unknown. MDH is the last step of Krebs cycle, restoring oxaloacetate levels to allow the continuous oxidation of acetyl-CoA in the mitochondria. During this reaction, reducing NADH equivalents are released, which are oxidized at the respiratory chain complex I (Dupourque and Kun 1969). MDH also participates on malate-aspartate shuttle, translocating electrons from cytosolic NADH to mitochondrial matrix to be oxidized at respiratory chain, since mitochondrial membranes are impermeable to this coenzyme. Increased MDH activity without a parallel increase of the other enzyme activities of the cycle could lead to an increase of NADH/NAD⁺ ratio, which could ultimately block the cycle, since NAD⁺ is also necessary for isocitrate dehydrogenase and alpha-ketoglutarate dehydrogenase activities. This effect on bioenergetics could also collaborate to the impairment of cognitive function, synaptic plasticity, dendritic spine density, and neurogenesis in the hippocampus, as well as neuronal loss caused by fructose (Cao et al. 2007, Stranahan et al. 2008, Ross et al. 2009, Stephan et al. 2010, Van der Borgh et al. 2011, Rafati et al. 2013).

Important differences in biochemical parameters after 12 h and 24 h of fructose exposure were observed. At present we cannot ascertain

the exact explanation for these differences. In this scenario, more studies should be carried out in order to clarify and better understand the present data. Additional study is also required regarding the nutritional status of rats receiving fructose acutely and chronically.

Concluding, we herein demonstrated that fructose exerts neurotoxic effects in cerebral cortex of rats submitted to an animal model of fructosemia. These data suggest that fructose toxicity might play a role in the neurological symptoms observed in patients affected by HIF.

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RESUMO

Frutose se acumula em tecidos e líquidos corporais de pacientes afetados por intolerância hereditária à frutose (HFI), doença causada pela deficiência da aldolase B. Nós investigamos o efeito da administração aguda de frutose sobre o perfil bioquímico e as atividades das enzimas do ciclo de Krebs em córtex cerebral de ratos jovens. Os ratos receberam uma administração subcutânea de NaCl (0,9 %; grupo controle) ou solução de frutose (5 μ mol/g; grupo tratado). Doze ou 24 horas após a administração, os animais sofreram eutanásia e os córtices cerebrais foram isolados. Também foram coletados sangue periférico (para obtenção de soro) e líquido cefalorraquidiano (LCR) dos animais. Foi observado que os níveis de albumina diminuíram e os níveis de colesterol aumentaram no LCR dos animais 12 horas após a administração de frutose. Adicionalmente, os níveis séricos de lactato aumentaram 12 h após a administração, em comparação como grupo controle. Além disso, a atividade da malato desidrogenase aumentou no córtex cerebral do grupo tratado 24 h após administração deste carboidrato. No presente trabalho, demonstramos que a administração de frutose altera

parâmetros bioquímicos em LCR e soro, bem como parâmetros bioenergéticos em córtex cerebral. Tais achados sugerem um papel para a frutose nas alterações cerebrais encontradas em pacientes com HFI.

Palavras-chave: dano cerebral, frutose, frutosemia, intolerância hereditária à frutose, ciclo de Krebs, metabolismo.

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