



## ***In vivo* and *in vitro* effects of fructose on rat brain acetylcholinesterase activity: an ontogenetic study**

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### **ABSTRACT**

Increased fructose concentrations are the biochemical hallmark of fructosemia, a group of inherited disorders on the metabolic pathway of this sugar. The main clinical findings observed in patients affected by fructosemia include neurological abnormalities with developmental delay, whose pathophysiology is still undefined. In the present work we investigated the *in vitro* and *in vivo* effects of fructose on acetylcholinesterase (AChE) activity in brain structures of developing rats. For the *in vitro* experiments, fructose was added at increasing concentrations to the incubation medium. It was observed that fructose provoked an inhibition of acetylcholinesterase activity in cerebral cortex of 30-day-old-rats, even at low concentrations (0.1 mM). For the *in vivo* experiments, rats were killed 1 h after a single fructose administration (5 µmol/g). Control group received the same volume of saline solution. We found that AChE activity was increased in cerebral cortex of 30- and 60-day-old rats receiving fructose administration. Finally, we observed that AChE activity was unaffected by acute fructose administration in cerebral cortex, striatum or hippocampus of 15- and 90-day-old rats. The present data suggest that a disruption in cholinergic homeostasis may be involved in the pathophysiology of brain damage observed in young patients affected by fructosemia.

**Key words:** acetylcholinesterase, brain, fructose, hereditary fructose intolerance.

### **INTRODUCTION**

Hereditary fructose intolerance (HFI) or fructosemia (OMIM ID: 229600) is an autosomal recessive

metabolic disease (Froesch et al. 1963, Steinmann 2007) caused by a deficiency of aldolase B (EC 4.1.2.13) activity in tissues in which this enzyme is crucial for dietary fructose metabolism (Hers and Joassin 1961, Wong 2009). The disease is also

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characterized by increased concentrations of fructose and fructose-1-phosphate in the blood of patients (Scriver et al. 2001). This deficiency may arise from various different mutations in the aldolase B gene situated on chromosome 9q22.3 (Lench et al. 1996, Coffee and Tolan 2010), most of which are point mutations resulting in single amino acid changes that decrease stability or catalytic activity (Bouteldja and Timson 2010). Symptoms appear in the newborn following weaning when fructose-containing foods are first introduced (Bouteldja and Timson 2010) and generally include abdominal pain, vomiting, and diarrhea. Children affected by these disorders may present with a general failure to thrive. Heavy and/or persistent intake of the sugar can lead to hypoglycemia, jaundice, progressive cirrhosis of the liver, renal tubular failure, metabolic acidosis, seizures, coma, and eventually death (Baerlocher et al. 1978, Cox 1993, Laméire et al. 1978, Morris 1968, Odièvre et al. 1978, Steinmann et al. 2001). Recently, a case has been reported with the association of hereditary fructose intolerance and poorly symptomatic Duchenne type muscular dystrophy (Paolella et al. 2012).

It has been previously speculated that the build-up of fructose-1-phosphate is the central element from which the spectrum of pathological pathways irradiates, as this compound fails to be cleaved into glycolysis or gluconeogenesis intermediates (Oberhaensli et al. 1987). The decrease on inorganic phosphate pool also results in impaired glycogenolysis, which, allied to the decreased ATP synthesis, disturbs all cellular processes that rely on phosphorylation or ATP (Oberhaensli et al. 1987, Van Den Berghe et al. 1973). However, the specific fructose toxicity is still poorly known. In the hope to clarify the contribution of fructose on the pathomechanisms of brain damage observed in HFI patients, the aim of the present work was to investigate the *in vivo* and *in vitro* effect of fructose on acetylcholinesterase (AChE) activity, an important enzyme involved in the homeostasis of cholinergic system (Igisu et al. 1994, Jha and Rizvi 2009), in different brain structures of young rats.

## MATERIALS AND METHODS

### REAGENTS

All chemicals were purchased from Sigma (St. Louis, MO, USA). Fructose was dissolved on the day of the experiments in the incubation medium used for each technique with pH adjusted to 7.4.

### ANIMALS

Fifteen-, thirty-, sixty- and ninety-day-old male Wistar rats obtained from the Central Animal House of Universidade do Extremo Sul Catarinense, Criciúma, SC – Brazil, were used. The animals were maintained on a 12:12 h light / dark cycle (lights on 07.00 - 19.00 h) in air conditioned constant temperature ( $22\text{ }^{\circ}\text{C} \pm 1^{\circ}\text{C}$ ) colony room, with free access to water and 20 % (w/w) protein commercial chow. The experimental protocol was approved by the Ethics Committee on Animal Research of the Universidade do Extremo Sul Catarinense (Protocol 076/2013-2) and followed the Guide for the Care and Use of Laboratory Animals (National Research Council, 2011). All efforts were made to minimize the number of animals used and their suffering.

### IN VITRO EXPERIMENTS

On the day of the experiments six animals were killed by decapitation without anesthesia and the cerebral cortex was rapidly excised on a Petri dish placed on ice. The brain structures were homogenized in a 100 mM phosphate buffer containing 0.1% Triton X-100, pH 7.5. The homogenates were centrifuged at  $800 \times g$  for 10 min at  $4\text{ }^{\circ}\text{C}$ . The supernatants were isolated and were preincubated for 1 h at  $37\text{ }^{\circ}\text{C}$  in the absence (control group) or presence of different concentrations of fructose (0.1 – 5 mM).

We always carried out parallel experiments with various blanks (controls) in the presence or absence of fructose and also with or without supernatants in order to detect artefacts caused by this sugar in the AChE assay.

### IN VIVO EXPERIMENTS

A total of thirty-six animals were divided into two groups for each age (12 animals of 15-, 30-, 60-, and 90-day-old; 6 animals per group): control group, which received a single injection of saline solution (0.9 g%), and fructose group, which received a single injection of fructose (5  $\mu\text{mol/g}$  body weight). One hour after the administration, the animals were killed by decapitation without anaesthesia, and the brains were rapidly excised on a Petri dish placed on ice. The cerebral cortex, striatum and hippocampus were peeled away from the white matter. Immediately after, the brain structures were kept at  $-70\text{ }^{\circ}\text{C}$  until being used for enzyme activity determination.

### DETERMINATION OF AchE ACTIVITY

AchE activity was determined by the method of Ellman et al. (1961). Acetylcholine hydrolysis rate was measured in an incubation medium containing acetylcholine 0.8 mM, 100 mM phosphate buffer (pH 7.5) and 1.0 mM DTNB. Fifty microliters of supernatants were added to the reaction mixture and preincubated for 3 min at  $25\text{ }^{\circ}\text{C}$ . The hydrolysis was monitored by the formation of the thiolate dianion of DTNB at 412 nm for 2–3 min (30 s intervals) at  $25\text{ }^{\circ}\text{C}$ . The results were expressed as  $\mu\text{mol}$  of hydrolysed acetylcholine  $\cdot\text{h}^{-1}\cdot\text{mg}$  of protein $^{-1}$  and all samples were run in duplicate (Zugno et al. 2008).

### PROTEIN DETERMINATION

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

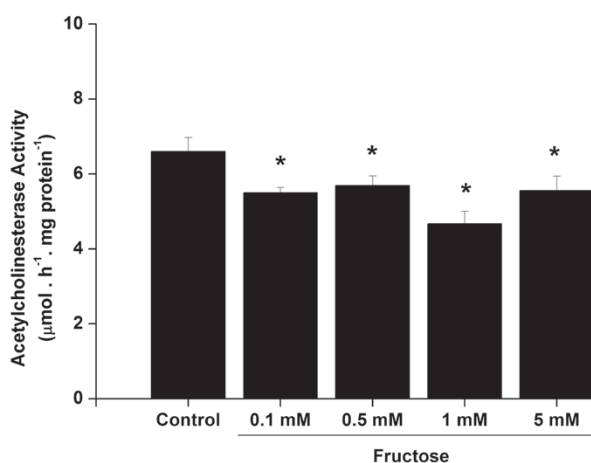
### STATISTICAL ANALYSES

Results are presented as mean  $\pm$  standard error of mean. Assays were performed in duplicate and the mean was used for statistical analyses. Data were analyzed using Student *t* test for independent samples when comparing two experimental groups (*in vivo* experiments) or one-way analysis of variance (ANOVA) followed by the post-hoc

Duncan multiple range test when *F* was significant when comparing three or more experimental groups (*in vitro* experiments). 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

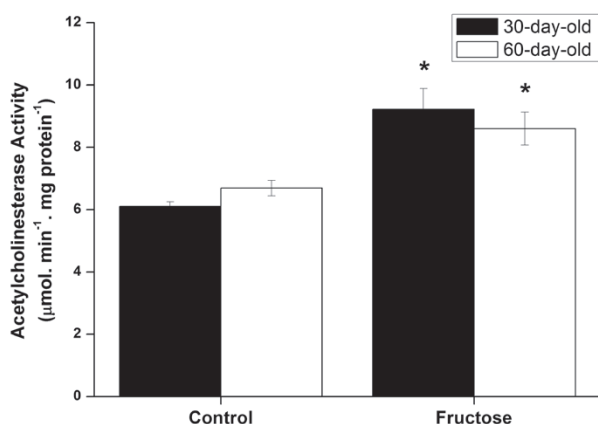
The present work investigated the *in vitro* effect of increasing fructose concentrations on AchE activity in cerebral cortex of young rats (30-day-old) (Figure 1). We observed that fructose induced a significant decrease in AchE activity even at low concentrations (0.1 mM and higher).



**Figure 1** - *In vitro* effect of fructose on acetylcholinesterase activity in cerebral cortex from 30-day-old rats. Values are means  $\pm$  standard error of mean for six independent experiments performed in duplicate and are expressed as  $\mu\text{mol}$  acetylcholine  $\cdot\text{h}^{-1}\cdot\text{mg}$  protein $^{-1}$ . \* $p < 0.05$  compared to control group (Duncan multiple range test).

The influence of acute fructose administration on AchE activity in cerebral cortex of rats with 30 and 60 days of life (Figure 2) were also assessed. It was observed that AchE activity was significantly increased in the brain of rats of both ages receiving fructose, as compared to control group.

The effects of acute fructose administration on AchE activity in various brain structures of 15- and



**Figure 2** - Effect of acute fructose administration (5 µmol/g) on acetylcholinesterase activity in cerebral cortex from 30-day-old rats (black bars) and 60-day-old rats (white bars). Values are means ± standard error of mean for six independent experiments performed in duplicate and are expressed as µmol acetylcholine · h<sup>-1</sup> · mg protein<sup>-1</sup>. \**p* < 0.05 compared to control group (Student's *t* test).

90-day-old rats (Table I and II, respectively) were finally assessed. AchE activity was not altered in cerebral cortex, striatum or hippocampus by this treatment in 15- and 90-day-old rats.

**TABLE I**  
Effect of acute fructose administration (5 µmol/g) on acetylcholinesterase activity in brain structures from 15-day-old rats.

Group	Cerebral Cortex	Striatum	Hippocampus
Control	7.69 ± 1.03	6.94 ± 0.97	7.45 ± 0.87
Fructose	7.02 ± 1.05	7.10 ± 0.99	7.30 ± 0.61

Values are mean ± standard error of mean for nine independent animals per group. Data were expressed as µmol acetylcholine · h<sup>-1</sup> · mg protein<sup>-1</sup>. No significant differences were detected between groups (Student *t* test for independent samples).

**TABLE II**  
Effect of acute fructose administration (5 µmol/g) on acetylcholinesterase activity in brain structures from 90-day-old rats.

Group	Cerebral Cortex	Striatum	Hippocampus
Control	3.61 ± 1.76	10.50 ± 3.00	6.12 ± 1.92
Fructose	2.44 ± 0.94	16.32 ± 10.73	5.15 ± 1.31

Values are mean ± standard error of mean for six independent animals per group. Data were expressed as µmol acetylcholine · h<sup>-1</sup> · mg protein<sup>-1</sup>. No significant differences were detected between groups (Student *t* test for independent samples).

## DISCUSSION

In all types of fructosemia, affected patients may present with CNS abnormalities (Scriver et al. 2001, Steinmann 2007, Wong, 2009, Bouteldja and Timson 2010). Currently, the pathophysiology of brain disturbances in this disease is postulated to be secondary to a fructose-1-phosphate accumulation. Considering that fructose is present at high concentrations in plasma and tissues of patients affected by fructosemia, and that very little is known regarding the direct toxicity of fructose, we investigated the effect of fructose on AchE activity in cerebral cortex of 30-day-old rats. It was initially observed that fructose provoked an inhibition of AchE activity *in vitro* at concentrations as low as 0.1 mM.

We then decided to investigate whether the effects observed *in vitro* for fructose also occur *in vivo*. Interestingly, AchE activity was increased in cerebral cortex of rats with 30 and 60 days of life receiving acute fructose administration.

Since the clinical onset of the neurological features in fructosemia occurs during the first infancy, we extended our investigation to evaluate the influence of acute fructose administration on AchE activity in various brain structures of suckling rats (15-day-old) and elder rats (90-day-old), without any significant alterations observed. These data point to an age-dependent effect elicited by acute fructose administration enhancing AchE activity. However, it cannot be ruled out that fructose may be metabolized faster in younger animals, as shown for other sugars, resulting in lower fructose in the different brain structures (Cuatrecasas and Segal 1965).

It has been demonstrated that alteration of the cholinergic system occurs during brain development (Herlenius and Lagercrantz 2004). In this context, it is feasible that signaling molecules may act by interfering on transcription and translation through a mechanism of feedback loop (Salgado et al. 2001, Keseler et al. 2005) modulating the levels of proteins, enzyme products, or other molecules related to the

action of the protein encoded by the gene considered (Krishna et al. 2006). In the present work, we observed that fructose inhibits AchE activity *in vitro* and enhances this enzyme activity when administered *in vivo*, indicating that a direct effect elicited by fructose enhancing AchE activity is unlikely. Therefore, it may be speculated that the *in vivo* increase of AchE activity occurs as a compensatory mechanism, resulting from interactions between genetic and metabolic networks. Alternatively, taking into account that oxidative stress was shown to increase AchE activity (Melo et al. 2003) and that fructose promotes protein oxidative damage and reactive oxygen species generation *in vivo* (Lee et al. 2009, Semchyshyn et al. 2011, Taleb-Dida et al. 2011), a putative mechanism could involve oxidative stress playing a role in this effect.

At present, we cannot ascertain the exact pathophysiological relevance of our findings. However, it should be mentioned that the findings observed in the present work occurred at concentrations even lower than those found in serum and tissues of patients affected by fructosemia (Levin et al. 1968). Moreover, it has been previously demonstrated that some metabolites, which accumulate in other inborn errors of the metabolism with neurological involvement, are also able to interfere on AchE activity (Ratnakumari et al. 1995, Schulpis et al. 2006, Zugno et al. 2008).

The present study provides evidence that fructose elicits a dual effect on AchE activity in cerebral cortex of rats. Considering that alterations on AchE activity has been related to progressive neurological decline (Beerli et al. 1995, García-Ayllón et al. 2008), and that increased fructose intake was recently appointed as a risk factor for dementia (Stephan et al. 2010), our data suggest that a disruption in the cholinergic system may be involved in the pathophysiology of the neurological symptoms observed in patients affected by fructosemia. However, more studies are necessary in order to investigate the influence of fructose on the cholinergic system, including those involved

in the maintenance of acetylcholine levels in the synaptic cleft (synthesis, release, degradation and reuptake), as well as on the quantity, distribution and functionality of cholinergic receptors.

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#### RESUMO

Concentrações elevadas de frutose são a principal característica bioquímica da frutosemia, um grupo de doenças hereditárias na via metabólica deste carboidrato. Os principais achados clínicos observados nos pacientes afetados pela frutosemia incluem anormalidades neurológicas com retardo do desenvolvimento, cuja fisiopatologia ainda não está definida. No presente trabalho investigou-se os efeitos *in vitro* e *in vivo* da frutose sobre a atividade da enzima acetilcolinesterase (AChE) em diferentes estruturas cerebrais de ratos em desenvolvimento. Para os experimentos *in vitro*, a frutose foi adicionada em concentrações crescentes ao meio de incubação. Observou-se que a frutose inibiu a atividade da AChE em córtex cerebral de ratos de 30 de vida, mesmo em baixas concentrações (0,1 mM). Para os experimentos *in vivo*, os ratos sofreram eutanásia 1 hora após uma administração única de frutose (5 µmol/g; subcutânea). O grupo controle recebeu o mesmo volume de solução salina. A atividade da AChE encontrou-se aumentada em córtex cerebral de ratos com 30 e 60 dias de vida que receberam administração de frutose. Finalmente, não se observou diferença significativa entre os grupos controle e frutose em cérebro de animais de 15 e 90 dias de vida. Os resultados do presente trabalho sugerem que um desequilíbrio na homeostase colinérgica pode estar envolvido na fisiopatologia do dano cerebral observado em pacientes jovens afetados pela frutosemia.

**Palavras-chave:** acetilcolinesterase, cérebro, frutose, intolerância hereditária frutose.

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