Fructose and NAFLD: metabolic implications and models of induction in rats[1]

Frutose e NAFLD: implicações metabólicas e modelos de indução em ratos

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ABSTRACT
PURPOSE: The increase in fructose consumption is paralleled by a higher incidence of obesity worldwide. This monosaccharide is linked to metabolic syndrome, being associated with hypertriglyceridemia, hypertension, insulin resistance and diabetes mellitus. It is metabolized principally in the liver, where it can be converted into fatty acids, which are stored in the form of triglycerides leading to NAFLD. Several models of NAFLD use diets high in simple carbohydrates. Thus, this study aimed to describe the major metabolic changes caused by excessive consumption of fructose in humans and animals and to present liver abnormalities resulting from high intakes of fructose in different periods of consumption and experimental designs in *Wistar* rats.
METHODS: Two groups of rats were fasted for 48 hours and reefed for 24 or 48 hours with a diet containing 63% fructose. Another group of rats was fed an diet with 63% fructose for 90 days.
RESULTS: Refeeding for 24 hours caused accumulation of large amounts of fat, compromising 100% of the hepatocytes. The amount of liver fat in animals refed for 48 hours decreased, remaining mostly in zone 2 (medium-zonal). In liver plates of *Wistar* rats fed 63% fructose for 45, 60 and 90 days it's possible to see that there is an increase in hepatocytes with fat accumulation according to the increased time; hepatic steatosis, however, is mild, compromising about 20% of the hepatocytes.
CONCLUSIONS: Fructose is highly lipogenic, however the induction of chronic models in NAFLD requires long periods of treatment. The acute supply for 24 or 48 hours, fasted rats can cause big changes, liver steatosis with macrovesicular in all lobular zones.
Key words: Fructose. Fatty Liver. Diet. Rats.

RESUMO
OBJETIVO: O aumento do consumo de frutose é concomitante a maior incidência mundial de obesidade. Este monossacarídeo está relacionado à Síndrome Metabólica, sendo vinculado à hipertrigliceridemia, hipertensão arterial, resistência à insulina e diabetes mellitus. É metabolizada principalmente no fígado, onde pode ser convertida em ácidos graxos, os quais serão estocados na forma de triglgérides ocasionando a esteatose hepática não alcoólica (NAFLD). Vários modelos de NAFLD utilizam dietas ricas em carboidratos simples. Desta forma, este trabalho teve como objetivos descrever as principais alterações metabólicas causadas pelo consumo excessivo de frutose em humanos e em animais e apresentar as alterações hepáticas decorrentes da alta ingestão de frutose em diferentes períodos de consumo e desenhos experimentais em ratos *Wistar*.
MÉTODOS: Dois grupos de ratos *Wistar* foram mantidos em jejum durante 48 horas e realimentados por 24 ou 48 horas com dieta contendo 63% de frutose. Outro grupo de ratos *Wistar* foi alimentado com 63% de frutose durante 90 dias.
RESULTADOS: A realimentação por 24 horas provocou acúmulo de grande quantidade de gordura. A quantidade de gordura hepática nos animais realimentados por 48 horas diminuiu, mantendo-se principalmente nas zona 2 (medio-zonal). Em fígados de ratos *Wistar* alimentados com 63% de frutose até 90 dias foi possível observar que há aumento de hepatócitos com acúmulo de gordura consequente ao aumento do tempo, no entanto a esteatose hepática é leve (20%).
CONCLUSÕES: A frutose é altamente lipogênica, no entanto a indução de NAFLD em modelos crônicos necessita de longos períodos de tratamento. A oferta aguda, por 24 ou 48 horas, a ratos mantidos em jejum é capaz de ocasionar grandes mudanças hepáticas, com presença de esteatose macrovesicular em todas as zonas lobulares
Descritores: Frutose. Fígado Gorduroso. Dieta. Ratos.

Introduction

Currently, non-alcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) are related to the increasing causes of liver disease and mortality and morbidity linked to diseases of the liver[1]. It is estimated that the prevalence of NAFLD in developed countries reaches 10% to 24% of the population[2], depending on the diagnostic criteria used[3], while the worldwide prevalence of NASH reaches 2% to 3% of the general population[3].

The difficulty in assessing the prevalence of NAFLD includes the absence of signs and symptoms, the low sensitivity of liver enzymes as indicators of the disease and the doubtful need of biopsy as gold standard for diagnosis[3].

The NAFLD can be defined as liver accumulation of lipids, primarily in the form of triglycerides, without ingestion of significant quantities of alcohol and with the exclusion of other known causes of steatosis, as some drugs and toxins[4].

The NAFLD is characterized as: type 1 - only steatosis, type 2 - steatosis plus inflammation, type 3 - steatosis plus hepatocellular injury and type 4 - steatosis plus sinusoidal fibrosis, Mallory bodies or both[4]. The NASH is considered the most serious form of NAFLD (types 3 and 4) and is also associated with cirrhosis and hepatocellular carcinoma[4].

The progression of NAFLD to NASH occurs on account of damage caused by lipid peroxidation and free-radical production[5]. Lipid peroxidation is accompanied by an inflammatory response and stellate cell activation, inducing fibrogenesis[6].

Day and James[7] proposed the "two hits" hypothesis to explain the progression of steatosis to inflammation, fibrosis and cirrhosis. The first "hit" is the steatosis, caused by imbalance between the formation and *turnover* of triglycerides. It is believed that insulin resistance (IR) affects the first "hit" of NAFLD as follows: it activates the adipocytokines secretion by adipocytes, alters the rate of synthesis and transport of triglycerides by hepatocytes and increases lipolysis in adipocytes that release free fatty acids (Non Esterified Fatty Acids - NEFA) in the portal circulation, exposing the liver to excessive levels of NEFAs. There is a decrease in mitochondrial function and increase in the rate of *de novo* synthesis and triglyceride levels. In the presence of NAFLD, other factors such as inflammation and increased oxidative stress probably lead to the progression to NASH, fibrosis and necrosis. The second "hit" is possibly caused by adipocytokines and reactive oxygen species, which activate stellate cells and increase fibrogenesis and lipid peroxidation[8].

Oxidative stress inhibits the proliferation of mature hepatocytes, resulting in an increase in oval cells and progenitor cells of non-differentiated hepatocytes, the presence of both cell types being related to the progression to fibrosis[9].

It is also discussed the role of factors that stimulate the synthesis of triglycerides, rather than only accumulation[10], and this because NEFAs, tumor necrosis factor - α (TNF-α) and adiponectin are directly linked to the progression of NAFLD to NASH. Published studies in humans report that the increase of TNF-α or decrease of adiponectin are associated with increased risk of progression of NAFLD to NASH[11]. TNF-α increases the formation of reactive oxygen species, promotes apoptosis of hepatocytes and inflammation in the liver, while adiponectin diminishes hepatic triglycerides accumulation by reducing the NEFA export from adipose tissue[12].

If insults are large enough to develop cirrhosis, about 33% of patients will die or develop morbid conditions[8].

The IR has an important role for development of NASH because of participation in intracellular processes such as the loss of the inhibitory effect of insulin on ß-oxidation. This mechanism induces intracellular oxidative stress. Patients with NASH have an increase in ß-oxidation and in hepatic oxidative stress. These changes are also present in patients with liver fat accumulation resulting from diseases such as obesity, malnutrition, intestinal malabsorption, endocrine metabolic diseases and thyroid disorders, but only in NASH there are mitochondrial defects in the structure[13].

Mitochondria are responsible for oxidative phosphorylation and ß-oxidation of fatty acids, processes that are the main sources of free radicals and, together with mitochondrial disorders, play a central role in the development of NASH[6]. The mitochondrial abnormalities associated with NAFLD include structural damage, depletion of mitochondrial DNA, decreased activity of respiratory chain complexes and reduced mitochondrial ß-oxidation. Electron microscopy revealed that in the presence of NAFLD, the mitochondria are larger and bulkier, less in number and the mitochondrial matrix has paracrystalline inclusion and lower density[14].

Moreover IR is considered as the most common risk factor for the development of NAFLD, and this one as a hepatic manifestation of the Metabolic Syndrome (MS), depending on the diagnostic criteria used[3].

Independently of weight gain, MS is a great predictor for NAFLD, so important that became recognized as the hepatic manifestation of this syndrome[8], since the prevalence of MS reaches 34% in the U.S. population, 53% in individuals with NAFLD and more than 88% in those with NASH[15,16].

This syndrome is strongly linked to steatosis, fibrosis and cirrhosis in obese adults in part for glucose intolerance and IR. Numerous studies have shown that obesity, type 2 diabetes mellitus, dyslipidemia, hypertension and IR are associated with NAFLD. It is also associated with reduced insulin sensitivity in liver and fat tissue of liver, and throughout the body, increasing rate of gluconeogenesis and reduced fatty acid oxidation. However, there is still no consensus on whether the IR is cause or consequence of steatosis[8].

Many patients with NAFLD are asymptomatic. In the absence of cirrhosis, hepatomegaly is the only physical finding in most patients, present in more than 75% of cases[3]. The use of liver biopsy for the diagnosis of NAFLD is controversial. Usual markers of NAFLD are alanine aminotransferase (ALT) and aspartate aminotransferase (AST), elevated levels of these being considered as a consequence of liver damage, fatty infiltration and inflammation. Serum levels of these enzymes are related to multiple components of MS[8].

Several studies have demonstrated the induction of NAFLD in rats by diets with large amounts of simple carbohydrates[17,18]. However, few are focused on dietary manipulation for effective reversal of metabolic damage generated by these diets[17].

Consumption of simple carbohydrates such as sucrose and fructose has increased steadily over the last 20 years[17]. The excess of glucose or fructose causes the synthesis of triacylglycerols that will accumulate in the liver. This accumulation is the cause of NAFLD[1,5].

Rats fed a diet high in simple carbohydrates (SC) developed dyslipidemia, weight gain, visceral adiposity and reduced insulin sensitivity[17]. Axen *et al.*[19] also reported higher levels of triglycerides in obese mice fed with SC compared to those fed a high-fat diet and, similarly, another study indicated that by replacing fat with carbohydrates the diet contributes to weight gain[20].

Ackerman *et al*.[1] reported the inuction of NAFLD in rats fed by fructose as carbohydrate source. The model of experimental induction of NAFLD through diets high in fructose is well studied, being able to cause hypertension, hypertriglyceridemia, hyperinsulinemia and IR in rats[21]. Supplementation with fructose for six days in healthy men caused an increased fasting glucose and endogenous glucose production[22]. Fructose may be the critical component associated with the risk of obesity and heart disease, and clinical studies confirm that sucrose, and fructose in particular, can induce weight gain and MS features[23].

Fructose consumption

The estimated consumption of fructose by the population is difficult to achieve because their consumption is not measured in most surveys or databases[24]. According to the *International Sugar Organization* (Sugar Year Book. London, 2008), South America and Oceania are the biggest consumers of sugar[24].

In the U.S. population, fructose consumption before 1900 came mainly from fruit and vegetable intake of about 15g/day; consumption increased for 24g/day in the period before World War II; around 1977 it reached 37g/day and in 1994 55g/day[15]. Tappy and Lee noted in a review that according to the *Third National Health and Nutrition Examination Survey*, currently it is estimated that fructose consumption by teenagers reaches 72.8g/day[24].

The large growth in consumption of fructose is due in great part to the development of techniques that allowed the extraction of starch from corn, its hydrolysis into glucose and the enzymatic isomerization of glucose to fructose, thereby producing large-scale syrup corn (High Fructose Corn Syrup - HFCS) after 1960's[25].

The HFCS presents good stability in acidified food and drink; being liquid, its transport and storage are easier than those of sucrose, and being derived from corn, HFCS has become one of the ingredients most used by food industry[26]. It consists of approximately 55% fructose and 45% glucose. It is widely used in soft drinks and processed juices. The largest consumers of fructose, according to *The Third National Health and Nutrition Examination Survey*[26], are adolescents and young adults.

It is important to consider that the increased consumption of free fructose was accompanied by a decrease in consumption of sucrose, in which fructose is linked to glucose[24]. Although Bray *et al*.[27] proposed that the increase in obesity was directly associated with increased consumption of HFCS, as they both grew in the period 1960 to 2000, we must consider that during this same period other factors also influenced the increase in obesity, such as increased consumption of total calories and fat[26].

Studies in humans with increased consumption of fructose showed weight gain and significant increase in triglycerides, systolic blood pressure and IR. Most of the diets provided 400 to 800kcal/day of fructose, values among the highest rates consumed in the United States[23].

Fructose metabolism

Fructose is a monosaccharide with the same chemical formula as glucose, the difference lying in its structure. It possesses in its second carbon a ketone group[28]. Figure 1 illustrates the structural formula of glucose and fructose.

Its metabolism differs from glucose's by not requiring insulin to enter the cell[28]. Absorption occurs via a specific transporter present in intestinal cells, the GLUT5, which increases in quantity according to levels of dietary fructose; in the liver its entrance occurs with GLUT2 transporter, being this transport by facilitated diffusion and independent of sodium. This transporter has high affinity on account of its chemical structure, existing competition with sorbitol due to their similarity. The liver retains at least 50% of the ingested fructose diet, and there fructose will be phosphorylated by frutoquinase into fructose-1-phosphate, which is then converted into glyceraldehyde and dihydroxyacetone phosphate. Glyceraldehyde will be later converted into glyceraldehyde-3-phosphate and participate in the *de novo* synthesis of fatty acids. Another portion of these substrates will be converted into lactate and will be part of neogluconeogenesis[28].

Fructose doesn't need phosphofructokinase to be metabolized, differently from glucose, which undergoes negative regulation by citrate and ATP, because they inhibit the enzyme[28]. Fructokinase is the first enzyme that converts fructose, producing fructose-1-phosphate, which will be converted to glyceraldehyde and dihydroxyacetone phosphate by ketosis-1-phosphate aldolase[29].

There are two sources of fructose to the body, the exogenous, supplied by the diet, and the endogenous, made from glucose via aldose reductase. The offer of fructose by each of these pathways depends on factors such as exogenous availability, transport rate and activity of aldose reductase and sorbitol dehydrogenase. These two enzymes constitute the metabolic pathway of polyols, responsible for the formation of fructose in the tissues; this formation is activated by hyperglycemia and forms fructose from sorbitol in the corneas, kidneys and peripheral nerves, for at these the excess glucose is converted to sorbitol by aldose reductase, which generates fructose by sorbitol dehydrogenase[29].

The fructose inability to stimulate insulin and leptin acutely and to inhibit ghrelin is a factor that admittedly affects the control of satiety in the central nervous system[23]. It also seems to decrease the basal metabolic rate, because mice that drank fructose water gained more weight than those who ate the same calories, but from starch[23].

Fructose and NAFLD

Fructose has been appointed as a factor linked to the increase of obesity in recent decades[24]. Its intake is also linked to

the severity of nonalcoholic fatty liver disease, being visible that NAFLD patients with larger fructose consumption had increased liver inflammation and fibrosis[30]. High-fructose diets constitute an experimental model for NAFLD. The animals begin to display characteristics of MS, including insulin resistance, hyperinsulinemia, hypertriglyceridemia and hypertension[28].

In animals, the induction of non-alcoholic fatty liver disease through the glut of simple carbohydrates such as sucrose and fructose, requires more time of experiment, compared to other models. With a shorter time it is already possible to see features of Metabolic Syndrome (MS) as the increase in dyslipidemia, insulin secretion and plasma free fatty acids, accumulation of triglycerides in the liver and enlarged adiposity[31]. On the other hand, some studies have reported the use of such diets for periods of up to 40 weeks. When offered for a short time, about five weeks, it constitutes a model to analyze the forms of treatment of MS, since it is possible to observe some of its constituents, such as dyslipidemia and increased body fat[32].

Methods

This study was approved by the Ethics Committee on Animal Research of the Faculty of Medicine of Ribeirão Preto. We used male *Wistar* rats from the Animal Service Campus USP - Ribeirão Preto and subsequently maintained in the Animal Department of Clinical Medicine, Faculty of Medicine of Ribeirão Preto, USP. The light-dark regime was kept constant at 12 to 12 hours and the temperature maintained at 24 ± 2º C.

All animals were handled in accordance with the recommendations of "The Guide for the Care and Use of Laboratory Animals" prepared by the "National Academy of Sciences" and published by "National Institutes of Health" (NIH publication, 1985, 140p).

The diets offered were prepared in accordance with what recommended the *American Institute of Nutrition*[33], with changing of the carbohydrate source, replacing starch and sucrose by fructose (Figure 1).

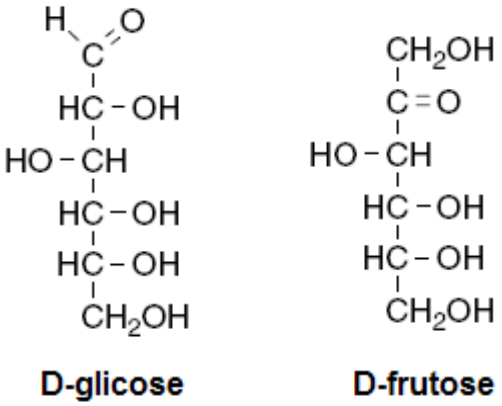

FIGURE 1 - Structural formula of D-glucose and D-fructose

For histological analysis, fragments of liver tissue were cut, being kept for 24 hours in a solution of 10% buffered formaldehyde. Shortly thereafter, this solution was replaced with 70% alcohol. After this step, the livers were immersed in paraffin and then cut with a thickness of 4m and stained with hematoxylin-eosin, in order to semi-quantitatively assess hepatic steatosis, which was classified as crosses, according to Oh *et al*.[34], with some modifications. The degree of steatosis was associated with morphological location (zone 1, 2 and 3) 0 (0%) 1-25% (1: little present in zone 3), 25-50% (2: zone 3); 50-75% (3: zones 2 and 3) and 75-100% (4: Zone 1, 2 and 3).

Results and Discussion

Figure 2 shows liver plates of *Wistar* rats that were fasted for 48 hours and refed for 24 (Figure 2b) and 48 hours (Figure 2c) with diet containing 63% fructose.

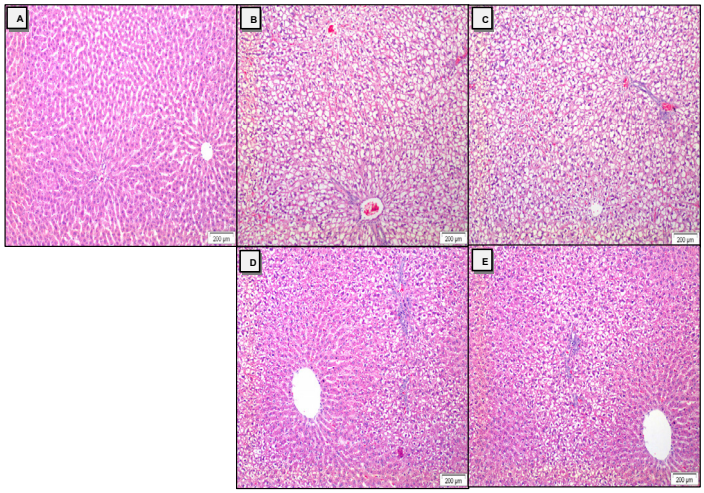

FIGURE 2 - Microphotographs of normal liver tissue (A) and animals fasted for 48 hours and refed with high-fructose diet for 24 hours (B and C) and 48 h (D and E). H & E, 10x

Refeeding for 24 hours caused accumulation of large amounts of fat, compromising 100% of the hepatocytes. The amount of liver fat in animals refed for 48 hours decreased, remaining mostly in zone 2 (medium-zonal). Figure 3 shows liver plates of Wistar *rats* fed 63% fructose for 45, 60 and 90 days. It's possible to see that there is an increase in hepatocytes with fat accumulation according to the increased time; hepatic steatosis, however, is mild, compromising about 20% of the hepatocytes.

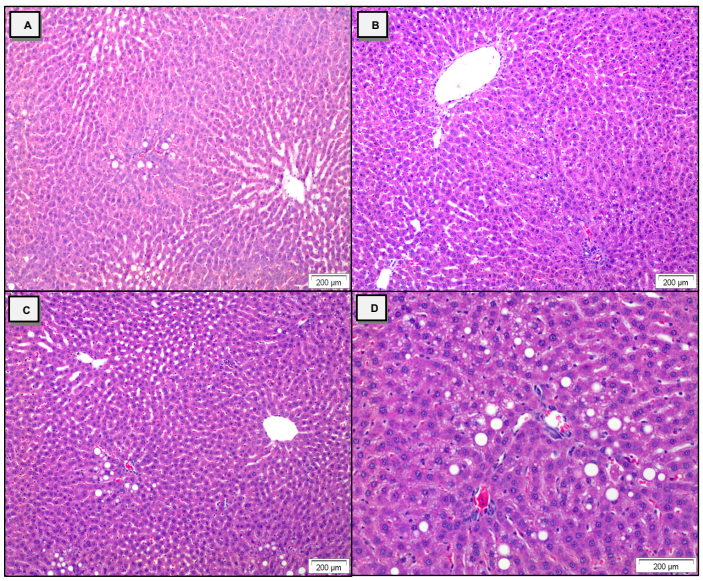

FIGURE 3 - Microphotographs of liver tissue showing histological aspects of animals refed with high-fructose diet for 45 days (A), 60 days (B) and 90 days (C and D). H&E, 10x (A, B and C) and 20x (D).

Refeeding with high-fructose diet caused a large accumulation of liver triacylglycerols; however, with the increase of the offer period of this diet, there were adaptive changes and steatosis has not presented itself so markedly, although it is still significantly present. In another study that evaluated the refeeding with 16 grams of carbohydrates (starch and sucrose in the same ratio) after 24 hours of fasting it was observed increase in liver volume, though these animals have shown a lower content of triglycerides compared to group refed with high-fat diet[35].

Delzenne *et al.*[36] also described the accumulation of hepatic fat in animals refed for 6 hours on high carbohydrate diet (40% sucrose and 40% starch) with the presence of mild steatosis microvesicular mainly in zone I, while the macrovesicular steatosis extended for the three zones.

The larger formation of acetyl-CoA by both fructose and sucrose, when they are offered in excess, causes a higher formation of fatty acids that will be stored in the form of triglycerides in the liver.

A study comparing the supply of sucrose and the supply of glucose and fructose showed that both diets were able to cause the MS, differing in the fact that glucose plus fructose induced higher formation of uric acid[37]. This is because fructose has the particularity of inducing higher formation of uric acid, compared to glucose and sucrose, however for this to occur in rats it is required a treatment with oxonic acid to uricase inhibition, an enzyme present in rats capable of converting uric acid into allantoin[37].

The fructose offered in diet to adult *Wistar* rats for 4 weeks, with 90 days of life, is more effective in producing the characteristics of MS compared to rats with 28 days of life, fed for 8 weeks. In adult animals there was an increase of fat liver, white adipose tissue, total cholesterol, HDL, LDL, triglycerides and serum insulin. Also offering 60% fructose in the diet was more effective than offering 10% fructose in water[32].

Conclusions

The oversupply of fructose in rats facilitates the appearance of several features of the Metabolic Syndrome. However, the establishment of NAFLD requires more time to experiment. This work was the first to show the effects on liver morphology of fructose refeeding in rats maintained in fasting for long periods. It's interesting to highlight that this model can be widely used in the search for metabolic targets for counteracting the effects of excess fructose.

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