Choline and Fructooligosaccharide: Non-alcoholic Fatty Liver Disease, Cardiac Fat Deposition, and Oxidative Stress Markers

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ABSTRACT: This study investigates the treatment of non-alcoholic fatty liver disease (NAFLD) in rats with choline and fructooligosaccharide (FOS). The healthy control group received standard diet. The other three groups consisted of animals with NAFLD. Group E_{str} received standard diet; group E_{cho} received standard diet plus choline (3 g/100 g diet); and group E_{fos} received standard diet plus FOS (10 g/100 g diet). Food intake, weight, urinary nitrogen, urinary ammonia, total cholesterol, serum triacylglyceride, liver and heart weights, tissue nitrogen, tissue fat, vitamin E, TBARS, and reduced glutathione (GSH) were measured in hepatic and heart tissue. Choline and FOS treatments resulted in total mean fat reduction in liver and heart tissue of 0.2 and 1.7 g, respectively. Both treatments were equally effective in reducing hepatic and cardiac steatosis. There were no differences in the TBARS level among experimental and control groups, indicating that the proposed treatments had no added protection against free radicals. While all experimental groups had increased vitamin E and GSH levels, choline treatment led to a significant increase compared to control.

KEYWORDS: hepatic steatosis, choline, fructooligosaccharide, liver fat, heart fat

CITATION: Haubert et al. Choline and Fructooligosaccharide: Non-alcoholic Fatty Liver Disease, Cardiac Fat Deposition, and Oxidative Stress Markers. *Nutrition and Metabolic Insights* 2015:8 1–6 doi:10.4137/NMI.S24385.

RECEIVED: January 30, 2015. **RESUBMITTED:** March 1, 2015. **ACCEPTED FOR PUBLICATION:** March 3, 2015.

ACADEMIC EDITOR: Joseph Zhou, Editor in Chief

TYPE: Original Research

FUNDING: This study was supported by the Conselho Nacional de Ciência e Tecnologia, CNPq, grant # 306824, and Fundação de Apoio ao Ensino e Pesquisa do Hospital das Clínicas de Ribeirão Preto da Universidade de São Paulo. The authors confirm that the funder had no influence over the study design, content of the article, or selection of this journal.

COMPETING INTERESTS: Authors disclose no potential conflicts of interest.

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Introduction

Nonalcoholic fatty liver disease (NAFLD) is defined as fatty infiltration of the liver. It is a common cause of chronic liver disease around the world and can progress to cirrhosis and liver failure.[1–3] NAFLD is often diagnosed on a presumptive basis in overweight, diabetic, or obese individuals without identifiable etiology, or when imaging studies suggest hepatic steatosis.[4–6] It has been recognized as a major health burden, and its high prevalence is probably due to the contemporary epidemics of obesity, unhealthy dietary pattern, and sedentary lifestyle.[7] Nice reviews on the issue can be found elsewhere.[4–9] However, controversy exists regarding the optimal recognition, diagnosis, and management of these conditions, and treatment recommendations are evolving.[4] Treatment of NAFLD aims to reduce risk factors through behavioral and medical approaches.[10–12] Several drugs and nutritional supplements, such as choline and fructooligosaccharide (FOS), have been proposed for the treatment of NAFLD without any scientific evidence of their efficacy.[13–15] Choline is a precursor of phospholipids, phosphatidylcholine, lysophosphatidylcholine, and sphingomyelin. In food, choline mainly occurs in the form of lecithin, but may also be free or in the form of a phospholipid.[16] Feeding choline to animals previously treated with a ketogenic diet recovers mitochondrial dysfunction and steatosis.[16] FOS belongs to a class of naturally occurring fructose oligosaccharides obtained from a wide variety of plants.[16] It also improves carbohydrate metabolism and reduces free-radical availability, as observed in diabetic animals.[17]

In view of the potential benefits of these supplements[18–22] for the treatment of NAFLD, the objective of this study was to investigate the efficacy of choline and FOS for the treatment of NAFLD and heart steatosis induced by a high carbohydrate diet. To test this hypothesis, we evaluated the supplementation of choline and FOS for rats with NAFLD and cardiac fat induced by a high carbohydrate diet. Therefore, the endpoint was to look for a decrease in fat tissue (hepatic and cardiac) at the end of the study. High carbohydrate (sucrose) diet is similar to food intake for most obese subjects, also known as a "western diet".[23] Choline and FOS were equally effective in reducing hepatic and cardiac steatosis. However, the proposed treatments had no added protection against free radicals.

Methods

The Ethics Committee for the Use of Experimental Animals of the Ribeirão Preto School of Medicine, São Paulo University (FMRP-USP), approved the study.

Forty-eight male Wistar rats weighing 250–320 g were maintained in the FMRP-USP animal facility. Animals were housed in individual metabolic cages for urine collection and maintained on a 12-h light–12-h dark cycle under conditions of natural ventilation and controlled temperature (24 ± 2°C). Food and water intake were checked daily. Weight was measured once a week in the morning, between 8:00 and 9:00, with a Filizola electronic scale with a maximum capacity of 1.5 kg, graduated in 1 g.

Animals were divided, at random, into four groups. Control animals (C) (n = 10) of normal weight without NAFLD received the standard AIN 93 diet[24] throughout 42 days of the experiment. This diet did not contain FOS but did contain 0.2 g choline/100 g diet.

NAFLD with hepatic steatosis (HS) was induced in 36 rats of the experimental groups (E) by offering a high carbohydrate (70% sucrose) diet for 21 days, the induction phase, as previously reported.[25] Experimental NAFLD with HS is often induced with choline- and/or methionine-deficient diets,[26] or alternatively a fructose-rich diet. The high carbohydrate diet was chosen because it more accurately reproduces a so-called western diet than the other options.[23,25]

Afterward, the experimental groups were randomly allocated to three groups (Fig. 1) for 21 more days, the experimental phase. Experimental group 1 animals (E_{str}) (n = 12) received the standard AIN 93 diet[24] (42% carbohydrate ground whole corn, 22% casein, 4% soy oil, and 0.2% choline, plus a salt and vitamin mix, methionine, and lysine). Experimental group 2 animals (E_{cho}) (n = 12) received the standard diet supplemented with 3 g choline/100 g. Experimental group 3 animals (E_{fos}) (n = 12) received the standard diet supplemented with 10 g FOS (Raftilose P95, Clariant S/A, Switzerland)/100 g. The usual human choline intake is around 300 mg, and the major food sources are eggs, soy foods, red meat, fish, and vegetables.[19] The usual FOS intake in animal studies is around 0.3 g.[18] The supplement amount chosen in the present protocol ensured that all animals ingested a large amount of the respective supplement.

On the first day of the experimental phase (22nd day), four animals from each of the experimental groups were selected at random and sacrificed by decapitation for blood, liver, and heart collection.[25,27] The remaining animals, including those from the control group, were sacrificed on the last day. Blood was centrifuged at 2500 rpm for 10 minutes and plasma was stored at –70°C. Urine samples were collected during 24 hours on the first and last days of the experimental phase and stored at –20°C. The liver and heart were washed in 0.9% NaCl solution, and the heart and the right liver lobe were isolated and weighed. A sample of the tissues was wrapped in aluminum foil, placed in liquid nitrogen, and stored at –70°C.

Biochemical determinations. Urinary nitrogen was determined by pyrochemiluminescence (Antek 720 and 771 nitrogen analyzers), a method consisting of oxidative pyrolysis at a temperature of 1050°C.[28] Urinary ammonia

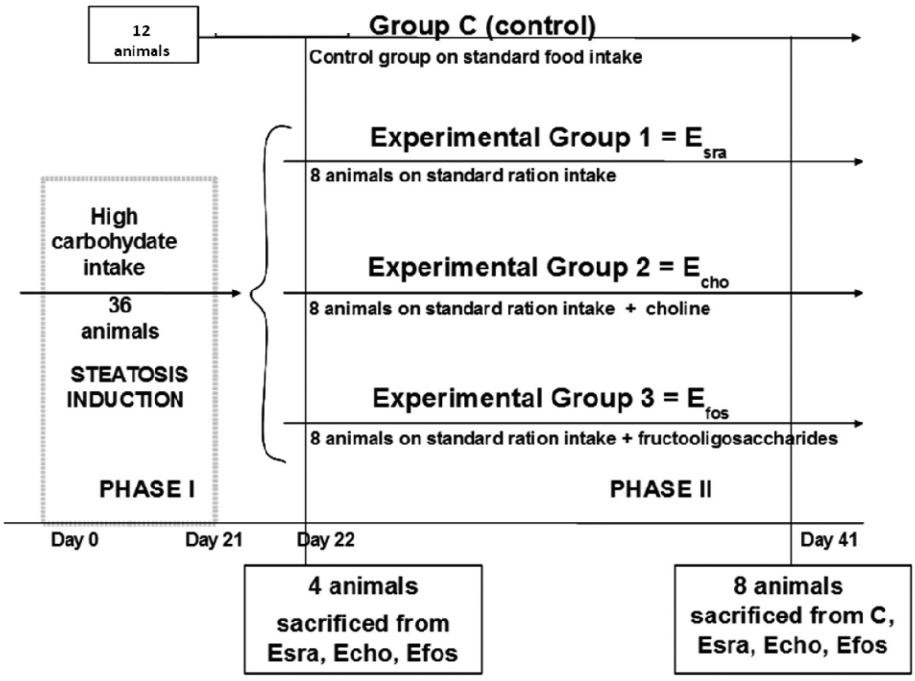

Figure 1. Protocol experimental design. Animals from experimental groups (E_{str}, E_{cho}, and E_{fos}) were previously subjected to a high carbohydrate (sucrose) diet for the induction of hepatic steatosis (see Ref. 10 for details).

Notes: Group C, control animals without steatosis receiving the standard diet without supplementation; Group E_{str}, animals with steatosis receiving the standard diet without supplementation; Group E_{cho}, animals with steatosis receiving the standard diet supplemented with choline; Group E_{fos}, animals with steatosis receiving the standard diet supplemented with FOS.

was determined by micro-diffusion,[29] which releases ammonia by alkalinization of 3-mL urine placed on the external part of a Conway disk with saturated potassium carbonate.[30] Total plasma cholesterol was determined by colorimetry (BT 3.000 plus, Wiener Lab),[31] and plasma triacylglyceride (TG) was determined by a colorimetric method based on enzymatic hydrolysis producing glycerol and fatty acids (BT 3.000 plus).[32]

For tissue analysis, the samples were first thawed, weighed, and dehydrated at 100°C for 24 hours. The micro-Kjeldahl method was then applied for the determination of tissue nitrogen.[32] Total liver and heart fat was determined by fat extraction with a Soxhlet apparatus (MA-487/6/250).[32]

Lipid peroxidation levels were measured by thiobarbituric acid reactive substances (TBARS)[33] quantification using a DU 640 Beckman spectrophotometer. Tissue vitamin E (α-tocopherol) content in liver and heart samples was determined by HPLC using a Shimadzu instrument (model LC10A)[34] equipped with a Shimadzu model SPD–10AV isocratic system and a UV–vis spectrophotometric detector. Reduced glutathione (GSH) was determined by spectrophotometry.[35]

Statistical analysis. Data are reported as mean ± SD and checked for normal distribution. The statistical differences between groups, paired and unpaired, were determined using a parametric or nonparametric univariate method, according to an appropriate statistical distribution. Two-way ANOVA, diet × time was applied when necessary. Data was analyzed using SAS 9.1 software, with the level of significance set at $P < 0.05$.

Table 1. Urinary nitrogen and ammonia excretion, cholesterolemia, and triacylglyceridemia values for the study groups at the beginning and end of the supplementation period.

	GROUPS	BEGINNING	END
Urinary nitrogen (g/day)	C	†	0.32 ± 0.08
	E_{str}	0.13 ± 0.08	0.22 ± 0.10*
	E_{cho}	0.13 ± 0.11	0.16 ± 0.03*
	E_{fos}	0.20 ± 0.25	0.21 ± 0.08*
Urinary ammonia (g/day)	C	†	0.035 ± 0.066
	E_{str}	0.016 ± 0.007	0.016 ± 0.008
	E_{cho}	0.012 ± 0.009	0.040 ± 0.020#
	E_{fos}	0.011 ± 0.008	0.021 ± 0.006
Cholesterolemia (mg/dL)	C	†	73 ± 12
	E_{str}	62 ± 11*	64 ± 15
	E_{cho}	66 ± 12	73 ± 13
	E_{fos}	60 ± 9*	57 ± 11*,#
Triacylglyceridemia (mg/dL)	C	†	80 ± 22
	E_{str} I	67 ± 17	49 ± 11*,#
	E_{cho}	63 ± 26	47 ± 8*,#
	E_{fos}	64 ± 28	51 ± 17*,#

Notes: Data are means ± standard deviation. Group C: control animals without steatosis receiving the standard diet without supplementation. Group E_{str}: animals with steatosis receiving the standard diet without supplementation. Group E_{cho}: animals with steatosis receiving the standard diet supplemented with choline. Group E_{fos}: animals with steatosis receiving the standard diet supplemented with FOS. †Control animals were sacrificed only at the end for data collection. *Significant difference compared to Group C (control). #Significant difference between the time points of the study (beginning and end).

Results

The mean daily diet intake was greater among C animals (195 ± 10 g, $P < 0.05$) compared to E_{str} (145 ± 9 g), E_{cho} (155 ± 10 g), and E_{fos} (156 ± 7 g) animals. At the end of the experiment, there was no difference in weight gain in E_{str}, E_{cho}, and E_{fos}, which was around 60 ± 10 g for all animals.

Urinary nitrogen was reduced in groups E_{str}, E_{cho}, and E_{fos} compared to control. However, no difference between the initial and final urinary nitrogen excretion was observed in the experimental animal groups (Table 1). There were also no significant differences in urinary ammonia excretion.

Serum cholesterol was similarly reduced for the experimental groups compared to the control, whereas only group E_{fos} showed a significant reduction of cholesterol after the experimental phase (Table 1). Serum TG was also reduced for the experimental groups compared to the control and had a similar reduction at the end of the experiment for all groups.

There were no differences in heart weight in control or experimental groups (Table 2). Liver weight did not change between the beginning and end of the experimental groups, but increased similarly between experimental and control groups ($P < 0.05$). No significant differences in heart or hepatic tissue nitrogen were detected among the experimental groups or between the beginning and end of the study (Table 2).

During the experimental phase, there was a reduction in hepatic fat in all experimental groups. However, the reduction was significant only in groups E_{cho} and E_{fos} ($P < 0.05$) (Table 2).

There was a significant increase of hepatic vitamin E for E_{cho} compared to the control group (Table 3). TBARS concentrations did not differ between the beginning and the end of the study, or compared to the control group (Table 3). There was an increase in GSH in all experimental groups, but only the choline supplementation group had a significant increase (Table 3).

Discussion

NAFLD with HS was induced with a high carbohydrate diet, or "western diet", similar to the food intake of obese individuals. Diet supplementation with FOS and choline was used to treat NAFLD with HS. Other diets that are rich in fructose[36] or poor in methionine and choline also lead to NAFLD with HS.[26] However, the obese diet does not have high fructose, nor is it poor in methionine and choline.[25] Moreover, the "western diet" in the present work agrees with those reported in other studies,[37,38] in which HS was detected after rats were fed a diet containing 50% sucrose for 21 days. The "western diet" is a high carbohydrate diet with 70% sucrose and efficiently induces HS, resulting in hepatic

Table 2. Weight, nitrogen, and hepatic and cardiac fat values for the study groups at the beginning and end of the supplementation period.

	GROUPS	BEGINNING	END
Heart weight (g)	C	†	1.20 ± 0.30
	E_{str}	1.24 ± 0.05	1.24 ± 0.13
	E_{cho}	1.33 ± 0.15	1.23 ± 0.16
	E_{fos}	1.20 ± 0.05	1.24 ± 0.21
Liver weight (g)	C	†	9.6 ± 1.1
	E_{str}	11.8 ± 0.8*	11.2 ± 1.8*
	E_{cho}	11.3 ± 1.5*	11.3 ± 1.3*
	E_{fos}	11.7 ± 0.9*	11.5 ± 2.0*
Hepatic nitrogen (g)	C	†	0.8 ± 0.3
	E_{str}	0.9 ± 0.5	0.9 ± 0.3
	E_{cho}	0.9 ± 0.1	1.0 ± 0.1
	E_{fos}	1.0 ± 0.05	0.9 ± 0.1
Hepatic fat (g)	C	†	0.52 ± 0.18
	E_{str}	0.84 ± 0.3*	0.60 ± 0.3#
	E_{cho}	1.58 ± 0.5*	0.70 ± 0.1#
	E_{fos}	1.25 ± 0.5*	0.70 ± 0.1#
Cardiac nitrogen (g)	C	†	0.11 ± 0.03
	E_{str}	0.12 ± 0.02	0.10 ± 0.03
	E_{cho}	0.12 ± 0.01	0.11 ± 0.01
	E_{fos}	0.11 ± 0.01	0.11 ± 0.02
Cardiac fat (g)	C	†	0.07 ± 0.01
	E_{str}	0.05 ± 0.03	0.05 ± 0.01
	E_{cho}	0.14 ± 0.03	0.08 ± 0.02#
	E_{fos}	0.12 ± 0.04	0.07 ± 0.01#

Notes: Data are means ± standard deviation. Group C: control animals without steatosis receiving the standard diet without supplementation. Group E_{str}: animals with steatosis receiving the standard diet without supplementation. Group E_{cho}: animals with steatosis receiving the standard diet supplemented with choline. Group E_{fos}: animals with steatosis receiving the standard diet supplemented with FOS. †Control animals were sacrificed only in end for data collection. *Significant difference compared to Group C (control). #Significant difference between the time points of the study (beginning and end).

Table 3. Oxidative stress marker values for the study groups at the beginning and end of the period of supplementation.

	GROUPS	BEGINNING	END
Hepatic vitamin E, α-tocopherol, (μmol/g tissue)	C	†	149 ± 90
	E_{str}	54 ± 14	249 ± 66#
	E_{cho}	96 ± 22	307 ± 82#,*
	E_{fos}	70 ± 6	217 ± 56#
TBARS (nmol/mg protein)	C	†	0.15 ± 0.021
	E_{str}	0.18 ± 0.029	0.18 ± 0.021
	E_{cho}	0.18 ± 0.027	0.18 ± 0.027
	E_{fos}	0.18 ± 0.013	0.17 ± 0.028
GSH (μmol/g protein)	C	†	33 ± 6
	E_{str}	23 ± 7	40 ± 8#
	E_{cho}	22 ± 1	46 ± 13#,*
	E_{fos}	25 ± 7	38 ± 14#

Notes: Data are means ± standard deviation. TBARS: thiobarbituric acid reactive substances. GSH: reduced glutathione. Group C: control animals without steatosis receiving the standard diet without supplementation. Group E_{str}: animals with steatosis receiving the standard diet without supplementation. Group E_{cho}: animals with steatosis receiving the standard diet supplemented with choline. Group E_{fos}: animals with steatosis receiving the standard diet supplemented with FOS. †Control animals were sacrificed only in end for data collection. *Significant difference compared to Group C (control). #Significant difference between the time points of the study (beginning and end).

lipid vacuoles.[25] Even though the amount of carbohydrate used in the present study is higher than the 50% sucrose diet, it is known to induce HS and is the amount found in the "western diet".[23,25]

Moreover, we showed that treatment with choline or FOS equally decreased the amount of liver and heart fat content. However, it did not reduce oxidative stress as indirectly measured by TBARS. Our data is in accordance with the literature, where it has been shown that FOS[39] or choline[40] reduces HS in rats. However, different from our protocol, the former study used a high fat diet to induce steatosis,[39] and suggested that the FOS supplementation could lead to epigenetic modifications because of the high fat supply. This could also be the case in the present work, where a high carbon diet also led to epigenetic modification.

In all the experimental groups, we tested the removal of the main harmful ingredient, ie, the rich carbohydrate diet was removed. This reflects the first step in counseling an obese patient. Animals in the experimental group consumed less food than the controls, but despite this, all animals gained a similar amount of weight during the experiment. Weight gain may be influenced by the HS, possibly its inflammatory state.[25,30] During steatosis induction, there are low amounts of choline, and this could aggravate the inflammatory process.[25] Low amounts of choline in maternal milk are also associated with inflammation.[41] This way, HS induction together with low levels of choline could lead to a major and nonspecific inflammatory process. Although this was not the aim of this study, we hypothesize increased inflammatory stress and high oxidative stress in this HS model.

Choline supplementation led to higher hepatic concentrations of vitamin E and GSH. Our finding supports studies that show a correlation between choline deficiency and low hepatic levels of vitamin E and GSH.[42] Male rats were used in the present study, but others have demonstrated that obese female rats exposed to similar conditions develop HS more frequently than male rats.[43] However, no differences were found for HS induction between male and female mice in models with methionine- or choline-deficient diet.[43]

We also demonstrated that supplementation with choline led to significant decreases of plasma TG compared to

control. Choline supplementation in combination with caffeine and carnitine has been shown to promote a reduction of serum TG levels and an increase in TG concentration in muscles.[44] Diabetic rats treated with choline did not decrease plasma fatty acids, suggesting that choline supplementation might not be indicated for serum TG reduction in diabetes.[45] We also showed that FOS was able to significantly reduce cholesterolemia compared to basal values. This effect has been previously reported and explained by an increase in steroids and lipid fecal excretion.[46] The literature shows that FOS also decreases serum TG levels;[10] however, we were unable to see a decrease in TG levels. FOS supplementation effects can be partially explained by modifications that FOS impose upon the intestinal microbiota.[9]

Animals supplemented with choline and FOS excreted smaller amounts of urinary nitrogen. However, a previous study showed that these nutrients do not change protein metabolism.[47] However, an increase in urinary nitrogen excretion was expected in choline-supplemented group because it is a nitrogen product, but this increase was not detected. Furthermore, an increase in urinary ammonia occurred only in the group supplemented with choline. This finding may be explained by the choline degradation pathway itself, which forms ammonia as an intermediate product.

The present study has some limitations inherent to the use of animal models for the study of NAFLD. We used a rat strain with no genetic modifications and a diet prepared using natural ingredients. We also used only TBARS as a stand-in for oxidative stress, and did not perform any measure of inflammation. In animal models of insulin resistance, an increased intake of fatty acids may lead to the development of NAFLD similar to that observed in humans. Since we induced HS with a high carbohydrate diet, further studies are needed to determine whether choline and FOS could have different effects in other experimental models of HS. Therefore, it is important to establish the beneficial effects and the appropriate amount of choline and FOS intake in order to avoid risks of toxicity or undesirable side effects.

Conclusions

Our findings confirm that controlled diet, choline, and FOS were equally effective in reducing hepatic steatosis.

Choline supplementation increased the hepatic levels of vitamin E and GSH, while FOS supplementation reduced serum cholesterol levels compared to basal levels.

Supplementation with choline or FOS did not modify TBARS levels. Clinical studies on humans should be carried out regarding the use of choline or FOS in cases of clinical NAFLD.

Author Contributions

Conceived and designed the experiments: NJBGBH, JSM, HV. Analyzed the data: NJBGBH, SFCC, VMMS, CMMMA, JFMM, HV. Wrote the first draft of the manuscript: NJBGBH, JSM. Contributed to the writing of the manuscript: SFCC, VMMS, GJP, AAJJ, CMMMA, JFMM, HV. Agree with manuscript results and conclusions: NJBGBH, JSM, SFCC, VMMS, GJP, AAJJ, CMMMA, JFMM, HV. Jointly developed the structure and argument for the paper: NJBGBH, JSM, SFCC, VMMS, GJP, AAJJ, CMMMA, JFMM, HV. Made critical revisions and approved final version: NJBGBH, JSM, SFCC, VMMS, GJP, AAJJ, CMMMA, JFMM, HV. All authors reviewed and approved of the final manuscript.

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