Histopathological and biochemical changes in the development of non-alcoholic fatty liver disease induced by high sucrose diet at different times

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Histopathological and biochemical changes in the development of non-alcoholic fatty liver disease induced by high sucrose diet at different times

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Running title: Changes in the development of NAFLD related to sucrose ingestion.

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Abstract

The high intake of sweetened-drinks is associated with obesity and insulin resistance. These pathologies are directly related to the development of non-alcoholic fatty liver disease (NAFLD), considered a condition of metabolic syndrome (MS). Due to their increasing worldwide prevalence, experimental animal models have been developed to gain a better understanding of its physiopathology; notwithstanding few studies have evaluated its progression in association with MS and ingestion of sweetened-drinks. Therefore, the aim of this study was to understand the pathophysiologic characteristics of NAFLD related to sucrose concentration and time of ingestion in rats. Wistar rats were divided into two groups with free access to either tap water or 30% sucrose, and euthanized at 12, 16 or 20 weeks; and other two groups with free access to 40% or 50% sucrose and were euthanized at 20 weeks. Biochemical parameters, serum cytokines and histology were performed. Ingestion of 30% sucrose induced liver steatosis until 16 weeks (grade 2), and 20 weeks (grade 3). While during 20 weeks, 40% sucrose induced grade 5 of non-alcoholic steatohepatitis (NASH), finally 50% sucrose induced grade 6 of NASH and fibrosis. Demonstrating that the more time of induction and concentration of sucrose ingestion, the higher grade of NAFLD.

Keywords: High sucrose, metabolic syndrome, NAFLD, NASH, rat model.
Introduction

The development of obesity and diabetes mellitus (DM) has been associated to high consumption of sugary drinks (ENSA0UT-MC 2016; Pandit et al. 2012; WHO 2016). Indeed, there is a direct relationship between the presence of obesity, insulin resistance or type 2 DM; and the development of non-alcoholic fatty liver disease (NAFLD). NAFLD: (1) is characterized by lipid accumulation in hepatocytes (>5%) (Dietrich and Hellerbrand 2014), (2) is considered the hepatic affection of metabolic syndrome (MS), and (3) promotes from progression of simple liver steatosis to non–alcoholic steatohepatitis (NASH) and, in more severe cases, to liver fibrosis, cirrhosis, and hepatocellular carcinoma (Dietrich and Hellerbrand 2014; Sanches et al. 2015).

Due to the increasing worldwide prevalence of NAFLD (Bellentani 2017), there is a growing interest in its study. Therefore, several animal models have been developed to induce this condition, with the purpose of studying the progression of the disease or to use them as experimental models to evaluate pharmacological therapies.

In this regard, several in vivo models have been developed, which can be divided into two groups: (1) those which have spontaneous or induced genetic modification; and (2) those induced by nutritional changes using different diets, (Cole et al. 2018; Jacobs et al. 2016; Sanches et al. 2015). However, there are few studies which evaluate the progression of NAFLD associated with metabolic syndrome (MS) and sugary drink ingestion.

Considering the aforementioned, we hypothesized that the consumption of water with high sucrose will allow the gradual development of NAFLD in male Wistar rats, with a greater degree of development of the disease depending on the time or water sucrose concentration consumed by animals. Therefore, the primary aim of this study was to determine the histopathological and
biochemical changes in the development of NAFLD induced by sucrose ingestion, associated with the presence of obesity and glucose intolerance. As secondary aims were: (1) to determine the influence of 30% sucrose ingestion during 12, 16 and 20 weeks, on body weight, food intake, triglycerides (TG), total cholesterol (TC), glucose intolerance, liver function, and the degree of hepatic steatosis or fibrosis in different times of induction of the disease; and (2) to determine the influence of 40% and 50% sucrose ingestion during 20 weeks in the same parameters.

Materials and methods

Ethical approval

The experimental protocol was approved by “Comité de Bioética de la Facultad de Ciencias Químico Biológicas” on June 1st of 2016. All animal procedures and protocols in the present investigation were followed by the regulation established by the Mexican Official Norm of the Use and Welfare of Laboratory Animals (NOM-062-ZOO-1999), and in accordance with the Guide to the Care and Use of Experimental Animals from the Canadian Council on Animal Care (CCAC).

Animals

Male Wistar rats of 80±10g (four weeks old, n=48) were used. Male Wistar rats were donated by the animal research facility located at Cinvestav Sede Sur. The animals were housed in plastic cages in a special temperature-controlled room (22±2°C, 50% humidity) on a 12/12 h light/dark cycle (with light beginning at 7:00 a.m.), with food (Purina Lab Diet 5012) and water freely available.

48 animals were divided into 4 groups. The first group (n=18) received tap water and the animals were euthanized after, 12 (n=6), 16 (n=6) or 20 (n=6) weeks of treatment. The second group (n=18) received 30% sucrose (wt/vol) and the animals were euthanized after 12 (n=6), 16 (n=6)
and 20 (n=6) weeks of treatment. The third and fourth group (n=6 each) received respectively 40% and 50% sucrose (wt/vol) and the animals were euthanized after 20 weeks of treatment. Body weight, food intake and oral glucose tolerance test (OGTT) were performed weekly. OGTT started four weeks prior to euthanasia and finished one day prior to euthanasia, to determine changes in glucose homeostasis. Next, the animals were euthanized, and blood samples, hepatic and adipose tissue were collected. Finally, the percentage by weight of liver and adipose tissue, as well as, serum TG, TC, alkaline phosphatase (ALP), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were determined. Besides, the liver histology stained with hematoxylin and eosin (H&E) and Masson trichrome (MT) was obtained (Fig. 1).

**Induction of obesity and glucose intolerance**

As previously reported (Balderas-Villalobos et al. 2013), the animal had *ad libitum* access to water with 30% sucrose for a period of 12, 16 or 20 weeks, and water with 40% and 50% sucrose for a period of 20 weeks. Each group at different times had a control group that received tap water *ad libitum* (Fig. 1).

**Assessment of glucose homeostasis**

Glucose homeostasis was evaluated with Oral Glucose Tolerance Test every week (starting four weeks before euthanasia, and ending one day before euthanasia). For this purpose, glycaemia was measured after 12 h fasting (time 0), 30, 60, 120 and 240 min after glucose administration (2 g/kg, p.o.) (Figure 1). Blood samples were collected from the tail. Glucose was measured using test strips and a glucometer (Accu-Chek Performa®, Roche of Mexico).

**Assessment of biochemical parameters for dyslipidemias**
After animals were euthanized adipose tissue from peritoneal cavity was excised and weighed, then intraabdominal fat was calculated using the next formula \[\left(\frac{\text{g tissue}}{\text{g body weight}}\right) \times 100\]. Furthermore, serum TG and TC were measured at the end of the study, by colorimetric assay using a commercial kit (TG Color GPO/PAP AA or Colestat enzymatic AA, Wiener Laboratories, Rosario, Argentina) following manufacturer instructions, using a plate reader (EliRead) at 37°C.

**Assessment of liver damage**

As a measurement of liver damage, after animals were euthanized the liver was removed and weighed \[\left(\frac{\text{g tissue}}{\text{g body weight}}\right) \times 100\]. Additionally, serum ALP, AST, and ALT activity were quantified at the end of the study (Fig. 1). ALP was quantified by enzymatic colorimetric assay using a commercial kit (ALP 405 AA, Wiener Laboratories, Rosario, Argentina) following manufacturer instructions; using plate reader (EliRead) at 37°C. Whereas, AST and ALT were quantified by enzymatic colorimetric assay using a commercial kit (GPT AA and GTO AA, Wiener Laboratories, Rosario, Argentina) following manufacturer instructions and; using a spectrophotometer with 340 nm filter (Spectronic Genesys 5) at 37°C. Serum values of ALP, AST and ALT are expressed as IU/L.

**Histopathological analysis**

After euthanasia, the liver was excised and one of the lobes was immersed in buffered formalin: formaldehyde 10% 100mL/L (J.T. Baker), NaH₂PO₄ 4g/L (Vetec), Na₂HPO₄ 6.5 g/L (Fermont); pH=7.4. Tissue sections were cut into pieces of 0.5 cm x 2.0 cm, were paraffin embedded (Leica Paraplast). Subsequently, the samples were cut using a microtome (Leica RM2125 RTS) with a thickness of 5-7 μm, placed on two slides per each one and stained with H&E or MT.
The determination of NAFLD degree in its development (from 0 to 8) was made according to the histological score system of the clinical research network of NASH (LaBrecque et al. 2012). This procedure was performed by counting the number of hepatocytes without steatosis, with steatosis and ballooning cells present in 50 fields in samples stained with H&E, analyzed at 40X. Fibrosis was determined independently (F0 to F4) (Diehl and Day 2017); using samples stained with MT and analyzed at 40X.

Assessment of serum cytokines

In order to prove an inflammatory process, serum interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α) and transforming grow factor beta (TGF-β) were analyzed using rat ELISA kits. The ELISA kits were obtained and used in accordance with directions of the manufacturer Elabscience (Houston, TX, USA).

Statistical analysis

The acquired values were subjected to statistical analysis and were expressed as the mean ± SD, and the number of animals was represented by n. In accordance with the normality of the data, an analysis of variance (ANOVA) was performed; then the differences among the changes in all the analyzed parameters were evaluated using Tukey post–hoc test. A p-value <0.05 was considered statistically significant. The results were analyzed using the statistical software Sigma plot, version 12.0 and were designed using GraphPad Prism version 5.0.

Results

Effect of sucrose ingestion on food intake and body weight

As shown in figure 2, 30% sucrose ingestion during 12 (395±53.7 g), 16 (369.6±66.5 g) and 20 (328.3±50.9 g) produced significant decrease (p<0.001, each; Fig. 2A) on mean weekly food intake from the second week to the last compared with all control group, by 12 (936.2±49.4 g), 16 (865±55.8 g), and 20 (963.6±74.1 g) weeks. In addition, the effect of 30% (328.3±50.9 g),
40% (573.1±45.5 g) and 50% (424.3±50.6 g) sucrose ingestion during 20 weeks was determined, noticing that the three concentrations of sucrose significantly decreased food intake compared to their respective control group (963.6±74.1 g) ($p<0.001$ each; Fig. 2B). Food intake in the group with 40% sucrose ingestion was higher than the group with 30% (573.1±45.5 vs 328.3±50.9 g; $p<0.001$) and with 50% (424.3±50.6 vs 328.3±50.9 g; $p<0.001$); while food intake in the group with 50% sucrose ingestion was higher than the group with 30% (424.3±50.6 vs 328.3±50.9 g; $p<0.001$).

Despite the decrease in food consumption, 30% sucrose ingestion did not modify body weight during 12 ($p=0.973$; Fig. 2C), 16 ($p=0.459$; Fig. 2D) and 20 ($p=0.594$; Fig. 2E) weeks, compared with its respective control groups. In contrast, 40% sucrose ingestion during 20 weeks increased significantly body weight from the week 9 to 20 ($p<0.05$; figure 2E) compared with its control group. Besides animals which consumed 40% sucrose had higher body weight from week 6 to 20 ($p<0.05$; figure 2E) compared with the group with 30% sucrose ingestion, and from week 6 to 17 ($p<0.05$; figure 2E) compared with the group with 50% sucrose ingestion. Finally, animals which consumed 50% sucrose had higher body weight from week 16 to 20 ($p<0.05$; figure 2E) compared with the control group, and from week 13 to 20 ($p<0.05$; Fig. 2E) compared with the group with 30% sucrose ingestion. Taken together, these results suggest that high sucrose ingestion, in addition to the ingestion of other nutritional components found in solid food is required to observe an increase in body weight.

Effect of sucrose ingestion on oral glucose tolerance

Groups that consume sucrose at 30% during twelve weeks (Fig. 3A–E) increased significantly blood glucose levels, at min 30 and 60, at week eight ($p<0.01$; Fig. 3A), nine ($p<0.01$; Fig. 3B) and ten ($p<0.01$; Fig. 3C) compared with the control group. While 30% sucrose ingestion only
increased blood glucose level, at min 30, at week eleven \((p<0.001; \text{Fig. 3D})\) and twelve
\((p<0.001; \text{Fig. 3E})\) compared with the control group.

Otherwise, regarding the groups that consume sucrose at 30% during sixteen weeks (Fig. 3F–J),
it increased significantly blood glucose levels, at min 30 at week twelve \((p<0.05; \text{Fig. 3F})\) and at
min 60 at week fourteen \((p<0.05; \text{Fig. 3H})\) compared with control group; and diminished at min
120 in both weeks (Fig. 3F, 3H). While sucrose ingestion did not induce changes on blood
glucose levels, at week thirteen \((p=0.102; \text{Fig. 3G})\), fifteen \((p=0.107; \text{Fig. 3I})\) and sixteen
\((p=0.302; \text{Fig. 3J})\) compared with the control group.

On the other hand, considering the groups that consumed 30%, 40% and 50% sucrose during
twenty weeks (Fig. 3K-O), we observed that 30% sucrose ingestion significantly increased blood
glucose levels, at min 30, 60 and 120 at week sixteen \((p<0.05; \text{Fig. 3K})\) and at min 60 at week
twenty \((p=0.035; \text{Fig. 3O})\). However, at weeks seventeen, eighteen and nineteen 30% sucrose
ingestion did not induce changes in blood glucose levels (Fig. 3L, 3M, 3N; respectively)
compared with the control group. While 40% sucrose ingestion significantly increased blood
glucose levels, at min 60 at week sixteen \((p<0.05; \text{Fig. 3K})\); at min 30 and 60 at week seventeen
\((p<0.05; \text{Fig. 3L})\); at min 60 and 120 at week eighteen \((p<0.05; \text{Fig. 3M})\); at min 30 at week
nineteen \((p=0.033; \text{Fig. 3N})\) and at min 60 at week twenty \((p<0.001; \text{Fig. 3O})\) compared with the
control group. Compared with 50% sucrose, 40% sucrose ingestion increased significantly blood
glucose levels at min 30 and 60 at week seventeen \((p<0.05; \text{Fig. 3L})\) and at min 30 at week
twenty \((p=0.019; \text{Fig. 3O})\).

Finally, 50% sucrose ingestion significantly increased blood glucose levels at min 30 at week
sixteen \((p<0.001; \text{Fig. 3K})\) at min 120 at week eighteen \((p<0.001; \text{Fig. 3M})\) and twenty \((p=0.025;\)
Fig. 3O) compared with control group. Taken together, these results suggest that sucrose
ingestion will exert a greater glucose intolerance at longer time of ingestion; and at higher concentration of ingested sucrose.

Effect of sucrose ingestion on percentage in liver weight and intraabdominal fat

The ingestion of sucrose at 30% failed to modify the percentage in liver weight ($p=0.099$; Fig. 4A) in any of the checkpoints, compared with each respective control group ($2.8\pm0.2$ vs $2.6\pm0.2$ (12 weeks), $2.5\pm0.1$ vs $2.6\pm0.2$ (16 weeks) and $2.6\pm0.1$ vs $2.5\pm0.2$% (20 weeks). On the other hand, 40% sucrose ($2.8\pm0.2$ vs $2.5\pm0.2$%; $p=0.044$; Fig. 4E) and 50% sucrose ($2.8\pm0.2$ vs $2.5\pm0.2$%; $p=0.014$; Fig. 4E) ingestion significantly increased the percentage in liver weight compared with group that consumed 30% sucrose. It is noted that the higher sucrose ingestion, the greater percentage in liver weight.

In contrast, 30% sucrose ingestion during twelve and sixteen weeks; both significantly increased the percentage of intraabdominal fat compared with their control groups ($5.4\pm0.8$ vs $4.2\pm1.2$% and $5.2\pm1.6$ vs $3.0\pm0.6$%, respectively $p<0.05$; Fig. 4B) and 30% sucrose ingestion during twenty weeks significantly increased the percentage of intraabdominal fat compared with control groups of all check points ($6.7\pm1.5$ vs $4.2\pm1.2$ (12 weeks), $3.0\pm0.6$ (16 weeks) and vs $1.4\pm0.9$ % (20 weeks); $p<0.001$; Fig. 4B). These results suggest that the longer consumption of 30% sucrose ingestion the greater increase in the percentage of intraabdominal fat. Besides, when the sucrose ingestion is compared at different concentrations with control group of twenty weeks, it was observed that all sucrose concentrations, increased significantly the percentage of intraabdominal fat compared with control group ($1.4\pm0.9$ vs $6.7\pm1.5$ (30%), vs $6.2\pm1.5$ (40%) and vs $5.7\pm1.6$ % (50%); $p<0.001$; Fig. 4F), suggesting that regardless of the concentration of sucrose ingestion during twenty weeks, it will be a similar effect on the percentage increase of intraabdominal fat compared with control group.
Effect of sucrose ingestion on biochemical parameters for dyslipidemias

The intake of sucrose at 30% significantly increased TG serum levels at week sixteen (112.1±28.8 mg/dL; p<0.05; Fig. 4C) and twenty (146.1±18.9 mg/dL; p<0.001; Fig. 4C) compared with all control groups (61.4±27.6 (12 weeks), 70.4±11.7 (16 weeks), 62.2±14.9 mg/dL (20 weeks)), besides 30% sucrose ingestion during twenty weeks significantly increased TG (146.1±18.9 vs 97.3±30.2 mg/dL; p=0.012; Fig. 4C) compared with the group that consumed 30% sucrose during twelve weeks. Otherwise, TG serum levels of 30% sucrose ingestion during twenty weeks were significantly higher than 40% sucrose (146.1±18.9 vs 93.0±31.3 mg/dL; p=0.001; Fig. 4G) and 50% sucrose (146.1±18.9 vs 95.9±11.7 mg/dL; p=0.002; Fig. 4G) ingestion during twenty weeks, while 50% sucrose ingestion during twenty weeks significantly increased TG (95.9±11.7 vs 62.2±14.9 mg/dL; p=0.047; Fig. 4G) compared with its control group.

In contrast, 30% sucrose ingestion failed to modify TC serum levels in any of the evaluated time (p=0.229; Fig. 4D) compared with any of the other groups. However, 50% sucrose ingestion during 20 weeks significantly increased TC (69.1±16.9 vs 49.5±11.4 mg/dL; p=0.04; Fig. 4H) compared the group of 40% sucrose ingestion.

Our results suggest that an increase of 30% sucrose ingestion on time is reflected in an increase in TG serum levels, without changes on TC serum levels. However, an increase in the concentration of sucrose ingestion apparently causes a decrease in body lipid accumulation, due to a decrease in TG serum levels; except that there is an increase in TC. This probably may be due to a change on lipid distribution in the body.

Effect of sucrose ingestion on hepatic enzymes
We found that ALP, AST and ALT serum levels of controls groups were influenced by age (Fig. 5A-C); noticing that ALP serum levels of control group were significantly diminished at week 20 compared with control group of 16 weeks (95.4±16.7 vs 155.9±20.9 IU/L; \( p=0.008 \); Fig. 5A); AST serum levels diminished throughout evaluation time (Fig. 5B), while ALT increased (Fig. 5C).

Moreover, 30% sucrose ingestion showed a tendency to increase ALP levels, although they were not significantly different in any of the evaluated times; that is, at week 12 (180.7±31.2 vs 132.8±38.6 IU/L; \( p=0.053 \); Fig. 5A); 16 (159.8±28.8 vs 155.9±20.9 IU/L; \( p=1.0 \); Fig. 5A) and 20 (142.4±23.0 vs 95.4±16.7 IU/L; \( p=0.060 \); Fig. 5A) compared with each control group. While ALP serum levels of the control group at week 20 were significantly lower compared with the group with 30% sucrose ingestion during 12 (95.4±16.7 vs 180.7±31.2 IU/L; \( p<0.001 \); Fig. 5A) and 16 (95.4±16.7 vs 159.8±28.8 IU/L \( p=0.004 \); Fig. 5A) weeks. However, when compared the effect of all sucrose concentrations during twenty weeks, it was observed that 30% sucrose ingestion increased significantly ALP levels compared with the control group (142.4±23.0 vs 95.4±16.7 IU/L; \( p=0.042 \); Fig. 5D).

In contrast, 30% sucrose ingestion decreased significantly AST serum levels (\( p<0.01 \); Fig. 5B) at week 12 (28.2±13.2 IU/L) compared with control group of 12 (61.5±20.3 IU/L) and 16 (59.1±11.0 IU/L) weeks; at week 16 (20.3±10.0 IU/L) compared with control group of 12, 16 (\( p<0.001 \); Fig. 5B) and 20 (26.0±6.6 vs 45±7.3 IU/L; \( p<0.05 \); Fig. 5B) weeks; and at week 20 (26.0±6.6 IU/L) compared with control group of 12 and 16 weeks (\( p<0.001 \); Fig. 5B). When the effect of 30%, 40% and 50% sucrose ingestion during twenty weeks were compared each other and with control group, data showed that 30% sucrose ingestion diminished significantly AST serum levels (\( p=0.001 \); Fig. 5E) compared with control group (26.0±6.6 vs 45±7.3 IU/L),
however they were significantly lower than AST serum levels on groups that consumed 40% sucrose (26.0±6.6 vs 44.6±7.4 IU/L; \( p=0.001 \); Fig. 5E) and 50% sucrose (26.0±6.6 vs 48.1±7.7 IU/L; \( p<0.001 \); Fig. 5E).

Lastly, 30% sucrose ingestion decreased ALT serum levels compared with each control group, however, this decrease was not statistically significant in any of the evaluated times; that is, at week 12 (7.2±3.4 vs 11.2±2.1 IU/L; \( p=0.465 \); Fig. 5C), 16 (7.2±4.1 vs 13.5±3.7 IU/L; \( p=0.074 \); Fig. 5C) and 20 (12.6±3.5 vs 15.1±5.3 IU/L; \( p=0.860 \); Fig. 5C) compared with each control group, but ALT serum levels of control group of 20 weeks were significantly higher than 30% sucrose ingestion during 12 (15.1±5.3 vs 7.2±3.4 IU/L; \( p<0.05 \); Fig. 5C) and 16 (15.1±5.3 vs 7.2±4.1 IU/L; \( p<0.05 \); Fig. 5C) weeks. When the comparison among different concentrations of sucrose ingestion was made, despite observing a tendency to increase with higher concentration sucrose ingestion, the ALT serum levels were not significantly different comparing each other \( (p=0.344 \); Fig. 5F).

**Histopathological changes induced by sucrose ingestion**

The group with 30% sucrose ingestion during 12 weeks (Fig. 6D-F) showed similar characteristics to those presented by the control group (Fig. 6A-C); those were, normal hepatocyte architecture was presented in parenchyma (Fig. 6A, 6D) with absence of steatosis (<5%) and inflammatory infiltrate; central veins (Fig. 6B, E) and portal structures (Fig. 6C, F). While, the group that intake 30% sucrose during 16 weeks (Fig. 6G-I) showed in hepatic parenchyma (Fig. 6G) and around central veins (Fig. 6H) moderate micro-vesicular steatosis (mean of 45.7%), with absence of inflammatory infiltrate; positioning it in grade 2 in the development of NAFLD (LaBrecque et al. 2012).
Besides, the group that intake sucrose at 30% during 20 weeks (Fig. 6J–L) showed in hepatic parenchyma (Fig. 6J) severe micro-vesicular steatosis (69.7%) and limited macro-vesicular steatosis (0.6%); in central veins (Fig. 6K) and portal structures (Fig. 6L) limited micro-vesicular steatosis, with absence of inflammatory infiltrate; with a steatosis mean of 70.3% (grade 3).

On the other hand, the group with 40% sucrose ingestion during 20 weeks (Fig. 6M-O) showed in hepatic parenchyma (Fig. 6M) severe micro–vesicular steatosis (71.7%) and limited macro-vesicular steatosis (5.8%), in central veins (Fig. 6N) and portal structures (Fig. 6O) moderate micro–vesicular steatosis, with a steatosis mean of 77.5%; also this group showed one ballooned cell per each field and one inflammatory infiltrate (grade 5). Finally, the group with 50% sucrose ingestion during 20 weeks (Fig. 6P-R) showed in hepatic parenchyma (Fig. 6P) severe micro–vesicular steatosis (70.5%) and moderate macro-vesicular steatosis (21.8%), in central vein (Fig. 6Q) and portal structures (Fig. 6R) moderate macro-vesicular steatosis, with a steatosis mean of 92.3%; two ballooned cell per each field and one inflammatory infiltrate (grade 6). It could be considered that the last two groups developed NASH, according to the histopathological findings. The group with 50% sucrose ingestion developed the highest degree of NAFLD.

When all samples were stained with MT, it became evident that there were no changes in the amount and distribution of collagen in liver histology in groups with 30% (Fig. 7D-L) and 40% (Fig. 7M-O) sucrose ingestion compared with control group (Fig. 7A-C). That is, all these groups showed the presence of collagen fibers in portal structures and large hepatic veins in a normal way, without showing significant accumulation in the hepatic parenchyma (F0). While the group with 50% sucrose ingestion (Fig. 7P-Q) was the only which developed portal fibrosis with few septa (F2).

Effect of sucrose ingestion on serum cytokines
The ingestion of 30% sucrose failed to modify IL-6 ($p=0.034$; Fig. 8A), TNF-α ($p=0.291$; Fig. 8B) and TGF-β ($p=0.121$; Fig. 8C) serum levels in any of the evaluated times compared with any of the other groups; with the exception of 30% sucrose ingestion during twenty weeks significantly increased serum levels of IL-6 compared with 30% sucrose ingestion during twelve weeks ($p=0.036$; Fig. 8A).

On the other hand, considering the groups that consumed 30%, 40% and 50% sucrose during twenty weeks (Fig. 8D-F), we observed that 40% sucrose ingestion significantly increased serum levels of IL-6 ($p<0.001$; Fig. 8C) and TNF-α ($p<0.001$; Fig. 8D) compared with control group and with group that consumed 30% sucrose. While 50% sucrose ingestion significantly increased serum levels of IL-6 compared with control group ($p<0.001$; Fig. 8C), and with the groups that consumed 30% sucrose ($p<0.001$; Fig. 8C) and 40% sucrose ($p=0.018$; Fig. 8C). Also, 50% sucrose ingestion significantly increased serum levels of TNF-α ($p<0.001$; Fig. 8D) and TGF-β ($p<0.001$; Fig. 8F) compared with control group, and with the groups that consumed 40% and 30% sucrose.

**Discussion**

Since the consumption of 30% sucrose for 16 or 18 weeks in male Wistar rats is an established model of MS (Balderas-Villalobos et al. 2013; Lima-Mendoza et al. 2014) and that NAFLD is the hepatic condition in MS, here we evaluated the effect of different concentrations of sucrose ingestion during different times on the development of NAFLD.

*Effect of sucrose ingestion on macroscopic parameters*

All sucrose concentrations during twenty weeks decreased significantly food intake in all checkpoints compared with their respective control group, this observation is similar to other reports (Lima-Mendoza et al. 2014; Packard et al. 2014). The above is attributed to the
consumption of drinks high in simple carbohydrates (such sucrose) and with sweet taste reduces significantly appetite and increase satiety (Kilpatrick et al. 2014), leading to a decrease in the ingestion of other nutritional sources found in solid food. Although we expected that the decrease in food intake was directly proportional to the concentration of ingested sucrose, our results showed a lower food intake on the group with 30% sucrose ingestion compared with 40% and 50%.

Despite the decrease in food consumption, 30% sucrose ingestion did not modify body weight, as previously demonstrated (Lima-Mendoza et al. 2014). This could be caused because the lack of ingestion of solid food is replaced by the carbohydrates ingested.

Effect of sucrose ingestion on glucose levels

The effect of consumption of sucrose at 30%, 40% and 50% during 20 weeks, suggests that glucose intolerance developed, and indirectly, suggested insulin resistance. These results are similar with previous reports in Long-Evans rats, which consumed 30% sucrose or 0.1% saccharin for 21 days, (Packard et al. 2014) or in Zucker rats (Davidson et al. 2014), where they showed an increase of glucose levels on OGTT with glucose intolerance.

Effect of sucrose ingestion on lipid metabolism

Our results show that the ingestion of sucrose at 30%, 40% and 50% led to the development of obesity and hypertriglyceridemia, two medical conditions of MS. These results of intraabdominal fat (obesity) and TG are in agreement with other reports (Balderas-Villalobos et al. 2013; Marin et al. 2016). Total cholesterol serum levels did not change, which also coincides with several studies, in NAFLD models with a diet rich in fat, carbohydrates or both (Aoun et al. 2010; Lima et al. 2016). However, it has been seen that there are modifications on levels of low-density lipoproteins (LDL) and high-density lipoproteins (HDL) (Marin et al. 2016).
The intraabdominal fat increase has been associated with different biologic mechanism including: (i) ingestion of sweetened drinks causes excess caloric intake; (ii) sweet liquids cause less thermogenesis leading to an increase in positive energy balance and as a consequence to an increase in adipose tissue; and (iii) intake of liquids with sucrose can lead to the development of insulin resistance and with this favors the increase of body weight and waist circumference which can lead to the development of obesity (Odegaard et al. 2012; Olsen et al. 2012).

Otherwise, it has been suggested that high sucrose ingestion affects negatively the balance of free radical production and antioxidant defense, which in turn leads to a higher susceptibility to peroxidation of lipids, causing damage to the organism (Busserolles et al. 2002).

**Effect of sucrose ingestion on liver damage**

In the present study, sucrose ingestion did not modify the percentage in liver weight in comparison to controls, although 40% and 50% sucrose ingestion increased significantly the percentage in liver weight compared with 30%. On the other hand, 30% sucrose ingestion during 20 weeks significantly increased ALP compared with control group. In contrast 40% and 50% sucrose ingestion did not modify statistically ALP, although they showed a tendency to increase when compared with the control group. In this respect, previous findings have demonstrated increases in ALP levels during the initial process of liver damage, although these increases were not statistically significant with respect to healthy group (Hall et al. 2017). Moreover, the fact that ALP levels decreased over the time in groups with sucrose at 30%, it might be due to a higher degree of malnutrition in animals as previously reported by Cho et al. (2007) in Sprague Dawley rats with a diet deficient in zinc.

An increase in serum levels of ALT and AST compared to healthy has been observed in murine models of NAFLD with severe damage (Marcolin et al. 2012; Rodriguez-Ramiro et al. 2016) or
clinical cases in humans (Hall et al. 2017), which have been attributed to the existence of
necrosis processes in hepatocytes which makes that enzymes be released into bloodstream. In
contrast, we found a decrease in transaminase levels in groups with 30% sucrose ingestion
compared with its respective control groups; but 40% and 50% sucrose ingestion increased
significantly AST, and a tendency to increase in serum levels of ALT compared with 30%
sucrose, during 20 weeks. Similarly, with that reported by Hall et al. (2017) who observed that
humans with early stages of NAFLD have decreased levels of transaminases and did not
distinguish and increase of them until the hepatic steatosis is >20%. We suggest, that the
decrease of transaminases could be, because in this rat model, liver damages is induced giving
water with sucrose leading to a lower solid food ingestion with the essential nutrients for its
development. In this regard, it has been reported that most forms of liver damage decrease
activity in the hepatocytes of the cytosolic and mitochondrial forms of AST and in alcoholic a
decrease in ALT activity is common; which is attributable to vitamin B6 (pyridoxal) deficiency
(Dufour 2005). In our study, no liver damage was induced with alcohol intake, however, the
decrease in solid food ingestion in rats reduced consequently the ingestion of pyridoxal in their
solid diet, leading to a deficiency of this vitamin and as consequence to a lower activity of
transaminases. In the case of 40% and 50% sucrose ingestion showed a tendency to increase
transaminase serum levels in a concentration-dependent manner; this could be because these
groups consumed more solid food and also had greater liver damage.

*Effect of sucrose ingestion on inflammatory process*

Our results on serum levels of cytokines (IL-6 and TNF-α) are associated to inflammatory
process in obesity (Basaranoglu et al. 2013) and from progression of simple liver steatosis to
NASH (Basaranoglu et al. 2013; Dietrich and Hellerbrand 2014; Sanches et al. 2015). Besides, it
is well known that TNF-\(\alpha\) increases endothelial permeability and the adherence of leukocytes to this monolayer by the induction of E-selectin, intracellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1); and also activates chemotaxis of leukocytes (Chimen et al. 2017). In turn, this is correlated with our histological samples, where the groups with 40% and 50% sucrose ingestion presented inflammatory infiltrates in liver and high levels of TNF-\(\alpha\).

Whereas the increase of TGF-\(\beta\) is related to the Kupffer cells activation, which is an important point to develop fibrosis (Chiang et al. 2011; Diehl and Day 2017; Peverill et al. 2014); as it was observed in the group with 50% sucrose ingestion.

**Possible mechanism of action of sucrose ingestion**

Overall, the results indicate that the 30%, 40% and 50% sucrose ingestion diminished the ingestion of food, because the sweet drinks and high concentrations of carbohydrates, acts on the central nervous system generating an increase in dopamine (Kampov-Polevoy et al. 2006) and in leptin (Kilpatrick et al. 2014; Pandit et al. 2012). This has been reported previously by different researchers (Castellanos Jankiewicz et al. 2015; Harris 2018; la Fleur et al. 2010) who observed an increase in leptin serum levels in Wistar rats as an effect of 30% sucrose ingestion, respectively. In spite of the fact that the leptin levels have been previously evaluated it would have been interesting to measure them in this study, however it was not possible and as a consequence it will be a perspective for future studies. Both effects induced by sweetened drinks resulting in an increase in satiety and hedonic responses to the sucrose ingestion (Figure 9). The diminish on food intake in the group with 50% sucrose ingestion compared with group with 40% sucrose ingestion, could be, because it has been seen that the higher concentration of sugar in
beverages, the higher hedonic responses, and as consequence leads to higher sucrose ingestion and less food intake.

The above mentioned, leads to a lower consumption of proteins, vitamins, and minerals, while the carbohydrates ingestion is higher; with the consequence that caloric intake is similar in the control group than of 30% sucrose, and therefore the animals body weight was similar; but caloric intake was higher in groups with 40% and 50% sucrose ingestion, therefore the body weight was higher in these groups compared with control group. Moreover, the lower solid food leads to deficiency of some nutrients and that might explain the decrease in ALT and AST levels in groups with 30% sucrose ingestion, with respect to the control group (Dufour 2005) (Figure 9).

It is important to mention that sucrose is a disaccharide composed of glucose and fructose that is cleaved by sucrase in the small intestine (Basaranoglu et al. 2013; Schultz et al. 2015). Therefore, we must consider the action of both monosaccharides. In this respect, it is well known that glucose is transported into cells by glucose transporter type-4 (GLUT-4), an insulin-dependent transport system; while fructose is transported into cells by carrier-mediated facilitated diffusion with GLUT-5. Conversely, fructose is metabolized principally by the liver and is exceptionally high compared to glucose; also its hepatic metabolism not only transforms it into glucose and stores it as glycogen faster than glucose, but also stimulates lipogenesis (Basaranoglu et al. 2015; Basaranoglu et al. 2013; Schultz et al. 2015).

By the way, sucrose ingestion increase the consumption of carbohydrates leading to hyperglycemia and the development of glucose intolerance, reported in our groups, and probably to insulin resistance. Insulin resistance produce a decreases in the effect of insulin on its receptor and leads to inhibition of substrate of insulin receptor, which consequently leads to an inhibition
in the metabolic pathway, as well as diminished of translocation of glucose transporter; in addition to an increase in \textit{de novo} lipogenesis. The increase of gluconeogenesis leads to an increase in glucose and, in turn, increases \textit{de novo} lipogenesis; both yield an increase in free fatty acids that are esterified as TG (Ahmed et al. 2015; Firneisz 2014), while fructose can induce lipogenesis in hepatocytes even before the development of insulin resistance (Schultz et al. 2015), leading to the development of liver steatosis, as was observed in the histology of the groups with 30\% sucrose ingestion during 16 and 20 weeks and 40\% and 50\% sucrose ingestion during 20 weeks. This also, can occur in other tissues, leading to an increase in visceral adipose tissue, an effect that was also reported in this study.

Fatty acids could lead to mitochondrial dysfunction, lipoperoxidation and reactive oxygen species (ROS) production; although it would have been interesting to measure ROS, it was not possible in this study, and as a consequence we suggest it as a perspective for future studies. These factors, in turn activate inflammation pathways, activating NF-κB and IKκB lead to the production of proinflammatory cytokines, chemotaxis and activation of Kupffer cells (Castro et al. 2014; López-Oliva and Muñoz-Martínez 2014) (Figure 9). This inflammatory process could be present in rats with 40\% and 50\% sucrose ingestion, due to the presence of inflammatory infiltrate cells in parenchyma and hepatic sinusoids and the increase in serum levels of IL-6 and TNF-α. Also, this liver damage, could be leading to an increase in serum levels of ALP, AST and ALT, but there are neutralized with the decrease produced due to nutrient deficiency, and this means that there were no differences when were compared with control levels.

On the other hand, it is known that activated Kupffer cells contributed to increase inflammation by the released of IL-6 and TNF-α and as consequence to necrosis of hepatocytes; also release ROS and TGF-β; as we observed an increase on serum levels of these cytokines. Therefore, ROS
and activated Kupffer cells could activate stellar liver cells, which undergo phenotypic
transdifferentiation to myofibroblast, taken together promote fibrosis (Chiang et al. 2011; Diehl
and Day 2017; Peverill et al. 2014). We suggest that not only this could be happening in the
group with 50% sucrose ingestion (because it was the only group with an increase in serum
levels of IL-6, TNF-α and TGF-β and with development of fibrosis), but also could be related
with our histological samples, where serum levels of IL-6 and TNF-α are directly proportional to
steatosis grade. The above mentioned is shown on figure 9.

Conclusion

This study demonstrates that sucrose ingestion in male Wistar rats could induce different grades
of the development of NAFLD, it is directly dependent from time of induction and concentration
of sucrose in water ingestion; where the highest time of induction by 30% sucrose ingestion
induce higher grade (grade 3) at 20 weeks than 12 or 16 weeks, leading to micro–vesicular
steatosis. While, 50% sucrose ingestion during 20 weeks induced the highest grade (grade 6) of
NAFLD of all of groups evaluated, allowing the development of NASH, with micro- and macro-
vesicular steatosis, ballooning cells, a little inflammatory infiltrate, and fibrosis (F2).

Competing interests

None declared.

Founding

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Author contributions

U.O. and E.A.: financial support; administrative support; final approval of manuscript. S.A.,
U.O. and E.A.: conception and design; analysis and interpretation of data; drafting of manuscript.
U.O.: critical revision; final approval of manuscript.

All authors have approved the final version of the manuscript and agree to be accountable for all
aspects of the work. All persons designated as authors qualify for authorship, and all those who
qualify for authorship are listed.

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Figure legends

**Figure 1. Experimental design.** All animals received standard diet *ad libitum* plus tap water (control group), 30%, 40% or 50% sucrose, respectively. OGGT was performed weekly starting four weeks before and finishing one day before the animals were euthanized. After animals were euthanized blood and tissue samples were obtained to make different assays. ALT, alanine aminotransferase; ALP, alkaline phosphatase, AST, aspartate aminotransferase; H&E, hematoxylin and eosin; %, percentage; MT, Masson trichrome; OGGT, oral glucose tolerance test; TC, total cholesterol; TG, triglycerides.

**Figure 2. Effect of sucrose consumption on food intake and body weight.** The upper panel shows the effect of 30% sucrose consumption on food intake in different times (A), and the effect of 30%, 40% and 50% sucrose consumption on food intake during 20 weeks (B). Each bar represents the mean±SD of n=6 each group, of weekly food intake. One way ANOVA and Tukey post–hoc test were performed. The bottom panel shows the effect of 30% sucrose consumption on body weight during 12 (C) and 16 weeks (D), and the effect of 30%, 40% and 50% sucrose consumption on body weigh during 20 weeks (E). Two way repeated measures ANOVA and Tukey post–hoc test were performed. *, p<0.05 vs control (12 weeks); #, p<0.05 vs control (16 weeks); †, p<0.05 vs control (20 weeks); &, p<0.05 vs 30% sucrose (12 weeks); $, p<0.05 vs 30% sucrose (16 weeks); ‡, p<0.05 vs 30% sucrose (20 weeks); ¡, p<0.05 vs 40% sucrose (20 weeks); ‡, p<0.05 vs 50% sucrose (20 weeks).
Figure 3. Temporal course of oral glucose tolerance test (OGTT) on groups that consumed sucrose at different times and concentrations. The upper panel shows OGTT from control group and group that 30% sucrose during 12 weeks (A-E). The middle panel shows OGTT from control group and group that consumed 30% sucrose during 16 weeks (F-J). The bottom panel shows OGTT from control group and groups that consumed 30%, 40% and 50% sucrose, during 20 weeks (K-O). Each point represents the mean±SD of n=6 each group, at 30, 60, 120 and 240 min when were determined blood glucose values after glucose administration (2 mg/kg, via p.o.). Time zero represents the basal values of fasting glucose. Two way repeated measures ANOVA and Tukey post–hoc test were performed. *, p<0.05 30% sucrose vs tap water; #, p<0.05 40% sucrose vs tap water; &, p<0.05 50% sucrose vs tap water; %, p<0.05 40% sucrose vs 50% sucrose.

Figure 4. Effect of sucrose ingestion on percentage in liver weight and on lipid metabolism. Upper panel shows the effect of 30% sucrose ingestion during 12, 16, and 20 weeks (A-D) and bottom panel shows the effect of 30%, 40% and 50% sucrose ingestion during 20 weeks (E-H), on percentage (%) in liver weight (A, E), % in adipose tissue weight (B, F), and serum levels of triglycerides (C, G) and total cholesterol (D, H). Each bar represents the mean±SD of n=6 each group, of each week. One way ANOVA and Tukey post–hoc test were performed. *, p<0.05 vs control (12 weeks); #, p<0.05 vs control (16 weeks); %, p<0.05 vs control (20 weeks); &, p<0.05 vs 30% sucrose (12 weeks); a, p<0.05 vs 30% sucrose (20 weeks); i, p<0.05 vs 40% sucrose (20 weeks); +, p<0.05 vs 50% sucrose (20 weeks).

Figure 5. Effect of sucrose ingestion on serum levels of hepatic enzymes. Upper panel shows the effect of 30% sucrose ingestion during 12, 16, and 20 weeks (A-C) and bottom panel shows the effect of 30%, 40% and 50% sucrose ingestion during 20 weeks (D-F), on serum levels of alkaline phosphatase (ALP) (A, D), aspartate aminotransferase (AST) (B, E) and alanine aminotransferase (ALT) (C, F). Each bar represents the mean±SD of n=6 each group, of each week.
week. One way ANOVA and Tukey post–hoc test were performed. *, p<0.05 vs control (12
weeks); #, p<0.05 vs control (16 weeks); %, p<0.05 vs control (20 weeks); i, p<0.05 vs 40%
sucrose (20 weeks); +, p<0.05 vs 50% sucrose (20 weeks).

**Figure 6. Effect of sucrose ingestion on hepatic tissue morphology.** Tissues stained with
hematoxylin and eosin (H&E) were observed at 40X. Hepatic parenchyma (A, D, G, J, M, P).
Central vein (B, E, H, K, N, Q). Portal structure (C, F, I, L, O, R). Control group, which
consumed tap water *ad libitum* (A-C). Group with 30% sucrose ingestion during 12 (D-F), 16
(G-I), and 20 (J-L) weeks. Group with 40% sucrose ingestion (M-O) and group with 50%
sucrose ingestion (P-R) during 20 weeks. The arrows point some hepatocytes with micro–
vesicular steatosis; the squares frame some hepatocytes with macro-vesicular steatosis and the
circles frame ballooning cells. In all pictures scale bar is 50 μm.

**Figure 7. Effect of sucrose ingestion on changes of collagen in hepatic tissue.** Tissues stained
with Masson trichrome (MT) were observed at 40X. Hepatic parenchyma (A, D, G, J, M, P).
Central vein (B, E, H, K, N, Q). Portal structure (C, F, I, L, O, R). Control group, which
consumed tap water *ad libitum* (A-C). Group with 30% sucrose ingestion during 12 (D-F), 16
(G-I), and 20 (J-L) weeks. Group with 40% sucrose ingestion (M-O) and group with 50%
sucrose ingestion (P-R) during 20 weeks. The arrows point fibrosis with septa. In all pictures
scale bar is 50 μm.

**Figure 8. Effect of sucrose ingestion on serum levels of interleukin 6 (IL-6), tumor necrosis
factor alpha (TNF-α) and transforming grow factor beta (TGF-β).** Upper panel shows the
effect of 30% sucrose ingestion during 12, 16, and 20 weeks (A-C) and bottom panel shows the
effect of 30%, 40% and 50% sucrose ingestion during 20 weeks (D-F), on serum levels of IL-6
(A, D), TNF-α (B, E) and TGF-β (C, F). Each bar represents the mean±SD of n=5 each group, of

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each week. One way ANOVA and Tukey post–hoc test were performed. &, \textit{p}<0.05 vs 30% sucrose (12 weeks); %, \textit{p}<0.05 vs control (20 weeks); \textit{a}, \textit{p}<0.05 vs 30% sucrose (20 weeks); \textit{i}, \textit{p}<0.05 vs 40% sucrose (20 weeks).

**Figure 9. Effect of 50% sucrose ingestion during 20 weeks.** The sucrose acts on central nervous system leading to a decrease of appetite and increase of satiety, at time, this has as consequence a higher intake of carbohydrates, and other nutrients intake deficiency. The high carbohydrate ingestion leads to an increase on adipose tissue; while nutrient deficiency could lead a decrease on serum levels of ALP, AST and ALT. Moreover, we suggest that chronic consumption of 50% sucrose leads to development of glucose intolerance and probably to insulin resistance, which leads to increase \textit{de novo} lipogenesis and with that an increase on FA that are esterified as TG and in liver bring to steatosis. This could induce mitochondrial dysfunction, lipoperoxidation and decrease in oxidative capacity, leading to an increase in ROS. These factors activate inflammation pathways, observed as an increase on IL-6 and TNF-\(\alpha\), Kupffer cells and stellar liver cells. Activated Kupffer cells contributed to increase inflammation (IL-6, TNF-\(\alpha\) and TGF-\(\beta\)) and necrosis; where this damage in liver might let increase the serum levels of hepatic enzymes, but that is neutralized with the decrease produced due to nutrients deficiency. Besides activated Kupffer cells can activate stellar liver cells, due to TGF–\(\beta\) and ROS, which are released, taken together promote fibrosis. ALT, alanine aminotransferase; ALP, alkaline phosphatase; AST; aspartate aminotransferase; FA, fatty acids; GLUT, glucose transporter; IRS, insulin receptor substrate; ROS; reactive oxygen species; TGF-\(\beta\), transforming growth factor beta; TG, triglycerides; TNF-\(\alpha\), tumor necrosis factor alpha.
Experimental design

182x115mm (300 x 300 DPI)
Effect of sucrose consumption on food intake and body weight

181x128mm (300 x 300 DPI)
Temporal course of oral glucose tolerance test (OGTT) on groups that consumed sucrose at different times and concentrations

181x138mm (300 x 300 DPI)
Effect of sucrose ingestion on percentage in liver weight and on lipid metabolism

181x110mm (300 x 300 DPI)
Effect of sucrose ingestion on serum levels of hepatic enzymes
Effect of sucrose ingestion on hepatic tissue morphology

170x236mm (300 x 300 DPI)
Effect of sucrose ingestion on changes of collagen in hepatic tissue

170x236mm (300 x 300 DPI)
Effect of sucrose ingestion on serum levels of interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α) and transforming growth factor beta (TGF-β)
Effect of 50% sucrose ingestion during 20 weeks

182x110mm (300 x 300 DPI)