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ARTICLE

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

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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 nonalcoholic 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 2 groups with free access to either tap water or 30% sucrose, and euthanized at 12, 16, or 20 weeks; and 2 additional groups were given free access to either 40% or 50% sucrose and were euthanized at 20 weeks. Biochemical parameters and levels of serum cytokines were measured, and histology was performed. Ingestion of 30% sucrose induced liver steatosis until 16 weeks (grade 2) and 20 weeks (grade 3). Meanwhile, during 20 weeks, 40% sucrose induced grade 5 of nonalcoholic steatohepatitis (NASH) and 50% sucrose induced grade 6 of NASH and fibrosis. This study demonstrated that increasing time of induction and concentration of sucrose ingestion resulted in a higher grade of NAFLD.

Key words: high sucrose, metabolic syndrome, NAFLD, NASH, rat model.

Résumé : La consommation élevée de boissons sucrées est associée à l'obésité et à la résistance à l'insuline. Ces pathologies sont directement liées à la présentation d'une surcharge en graisse du foie non alcoolique (ou NAFLD pour « nonalcoholic fatty liver disease »), état considéré comme faisant partie du syndrome métabolique (SM). Comme la prévalence de ces états est en croissance à l'échelle mondiale, des modèles expérimentaux animaux ont été mis au point en vue d'en mieux comprendre la physiopathologie; malgré tout, peu d'études ont porté sur son évolution en association avec le SM et l'ingestion de boissons sucrées. Par conséquent, cette étude avait pour but de comprendre les caractéristiques physiopathologiques de la NAFLD en lien avec la concentration de saccharose et la durée de l'ingestion chez le rat. Nous avons réparti des rats Wistar dans 2 groupes avec un accès libre à l'eau du robinet ou à de l'eau contenant du saccharose à 30 %, et nous les avons euthanasiés après 12, 16 ou 20 semaines; les rats de 2 autres groupes avaient accès à du saccharose à 40 ou à 50 % et étaient euthanasiés après 20 semaines. Nous avons mesuré des paramètres biochimiques et des taux de cytokines sériques, ainsi que procédé à une évaluation de l'histologie. L'ingestion de saccharose à 30 % engendrait une stéatose hépatique à 16 semaines (grade 2) et à 20 semaines (grade 3). Entre-temps, sur 20 semaines, le saccharose à 40 % entraînait une stéatohépatite non alcoolique (ou NASH pour « nonalcoholic steatohepatitis ») de grade 5, et le saccharose à 50 % entraînait une NASH de grade 6 avec de la fibrose. Cette étude montrait que le grade de la NAFLD augmentait en fonction de la durée de l'induction et de la teneur en saccharose de la boisson ingérée. [Traduit par la Rédaction]

Mots-clés : teneur élevée en saccharose, syndrome métabolique, NAFLD, NASH, modèle chez le rat.

Introduction

The development of obesity and diabetes mellitus has been associated with high consumption of sugary drinks (ENSANUT-MC 2017; Pandit et al. 2012; WHO 2016). Indeed, there is a direct relationship between the presence of obesity, insulin resistance, or type 2 diabetes mellitus; and the development of nonalcoholic fatty liver disease (NAFLD). NAFLD is characterized by lipid accumulation in hepatocytes (>5%) (Dietrich and Hellerbrand 2014); considered the hepatic affection of metabolic syndrome (MS); and promotes from progression of simple liver steatosis to nonalcoholic 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. There-

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fore, 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 2 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 that evaluate the progression of NAFLD associated with MS and sugary drink ingestion.

Considering the etiology and background of this disease, 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. The 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 1 June 2016. All animal procedures and protocols in the present investigation followed the regulations 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 weighing 80 ± 10 g (4 weeks old, n = 48) were used. The 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 h light – 12 h dark cycle (with light beginning at 0700), with food (Purina Lab Diet 5012) and water freely available.

Forty-eight 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 groups (n = 6 each) received 40% and 50% sucrose (wt/vol), respectively, 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 4 weeks prior to euthanasia and finished 1 day prior to euthanasia, to determine changes in glucose homeostasis. Next, the animals were euthanized, and blood samples and 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. In addition, 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 animals 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 OGTT every week (starting 4 weeks before euthanasia, and ending 1 day before euthanasia). For this purpose, glycaemia was measured after 12 h fasting (time 0) and 30, 60, 120, and 240 min after glucose administration (2 g/kg, p.o.) (Fig. 1). Blood samples were collected from the tail. Glucose was measured using test strips and a glucometer (Accu-Chek Performa; Roche, Mexico).

Assessment of biochemical parameters for dyslipidemias

After animals were euthanized, adipose tissue from peritoneal cavity was excised and weighed, then intra-abdominal fat was calculated using the following formula: (tissue weight/body weight) × 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 [(tissue weight/body weight) × 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. 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% 100 mL/L (J.T. Baker), NaH_2PO_4 4 g/L (Vetec), Na_2HPO_4 6.5 g/L (Fermont); pH 7.4. Tissue sections were cut into pieces of 0.5 cm × 2.0 cm, and 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 2 slides per sample 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, hepatocytes with steatosis, and ballooning cells present in 50 fields in samples stained with H&E, analyzed at 40×. Fibrosis was determined independently (F0 to F4) (Diehl and Day 2017), using samples stained with MT and analyzed at 40×.

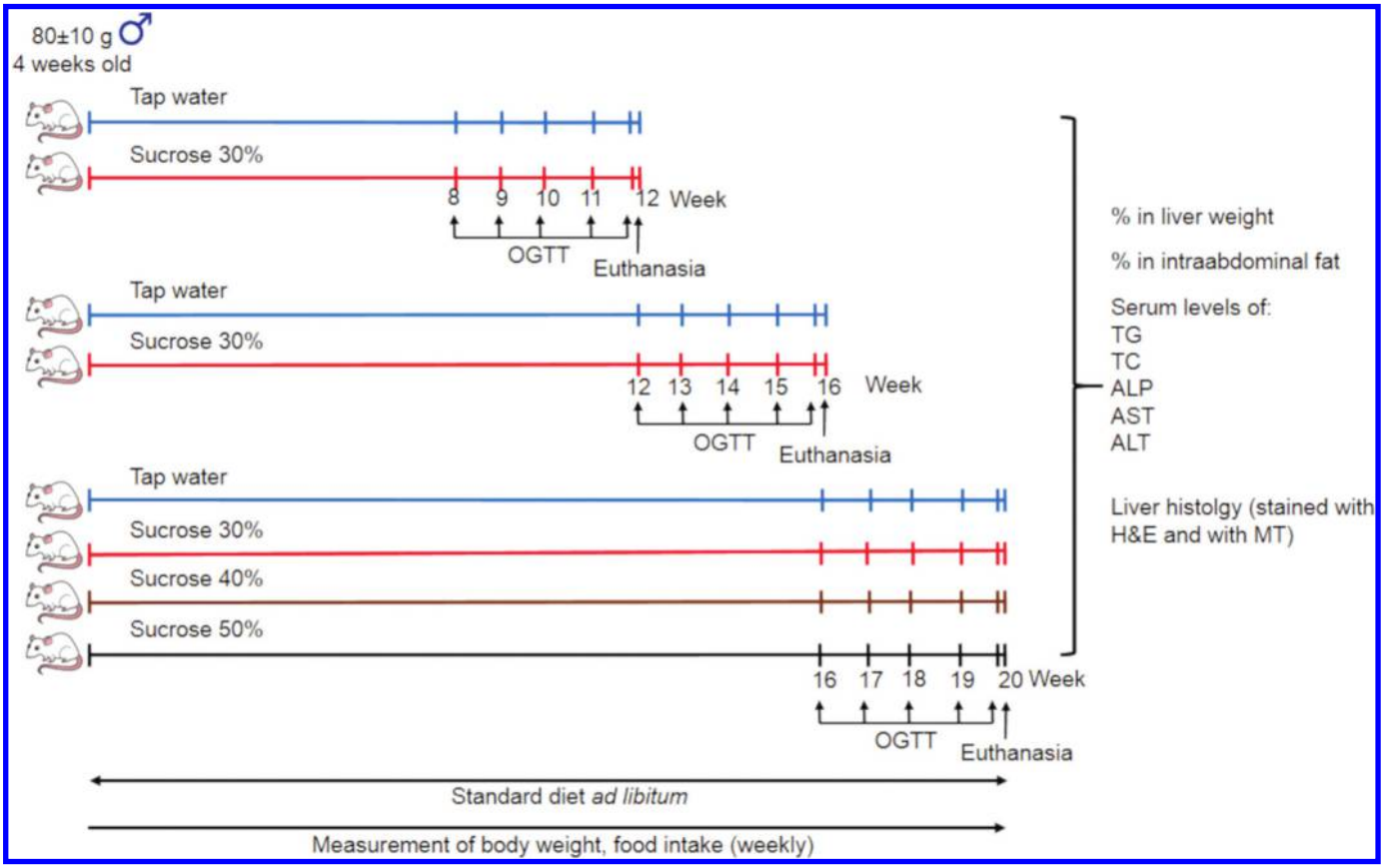
Assessment of serum cytokines

To prove an inflammatory process, serum interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), and transforming growth 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, Texas, 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 statis-

Fig. 1. Experimental design. All animals received standard diet ad libitum plus tap water (control group), 30%, 40%, or 50% sucrose, respectively. Oral glucose tolerance test (OGTT) was performed weekly starting 4 weeks before and finishing 1 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; TC, total cholesterol; TG, triglycerides. [Colour online.]



tically 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 Fig. 2, 30% sucrose ingestion during 12 (395 ± 53.7 g), 16 (369.6 ± 66.5 g), and 20 (328.3 ± 50.9 g) weeks 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 groups, 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 3 concentrations of sucrose significantly decreased food intake compared with 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; $p < 0.001$).

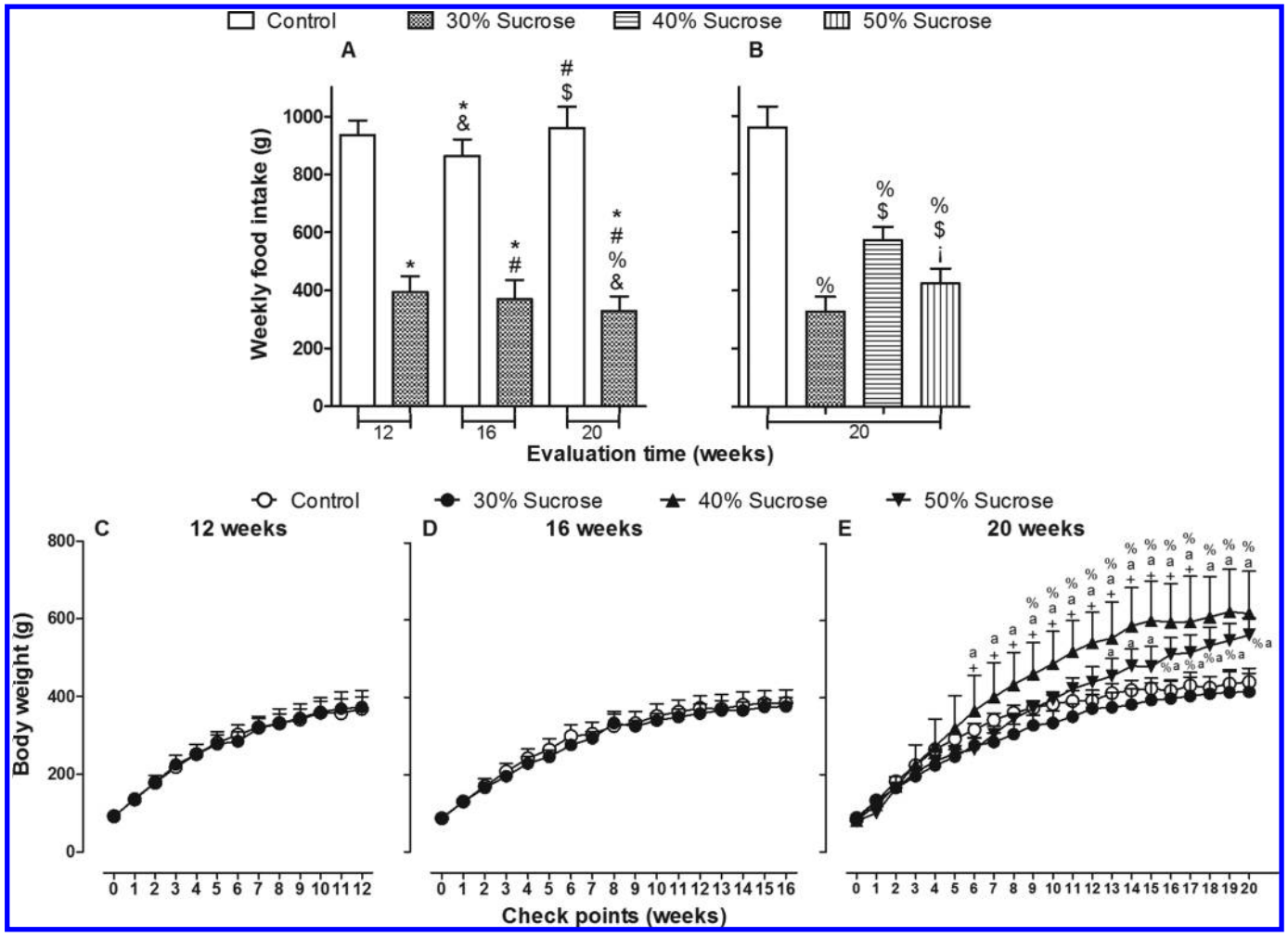
Despite the decrease in food consumption, 30% sucrose ingestion did not modify body weight during 12 ($p = 0.973$; Fig. 2C), 16 ($p = 0.459$; Fig. 2D), and 20 ($p = 0.594$; Fig. 2E) weeks, compared with its respective control groups. In contrast, 40% sucrose ingestion during 20 weeks increased significantly body weight from week 9 to week 20 ($p < 0.05$; Fig. 2E) compared with its control group. In addition, animals that consumed 40% sucrose had higher body weight from week 6 to week 20 ($p < 0.05$; Fig. 2E) compared with the group with 30% sucrose ingestion, and from week 6 to week 17 ($p < 0.05$; Fig. 2E) compared with the group with 50% sucrose ingestion. Finally, animals that consumed 50% sucrose had higher body weight from week 16 to week 20 ($p < 0.05$; Fig. 2E) compared with the control group, and from week 13 to week 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 12 weeks (Figs. 3A–3E) increased significantly blood glucose levels, at min 30 and 60, at weeks 8 ($p < 0.01$; Fig. 3A), 9 ($p < 0.01$; Fig. 3B), and 10 ($p < 0.01$; Fig. 3C) compared with the control group. While 30% sucrose ingestion only increased blood glucose level, at min 30, at weeks 11 ($p < 0.001$; Fig. 3D) and 12 ($p < 0.001$; Fig. 3E) compared with the control group.

Otherwise, regarding the groups that consume sucrose at 30% during 16 weeks (Figs. 3F–3J), it increased significantly blood glucose levels, at min 30 at week 12 ($p < 0.05$; Fig. 3F) and at min 60 at week 14 ($p < 0.05$; Fig. 3H) compared with control group; and diminished at min 120 in both weeks (Figs. 3F and 3H). Sucrose ingestion did not induce changes on blood glucose levels, at weeks 13 ($p = 0.102$; Fig. 3G), 15 ($p = 0.107$; Fig. 3I), and 16 ($p = 0.302$; Fig. 3J) compared with the control group.

Fig. 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 weight 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); [a], $p < 0.05$ vs. 30% sucrose (20 weeks); ¡, $p < 0.05$ vs. 40% sucrose (20 weeks); +, $p < 0.05$ vs. 50% sucrose (20 weeks).



On the other hand, considering the groups that consumed 30%, 40%, and 50% sucrose during 20 weeks (Figs. 3K–3O), we observed that 30% sucrose ingestion significantly increased blood glucose levels, at min 30, 60, and 120 at week 16 ($p < 0.05$; Fig. 3K) and at min 60 at week 20 ($p = 0.035$; Fig. 3O). However, at weeks 17, 18, and 19, 30% sucrose ingestion did not induce changes in blood glucose levels (Figs. 3L, 3M, and 3N; respectively) compared with the control group. We observed that 40% sucrose ingestion significantly increased blood glucose levels, at min 60 at week 16 ($p < 0.05$; Fig. 3K); at min 30 and 60 at week 17 ($p < 0.05$; Fig. 3L); at min 60 and 120 at week 18 ($p < 0.05$; Fig. 3M); at min 30 at week 19 ($p = 0.033$; Fig. 3N); and at min 60 at week 20 ($p < 0.001$; 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 17 ($p < 0.05$; Fig. 3L) and at min 30 at week 20 ($p = 0.019$; Fig. 3O).

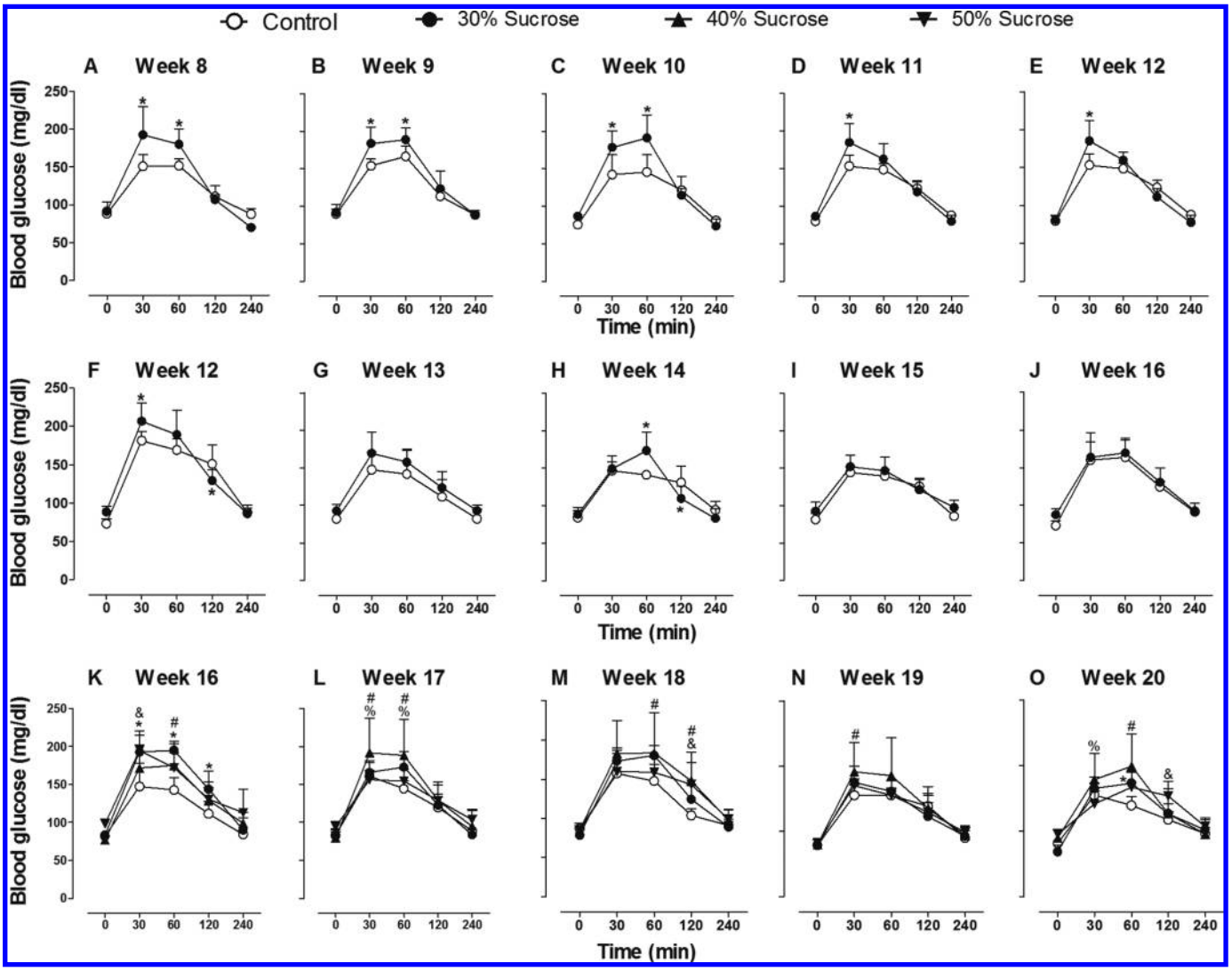
Finally, 50% sucrose ingestion significantly increased blood glucose levels at min 30 at week 16 ($p < 0.001$; Fig. 3K) at min 120 at weeks 18 ($p < 0.001$; Fig. 3M) and 20 ($p = 0.025$; Fig. 3O) compared with control group. Taken together, these results suggest that sucrose ingestion will exert a greater glucose intolerance at longer time of ingestion; and at higher concentration of ingested sucrose.

Effect of sucrose ingestion on percentage in liver weight and intra-abdominal 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 ± 0.2 vs. 2.6 ± 0.2 (12 weeks), 2.5 ± 0.1 vs. 2.6 ± 0.2 (16 weeks), and 2.6 ± 0.1 vs. 2.5 ± 0.2% (20 weeks)). On the other hand, 40% sucrose (2.8 ± 0.2 vs. 2.5 ± 0.2%; $p = 0.044$; Fig. 4E) and 50% sucrose (2.8 ± 0.2 vs. 2.5 ± 0.2%; $p = 0.014$; 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 12 and 16 weeks; both significantly increased the percentage of intra-abdominal fat compared with their control groups (5.4 ± 0.8 vs. 4.2 ± 1.2% and 5.2 ± 1.6 vs. 3.0 ± 0.6%, respectively, $p < 0.05$; Fig. 4B) and 30% sucrose ingestion during 20 weeks significantly increased the percentage of intra-abdominal fat compared with control groups of all check-

Fig. 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 consumed 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.



points (6.7 ± 1.5 vs. 4.2 ± 1.2 (12 weeks), vs. 3.0 ± 0.6 (16 weeks) and vs. 1.4 ± 0.9% (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 intra-abdominal fat. In addition, when the sucrose ingestion is compared at different concentrations with control group of 20 weeks, it was observed that all sucrose concentrations, increased significantly the percentage of intra-abdominal fat compared with control group (1.4 ± 0.9 vs. 6.7 ± 1.5 (30%), vs. 6.2 ± 1.5 (40%) and vs. 5.7 ± 1.6% (50%); $p < 0.001$; Fig. 4F), suggesting that regardless of the concentration of sucrose ingestion during 20 weeks, it will be a similar effect on the percentage increase of intra-abdominal fat compared with control group.

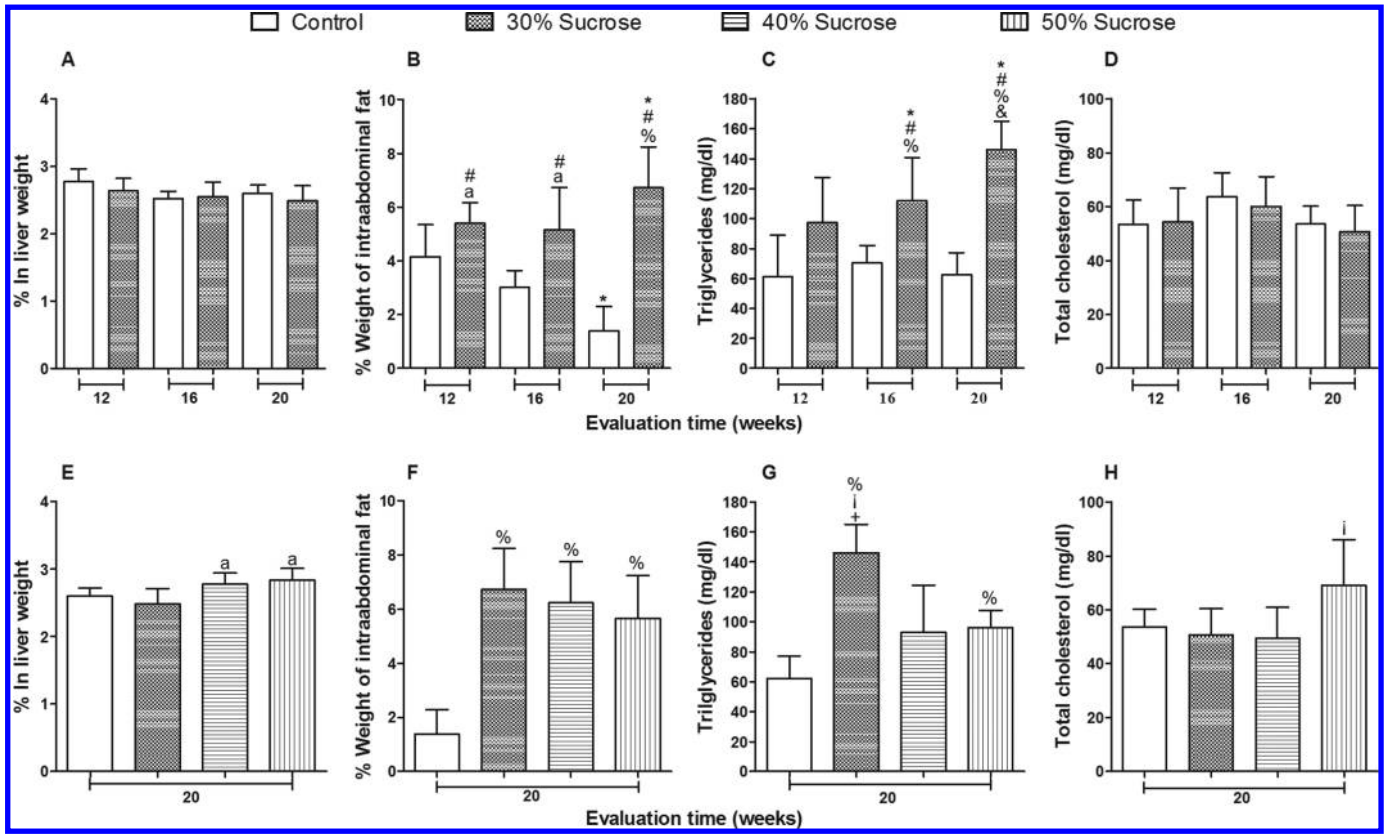
Effect of sucrose ingestion on biochemical parameters for dyslipidemias

The intake of sucrose at 30% significantly increased TG serum levels at weeks 16 (112.1 ± 28.8 mg/dL; $p < 0.05$; Fig. 4C) and 20 (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)); in addition, 30% sucrose ingestion during 20 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 12 weeks. Otherwise, TG serum levels of 30% sucrose ingestion during 20 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 20 weeks, while 50% sucrose ingestion during 20 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 times ($p = 0.229$; 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 with the group of 40% sucrose ingestion.

Our results suggest that a longer time in 30% sucrose ingestion leads to a greater increase in TG serum levels, but without

Fig. 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 intra-abdominal fat 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); ¡, $p < 0.05$ vs. 40% sucrose (20 weeks); +, $p < 0.05$ vs. 50% sucrose (20 weeks).



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 may be due to a change on lipid distribution in the body.

Effect of sucrose ingestion on hepatic enzymes

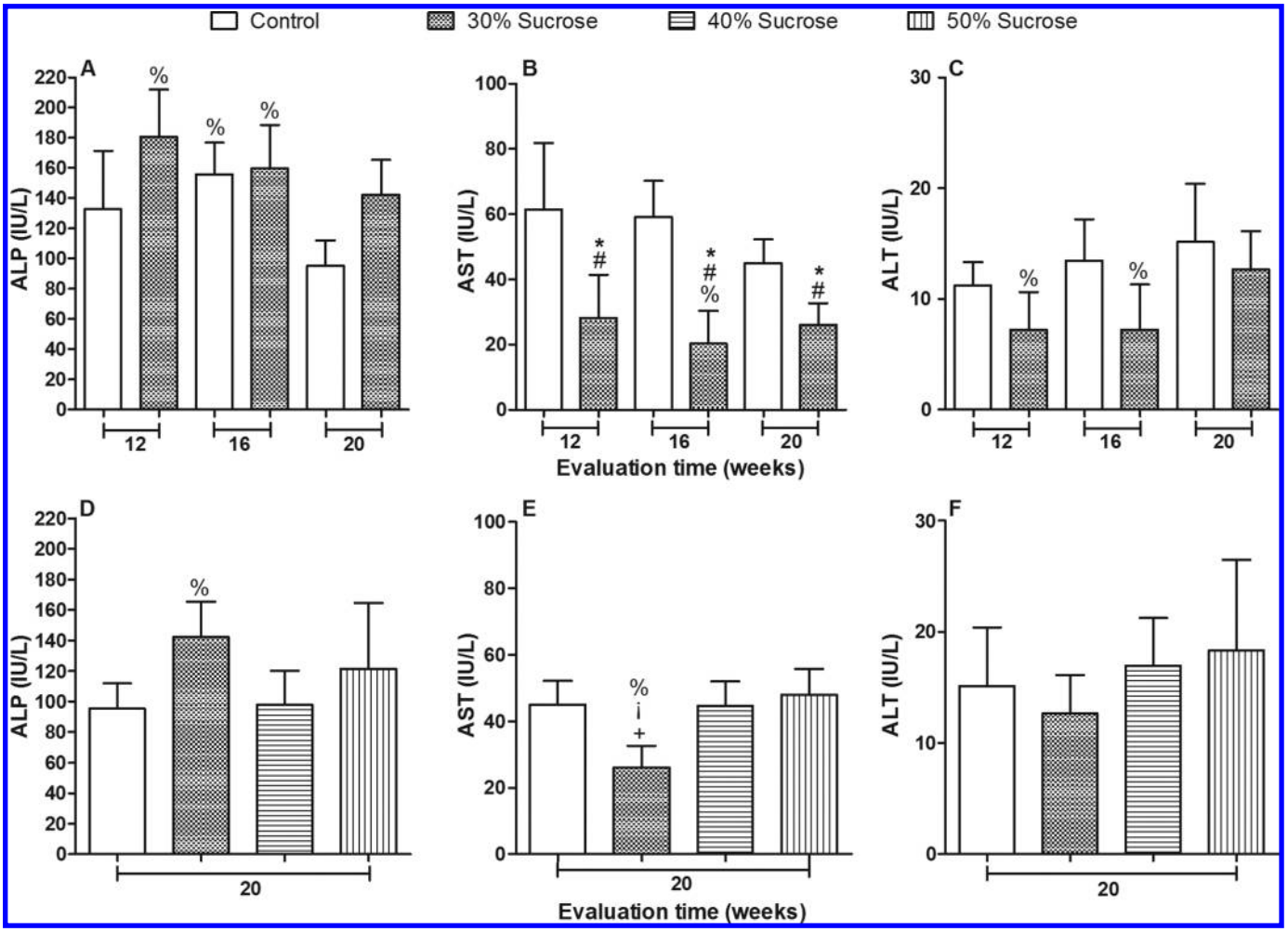
We found that ALP, AST, and ALT serum levels of controls groups were influenced by age (Figs. 5A–5C); noticing that ALP serum levels of control group were significantly diminished at week 20 compared with control group of 16 weeks (95.4 ± 16.7 vs. 155.9 ± 20.9 IU/L; $p = 0.008$; Fig. 5A); AST serum levels 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. 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 comparing the effect of all sucrose concentrations during 20 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 20 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 weeks 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).

Fig. 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. 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. 40% sucrose (20 weeks); +, $p < 0.05$ vs. 50% sucrose (20 weeks).



Histopathological changes induced by sucrose ingestion

The group with 30% sucrose ingestion during 12 weeks (Figs. 6D–6F) showed similar characteristics to those presented by the control group (Figs. 6A–6C); those were normal hepatocyte architecture was presented in parenchyma (Figs. 6A and 6D) with absence of steatosis (<5%) and inflammatory infiltrate; central veins (Figs. 6B and 6E) and portal structures (Figs. 6C and 6F).

The group with 30% sucrose ingestion during 16 weeks (Figs. 6G–6I) showed in hepatic parenchyma (Fig. 6G) and around central veins (Fig. 6H) moderate microvesicular steatosis (mean of 45.7%), with absence of inflammatory infiltrate; positioning it in grade 2 in the development of NAFLD (LaBrecque et al. 2012).

In addition, the group with 30% sucrose ingestion during 20 weeks (Figs. 6J–6L) showed in hepatic parenchyma (Fig. 6J) severe microvesicular steatosis (69.7%) and limited macrovesicular steatosis (0.6%); in central veins (Fig. 6K) and portal structures (Fig. 6L) limited microvesicular 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 (Figs. 6M–6O) showed in hepatic parenchyma (Fig. 6M) severe microvesicular steatosis (71.7%) and limited macrovesicular steatosis (5.8%), in central veins (Fig. 6N) and portal structures (Fig. 6O) moderate microvesicular steatosis, with a steatosis mean of 77.5%; also this group showed 1 ballooned cell per field and 1 inflammatory infiltrate (grade 5). Finally, the group with 50% sucrose ingestion during 20 weeks (Figs. 6P–6R) showed in hepatic parenchyma (Fig. 6P) severe microvesicular steatosis (70.5%) and moderate macrovesicular steatosis (21.8%), in central vein (Fig. 6Q) and portal structures (Fig. 6R) moderate macrovesicular steatosis, with a steatosis mean of 92.3%; 2 ballooned cells per field and 1 inflammatory infiltrate (grade 6). It could be considered that the last 2 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% (Figs. 7D–7L) and 40% (Figs. 7M–7O) sucrose ingestion compared with control group (Figs. 7A–7C). 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). The group with 50% sucrose ingestion (Figs. 7P–7R) was the only that 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)

Fig. 6. Effect of sucrose ingestion on hepatic tissue morphology. Tissues stained with hematoxylin and eosin (H&E) were observed at 40×. 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 to some hepatocytes with microvesicular steatosis, the squares frame some hepatocytes with macrovesicular steatosis, and the circles frame ballooning cells. Scale bars = 50 μm. [Colour online.]

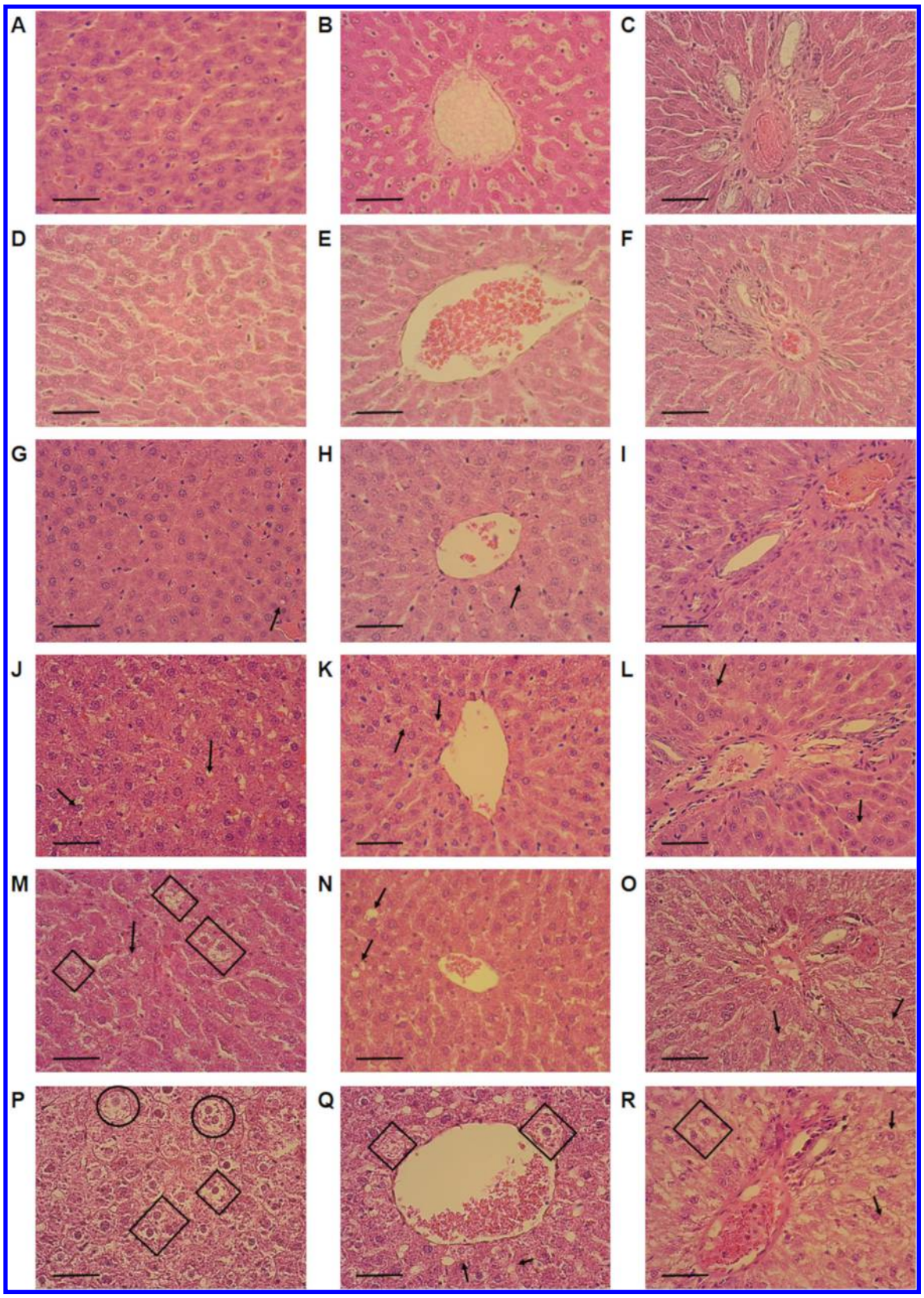


Fig. 7. Effect of sucrose ingestion on changes of collagen in hepatic tissue. Tissues stained with Masson trichrome (MT) were observed at 40×. Hepatic parenchyma (A, D, G, J, M, P). Central vein (B, E, H, K, N, Q). Portal structure (C, F, I, L, O, R). Control group, which consumed tap water ad libitum (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 to fibrosis with septa. Scale bars = 50 μm. [Colour online.]

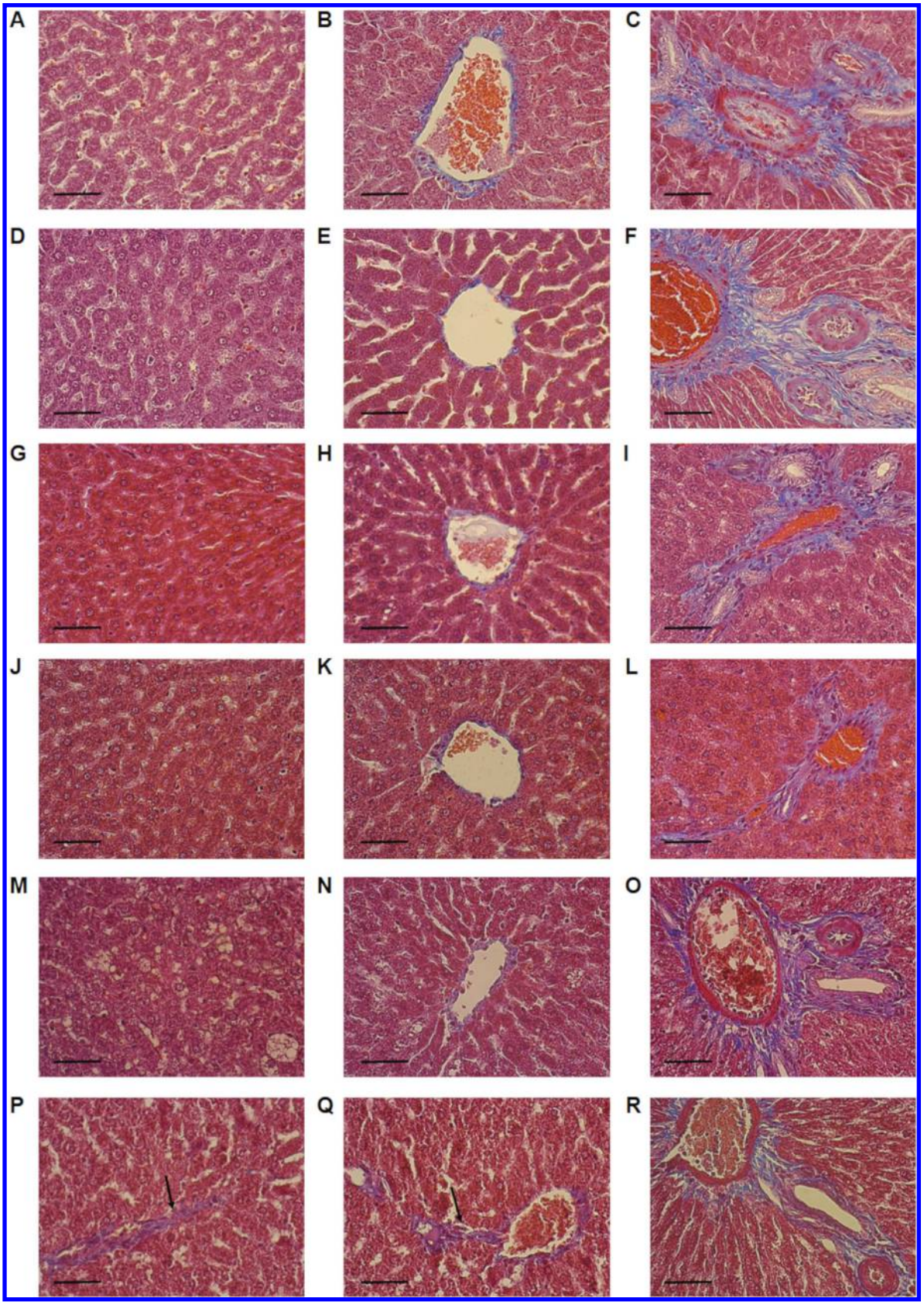
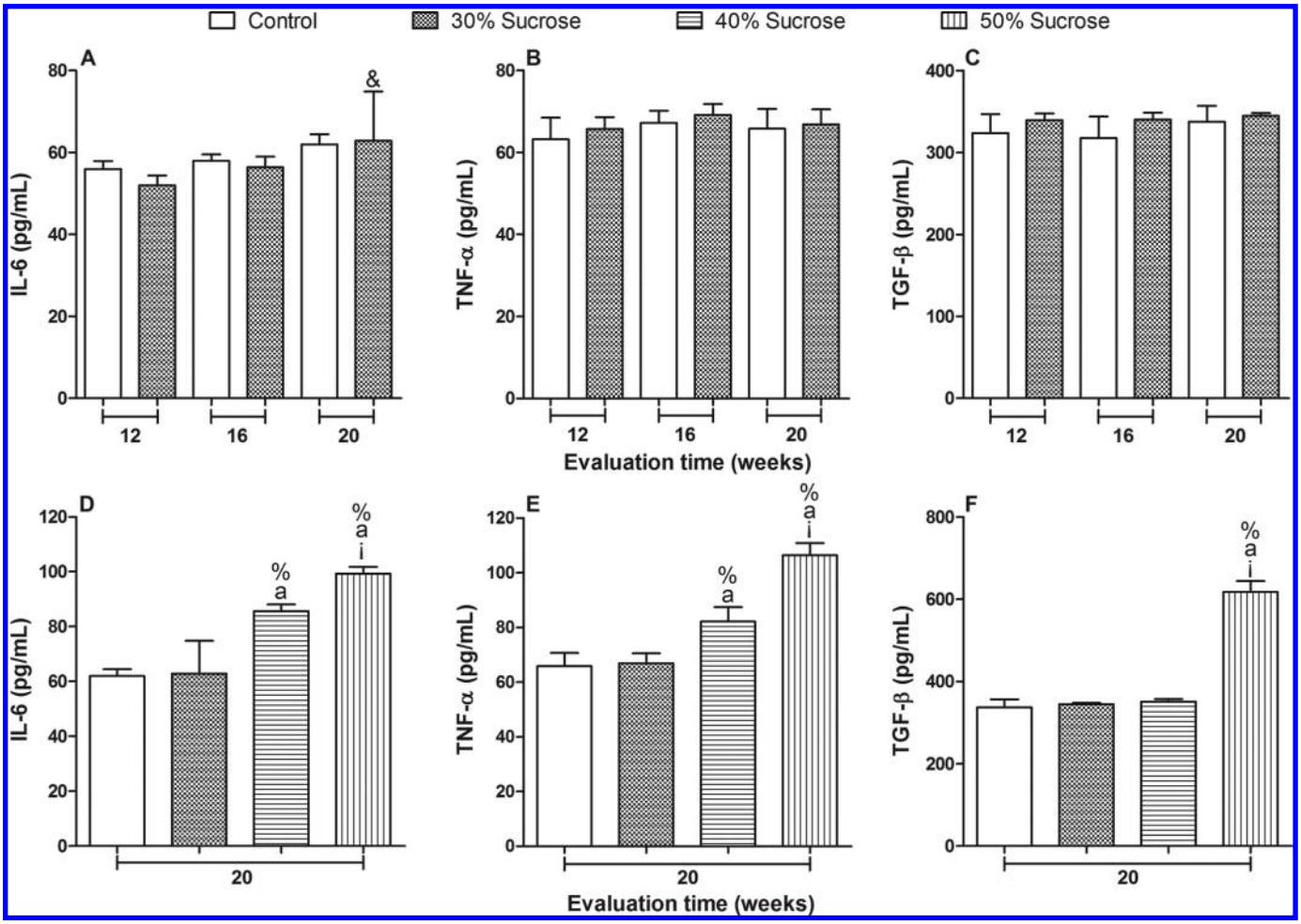


Fig. 8. Effect of sucrose ingestion on serum levels of interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α), and transforming grow factor beta (TGF-β). Upper panel shows the effect of 30% sucrose ingestion during 12, 16, and 20 weeks (A–C) and bottom panel shows the effect of 30%, 40%, and 50% sucrose 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 each week. One-way ANOVA and Tukey post hoc test were performed. &, $p < 0.05$ vs. 30% sucrose (12 weeks); %, $p < 0.05$ vs. control (20 weeks); [a], $p < 0.05$ vs. 30% sucrose (20 weeks); ¡, $p < 0.05$ vs. 40% sucrose (20 weeks).



serum levels in any of the evaluated times compared with any of the other groups; with the exception of 30% sucrose ingestion during 20 weeks significantly increased serum levels of IL-6 compared with 30% sucrose ingestion during 12 weeks ($p = 0.036$; Fig. 8A).

On the other hand, considering the groups that consumed 30%, 40%, and 50% sucrose during 20 weeks (Figs. 8D–8F), 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

As 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 20 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 in the group with 30% sucrose ingestion compared with 40% and 50% sucrose ingestion.

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 that 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, 2 medical conditions of MS. These results of intra-abdominal fat (obesity) and TG are in agreement with other reports (Balderas-Villalobos et al. 2013; Marin et al. 2016). TC 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 and high-density lipoproteins (Marin et al. 2016).

The intra-abdominal 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 with 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 with healthy animals 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, in turn, this process generates the release of ALP, ALT, and AST 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 an increase of them only when 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). In addition, it is well known that TNF-α increases endothelial permeability and the adherence of leukocytes to this monolayer by the induction of E-selectin, intracellular adhesion molecule 1 and vascular cell adhesion molecule 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-α.

The increase of TGF-β 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.

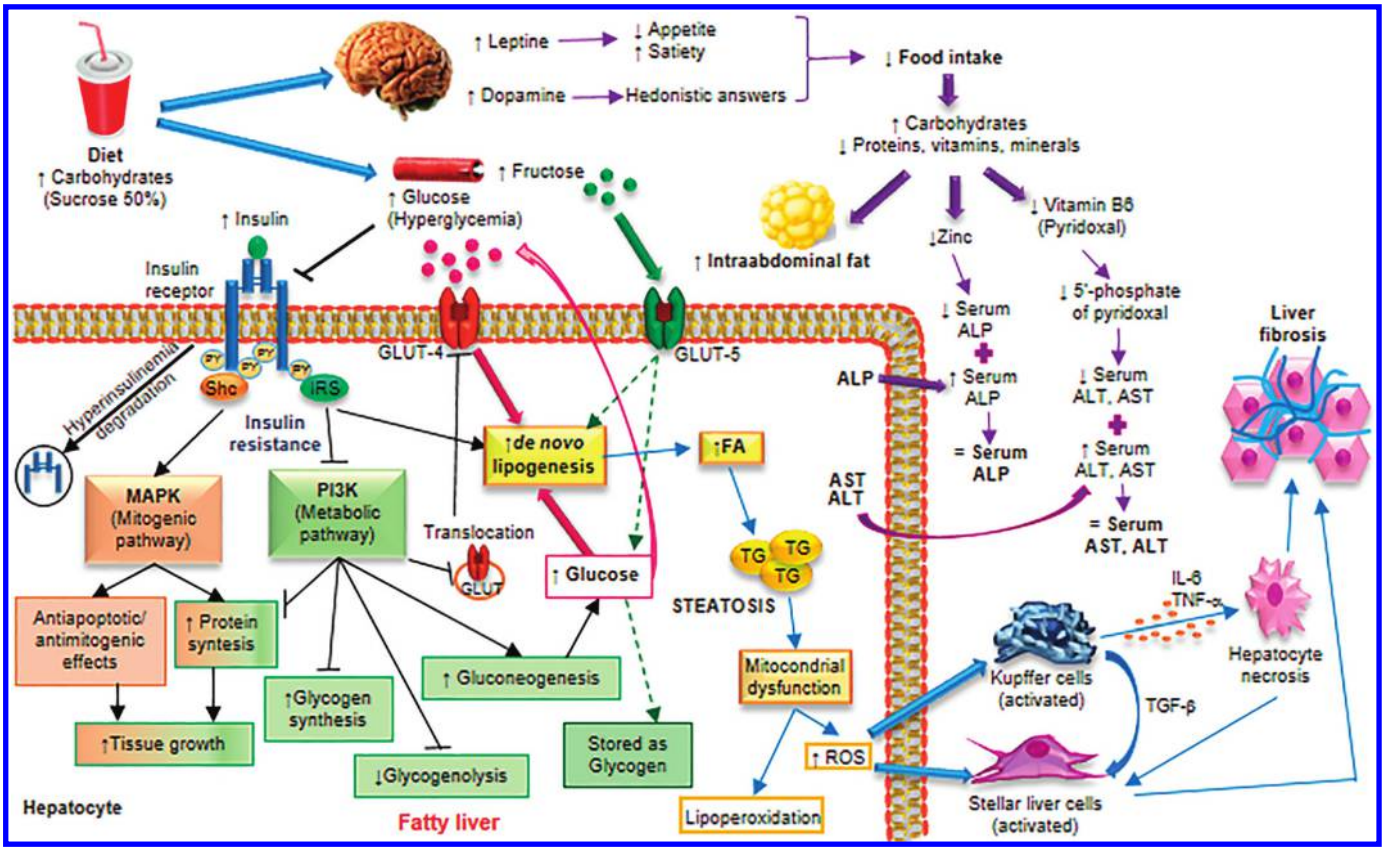
Possible mechanism of action of sucrose ingestion

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 (Fig. 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 decrease in food intake induced by sucrose ingestion, 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. The 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) (Fig. 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

Fig. 9. Effect of 50% sucrose ingestion during 20 weeks. The sucrose acts on central nervous system leading to a decrease of appetite and an increase of satiety, that 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 alkaline phosphatase (ALP), aspartate aminotransferase (AST), and alanine aminotransferase (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 de novo lipogenesis and with that an increase on fatty acids (FA) that are esterified as triglycerides (TG) and in liver leads to the development of steatosis. This could induce mitochondrial dysfunction, lipoperoxidation, and decrease in oxidative capacity, leading to an increase in reactive oxygen species (ROS). These factors activate inflammation pathways, observed as an increase on interleukin 6 (IL-6) and transforming growth factor beta (TNF-α), Kupffer cells and stellar liver cells. Activated Kupffer cells contributed to increase inflammation (IL-6, TNF-α, and transforming growth factor beta (TGF-β)) 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-β and ROS, which are released, taken together promote fibrosis. GLUT, glucose transporter; IRS, insulin receptor substrate; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositol 3-kinase. [Colour online.]



transport system; while fructose is transported into cells by carrier-mediated facilitated diffusion with glucose transporter type-5 (GLUT-5). Conversely, fructose is metabolized principally by the liver and is exceptionally high compared with 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 de novo lipogenesis. The increase of gluconeogenesis leads to an increase in glucose and, in turn, increases 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 IKKβ lead to the production of proinflammatory cytokines, chemotaxis, and activation of Kupffer cells (Castro et al. 2014; López-Oliva and Muñoz-Martínez 2014) (Fig. 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 release of IL-6 and TNF-α and as consequence to necrosis of hepatocytes; also release ROS and TGF-β; as we observed an increase in 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 description of 50% sucrose ingestion is shown in Fig. 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 microvesicular 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).

Conflict of interest

The authors declare that there is no conflict of interest associated with this work.

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