IJC Metabolic & Endocrine 9 (2015) 12–21

Contents lists available at ScienceDirect

IJC Metabolic & Endocrine

journal homepage: http://www.journals.elsevier.com/ijc-metabolic-and-endocrine

Liquid fructose supplementation in LDL-R$^{-/-}$ mice fed a western-type diet enhances lipid burden and atherosclerosis despite identical calorie consumption☆,☆☆

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ARTICLE INFO

Article history:
Received 9 June 2015
Received in revised form 9 September 2015
Accepted 6 October 2015
Available online 13 October 2015

Keywords:
Sugar-sweetened beverages
CVD
Lipid metabolism
Macrophages
NAFLD
Inflammation

ABSTRACT

Background: Studies on humans have related the high consumption of fructose, especially in the form of sugar-sweetened beverages, to obesity, fatty liver, and hypercholesterolemia, all risk factors for atherosclerosis, and cardiovascular disease. We aimed to determine whether supplementation of liquid fructose (SLF), in either a normal, healthy chow or a Western-style chow, promoted the appearance of atherosclerosis in a rodent model.
Methods: LDL receptor knockout mice were fed for twelve weeks with normal chow, normal chow plus *ad libitum* 15% fructose solution, Western chow, or Western chow plus *ad libitum* 15% fructose solution (W + F). Food and liquid intake and body weight were periodically monitored. At the end of the study, plasma and hepatic lipids, liver histology and expression of genes related to lipid handling were analyzed and histological and immunohistological analyses of atherosclerosis at the aortic origin was performed.
Results: Total calorie intake was significantly increased in Western-fed *vs* normal chow-fed mice, but was not modified by SLF. SLF significantly increased body weight, visceral adiposity, plasma lipids and liver cholesterol content in Western-fed mice, probably due to an increase in *de novo* lipid synthesis. Aortic atherosclerotic total lesion area was significantly correlated to plasma lipids, being highest in W + F mice. Further, SLF induced higher immunostaining for macrophages and oxidized-LDL receptor, independently of lesion area and caloric burden.
Conclusions: SLF, without changing total calorie intake, increases atherosclerosis, visceral adipose tissue and cholesterol burden in a background of overweight LDL receptor knockout mice consuming an unhealthy, Western-type solid rodent chow.

1. Introduction

A sedentary lifestyle and unhealthy dietary habits are fueling the epidemic of metabolic diseases, such as type 2 diabetes and obesity, and the associated cardiovascular diseases related to atherosclerosis [1]. One of the most prevalent dietary changes over recent decades has been the increasing consumption of simple sugars, mainly in the form of sweetened beverages, especially among low income populations all over the world [2]. Together with glucose, fructose in the form of sucrose or high fructose corn syrup (HFCS) is one of the main sweeteners used in the production of sodas and baked foods. Numerous studies in recent years have related the high consumption of fructose or simple sugars to ectopic fat deposition [3], hypercholesterolemia [4], and

☆ This work was supported by grants from the Fundació Privada Catalana de Nutrició i Lípids, the Ministry of Economy and Competition [SAF2013-42982-R, FIS PI11/00315] and European Commission FEDER [SAF2013-42982-R] funds. Miguel Baena and Gemma Sangüesa were supported by FPI and FPU grants, respectively, from the Spanish Ministry of Science and Innovation. We are a Consolidated Research Group of the Autonomous Government of Catalonia [SGR09-00413; SGR13-00066].
☆☆ No potential conflict of interest, including related consultancies, shareholdings and funding grants.
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http://dx.doi.org/10.1016/j.ijcme.2015.10.002

diabetes [5] in humans. As a consequence, the high consumption of fructose or simple sugars, especially in the form of sugar-sweetened beverages, has also been related to cardiovascular disease and mortality [6,7,8].

Due to metabolic similarities, the rat is a good model for studying changes induced by fructose consumption in humans [9]. Fructose supplementation of rats' drinking water (10% weight/volume), provides a pattern of fructose consumption similar to that of the upper quintile of human populations consuming high quantities of sugar-sweetened beverages [10]. In previous studies, the use of this model enabled us to demonstrate that fructose, but not glucose, induces hypertriglyceridemia and fatty liver by simultaneously increasing liver fatty acid synthesis and reducing liver fatty acid oxidation [11,12]. Moreover, at least in female Sprague–Dawley rats, metabolic changes are accompanied by alterations in liver glucose handling, with a marked reduction in insulin receptor substrate-2 and silent regulator of translation-1 expression and activity [13,14]. These fructose-induced metabolic alterations observed in rat are well known risk factors for the development of atherosclerosis and related cardiovascular diseases in humans.

Consequently, we studied the influence of liquid fructose ingestion in atherogenesis. As rats are resistant to atherosclerosis, we studied the effect of liquid fructose supplementation in a murine model of dietary-induced atherosclerosis, LDL receptor-deficient ($LDLR^{-/-}$) mice [15]. We used two different solid dietary substrates, a standard "healthy" rodent chow and an "unhealthy", Western-type rodent chow rich in saturated fat, refined carbohydrates and cholesterol. Supplementation of liquid fructose in $LDLR^{-/-}$ mice fed a standard rodent show induced a non significant increase in plasma lipid and liver cholesterol content, but failed to significantly increase atherosclerosis. However, liquid fructose supplementation significantly increased atherosclerosis and liver and plasma lipid content in $LDLR^{-/-}$ mice fed Western-type diet, despite ingesting exactly the same amount of calories as $LDLR^{-/-}$ mice on Western-type diet only.

2. Materials and methods

2.1. Animals and experimental design

Male $LDL^{-/-}$ mice (C57BL/6 N) were purchased from Charles River (France) and maintained with water and food *ad libitum* at constant humidity and temperature with a light/dark cycle of 12 h. After three weeks of acclimatizing, animals were randomly separated into four groups of 10 mice each which received: 1. control rodent diet without supplementary sugar: control group (C); 2. control rodent diet supplemented with 15% weight/volume fructose in drinking water: fructose-supplemented group (F); 3. Western-type diet without supplementary sugar: Western group (W); 4. Western-type diet supplemented with 15% weight/volume fructose in drinking water: Western plus fructose group (W + F). The composition of control (2018 Teklad Global 18% protein, Harlan Laboratories) and Western-type (D12079B Open Source Diets, Research Diets, Inc.) diets is detailed in Table 1.

During a feeding period of 12 weeks, consumed food and beverage were measured every two days and body weight once a week. At the end of the study, animals were sacrificed under intraperitoneal ketamine (100 mg/kg)/xylazine (10 mg/Kg) anesthesia between 9 and 10 a.m. after being fasted for 2 h. All procedures were conducted in accordance with the guidelines established by the University of Barcelona's Bioethics Committee, as stated in Law 5/1995 (21st July) from the Generalitat de Catalunya. These guidelines follow the Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

Table 1
Composition of experimental diets.

	Control diet	Western-type diet
Kcal/g	3.1	4.7
Protein	18.6%	20%
Carbohydrates	60%	54%
Sucrose	0%	34%
Insoluble fiber	14.7%	5%
Lipids	6.2%*	21%**
cholesterol	0%	0.21%

* Mainly unsaturated fat.
** 20% saturated fat from milk plus 1% corn oil.

2.2. Sample preparation

Blood samples were obtained by intracardiac punction at the time of death and collected in micro tubes containing anticoagulant as additive (Sarstedt AG & Co, Nümbrecht, Germany). Plasma was obtained by centrifugation and stored at −80 °C until used. Liver tissue was excised and fractionated. 10–100 mg were immediately frozen in liquid N2 and stored at −80 °C until used for total RNA extraction. Another portion for hepatic histological analysis was obtained. An additional section of liver tissue (100 mg) was perfused and stored at −80 °C for quantifying liver lipids content. The upper atrial portions of hearts, including the aortic valve, were excised and used for histological analysis, as described below.

2.3. Leptin and lipid analysis

Plasma leptin and lipid levels were determined at the end of treatment. Plasma leptin levels were determined with the RL83K RIA kit from Linco Research (St. Charles, MO). Plasma triglycerides levels were measured by Accutrend® Plus System glucometer (Cobas, Roche Farma, Barcelona, Spain) using a specific test strips. Plasma cholesterol was determined by Cholesterol CHOD-POD colorimetric test from Spinreact (Girona, Spain).

Liver lipids were extracted according to Bligh & Dyer [16] method using the homogenate fraction. The lipid extract was evaporated under a stream of nitrogen gas and dissolved in absolute ethanol. Triglycerides, total and free cholesterol contents in liver were determined by using colorimetric tests: Triglycerides-LQ n° 41,030, Cholesterol CHOD-POD n° 1,001,091 and Free-Cholesterol-LQ n° 41,035 from Spinreact (Girona, Spain), respectively.

Size fractionation of plasma lipoproteins was performed by fast performance liquid chromatography (FPLC) of pooled filtered plasma samples using a Superose 6® column as previously described [17]. In each fraction, total cholesterol and triglyceride content was assayed.

2.4. RNA isolation and quantitative real-time PCR

Total RNA was isolated from 60 mg of liver tissue using Trizol^{R} reagent (Invitrogen™, Thermo-Fisher Scientific Inc., MA, USA), in accordance with the manufacturer's guidelines. Single stranded cDNA was synthesized by mixing 1 μg of liver total RNA, 125 ng of random hexamers (Roche Farma, SA, Madrid, Spain,) as primers in the presence of 5× First-Strand Buffer, 10 mM dithiothreitol (DTT), 200 units of Moloney murine leukemia virus reverse transcriptase (M-MLV RT), 20 units of RNase OUT from Invitrogen™ and 0.5 mM of each dNTP (Sigma-Aldrich, St. Louis, MO, USA) in a total volume of 20 μl. Samples were incubated at 37 °C for 60 min in MJ Mini™ Personal Thermal Cycler (Bio-Rad, Hércules, CA, USA).

The PCR reaction was carried out in StepOnePlus™ Real-Time PCR System Thermal Cycling Block (Applied Biosystem, Foster City, CA, USA). 20 μL of reaction mixture contained: Power SYBR® Green PCR Master Mix (Applied Byosystems), 100 nM of each specific primer (including forward and reverse primers) and 10 ng of cDNA for each gene. After an initial denaturation at 95°C for 10 min, 40 cycles of amplification were done. PCR reactions were performed in duplicate and normalized to a housekeeping gene, the TATA box binding protein (*tbp*) gene, using the $2^{-\Delta\Delta Ct}$ method. The GenBank™ number, primer sequences and PCR product length are listed in Supplemental Table 1.

2.5. miRNA determination

Total RNA, including small RNAs, was isolated using the miRNeasy minikit in accordance with the manufacturer's protocol. For miRNA quantification, miRNAs were reverse-transcribed using miScript II reverse transcription kit. Specific primers for each miRNA were used and real time PCR was performed using the miScript SYBR Green PCR kit on a 7900 HT Fast Real-Time PCR System (Applied Biosystems). All reagents and kits used were from Qiagen (Las Matas, Madrid, Spain).

Relative expression of miRNAs was calculated by the $2^{-\Delta\Delta Ct}$ method using RNU6 for normalization. Only validated miRNAs for the target genes were analyzed. Selection of validated miRNAs was assessed using the MirWalk 3 [18] and miRTarbase 4 [19] databases.

2.6. Histological analysis

For hepatic histological analysis of Oil-Red O stained sections, the liver tissue was perfused and fixed in 10% paraformaldehyde solution before processing for paraffin embedding. Images were acquired with an Olympus BX71 microscope equipped with a DP72 camera and analyzed by a registered pathologist at BioBanc (Banc de tumors–IDIBAPS, Barcelona Spain), who was unaware of the treatment groups. The area of positive staining for Oil-Red O was calculated as a percentage of stained cells/total section area in each sample.

For heart histological analysis, the upper portion of the heart was excised, and placed in cryomolds (Cryomold Biopsy — Tissue Tek from Sakura Finetek Europe B.V., Netherlands) preserved in Tissue-Tek O.T.C. embedding medium, followed by freezing in dry ice-cooled isopentane. Eight micrometer serial sections of heart segments obtained in a cryostat (Leyca® CM-1900) at −24 °C, were prepared and stained with Oil-Red O and Hematoxilin-Eosin stain for histological evaluation of cardiac structures. Lesion area was quantified following the standard procedure by using *Image-J* software.

2.7. Immunohistochemistry studies

The immunohistochemistry analysis was performed basically as previously described by Zhang and Ramos [20]. Serial frozen sections of heart were examined for macrophage infiltration, inflammation and oxidative stress using immunohistochemistry stain with several monoclonal antibodies: F4/80 (Santa Cruz Biotechnology Inc. Heidelberg, Germany; reference product: sc-377,009), LOX1, p-NF_{κ}B-P65, RAGE (AbCam, Bristol, UK; reference product: Ab60178, Ab131109, Ab3611, respectively) and Anti-rabbit Alexa Fluor 594 (reference: A11012 from Invitrogen, Life Technologies) used as a secondary antibody.

The slides were thawed at room temperature for 2 h, and fixed with acetone for 10 min. After 30 min of treatment with the blocking solution (1% gelatin in PBS solution), tissue sections were incubated at room temperature with primary antibody (dilution 1:1000) for 1 h. Slides were washed with PBS twice and incubated with antirabbit Alexafluor 594 secondary antibody for 1 h. After two washes with PBS, 20 μl of Fluoromount-G (e-Bioscience, San Diego, CA, USA) were added to the slides and covered. The images of the tissue sections were recorded using a fluorescence microscope and their color intensity was measured by *Photoshop* (Adobe Photoshop 7.0 software).

2.8. Statistical methods

Results are expressed as the mean of n values ± standard deviation. Plasma and lipid samples were assayed in duplicate. Significant differences between values from control, fructose and western groups were established by the one-way ANOVA test and Bonferroni post-test for selected comparisons; significant differences between values from western and western + fructose group were established by the unpaired t-test (GraphPad Software V5). When the number of samples was too small or variance was not homogeneous, a non-parametric test was performed. The level of statistical significance was set at $P \leq 0.05$.

3. Results

3.1. Liquid fructose supplementation in solid diet increases plasma lipids in $LDLR^{-/-}$ mice

Zoometric parameters and plasma cholesterol, triglyceride and leptin concentrations are shown in Table 2. $LDLR^{-/-}$ mice fed a solid Western-type diet showed an increased body weight during and at the end of treatment (×1.11 and ×1.24 fold, respectively, *vs* Control (C) group). Although both the C and Western (W) groups of mice consumed equal amounts of solid diet, the high energy density of the Western diet yielded a ×1.49 fold increase in the amount of energy consumed by W mice. Furthermore, W mice showed increased concentrations of plasma cholesterol (×2.62 fold), triglyceride (×2.92 fold) and leptin (×3.56 fold) *vs* C animals. Liquid fructose supplementation in standard (F) or western (W + F) solid diet-fed animals increased concentration of plasma cholesterol (×1.41 and ×1.73 fold *vs* C and W groups, respectively) and triglyceride (×1.31 and ×1.39 fold *vs* C and W groups, respectively), although only W + F samples achieved statistical significance. Fast protein liquid chromatography (FPLC) analysis (Fig. 1) indicated that triglycerides were transported in triglyceride-rich lipoproteins (chylomicrons and VLDL), while cholesterol shifted progressively from HDL and LDL (C samples) to triglyceride-rich lipoproteins (W and W + F samples). W + F mice showed an increased body weight during and at the time of sacrifice (×1.11 and 1.19 fold *vs* W, respectively), mainly due to an increased percentage of visceral fat (×2.03 fold *vs* W). These fructose-induced changes in W + F mice were apparent without significant changes in the total amount of energy consumed, due to a compensatory reduction in the amount of ingested solid food (×0.83 fold *vs* W), together with a reduction in the ingested volume of fructose solution (×0.77 fold *vs* W).

3.2. Liquid fructose supplementation in $LDLR^{-/-}$ mice alters hepatic cholesterol homeostasis independently of the type of solid diet consumed

Histological analysis of Oil-Red O (ORO) liver sections showed no differences among the different dietary groups in the percentage of steatotic liver cells, which was always higher than 85–90% (Fig. 2A). Only livers from the W + F group had micro- and macrovesicular steatosis in all samples studied. Accordingly, liver triglyceride content was unchanged in F and W groups with respect to C values, while livers from W + F animals showed a ×1.55 fold increase in triglyceride content *vs* W animals (Fig. 2B). In contrast, there was a progressive increase of total cholesterol content in liver among all dietary interventions (×1.22 and ×2.54 fold *vs* C for F and W groups respectively), which was further increased by the addition of fructose to the Western diet (×1.72 fold W + F *vs* W). This change was mainly observed in the cholesteryl-ester fraction, which was almost absent in the control group and followed a similar pattern to that of total cholesterol (Fig. 2B).

We analyzed the liver expression of genes related to fatty acid and cholesterol synthesis and metabolism (Fig. 2C). The Western diet did not change the mRNA levels of genes related to fatty acid synthesis (fatty acid synthase *fas*, stearoyl-CoA desaturase 1 *scd1*, and liver pyruvate kinase *l-pk*), with respect to control animals, but it did increase the mRNA levels of genes related to fatty acid oxidation (liver carnitine palmitoyltransferase 1 *lcpt-1* and peroxisome proliferator activated receptor α *pparα*), as well as reducing those related to cholesterol synthesis (hydroxymethylglutaryl-Coenzyme A reductase *hmgcrd*), and increasing the expression of genes related to lipoprotein uptake (scavenger receptor-class B1 *sr-b1*, low density receptor related protein 1 *lrp1*, scavenger receptor-class A1 *sr-a1*, and cluster of differentiation 36 *cd36*). Fructose supplementation in both control and western diets had the opposite effect, increasing liver mRNA levels of fatty acid and

Table 2
Zoometric parameters and plasma analytes of LDLR$^{-/-}$ mice exposed to four different dietary regimes for 84 days.

Parameters	C	F	W	W + F
Final body weight (g)	26.6 ± 2.4	26.5 ± 4.9	33.0 ± 5.8##	39.2 ± 2.7*
Liver weight (g)	1.38 ± 0.31	1.37 ± 0.19	1.60 ± 0.27	2.35 ± 0.85**
% Liver/body weight	4.9 ± 0.4	5.3 ± 0.9	4.9 ± 0.6	6.1 ± 1.2
VAT weight (g)	0.88 ± 0.38	1.08 ± 0.36	1.60 ± 1.02	3.25 ± 0.39**
% VAT/body weight	3.1 ± 1.1	3.7 ± 1.0	4.2 ± 2.3	8.0 ± 0.6*
AUC weight (g/[animal × 84 days])	161 ± 12	160 ± 17	179 ± 21#	199 ± 16*
AUC solid food intake (g/[animal × 84 days])[a]	271 ± 4	180 ± 18ǂ	266 ± 37	221 ± 1
AUC liquid intake (ml/[animal × 84 days])[a]	448 ± 26	646 ± 60ǂ	477 ± 6	369 ± 12*
Ingested solid kcal (animal × 84 days)[a]	840 ± 13	558 ± 56	1252 ± 176#	1041 ± 3
Ingested liquid kcal (animal × 84 days)[a]	0	388 ± 36	0	221 ± 7
Total ingested kcal (animal × 84 days)[a]	840 ± 13	946 ± 20	1252 ± 176#	1262 ± 11
Plasma cholesterol (mg/dL)	291 ± 14	410 ± 98	772 ± 272##	1337 ± 312*
Plasma triglycerides (mg/dL)	116 ± 16	151 ± 30	339 ± 146##	473 ± 86&
Plasma leptin (ng/mL)	4.8 ± 2.4	5.5 ± 2.9	17.1 ± 5.0##	20.5 ± 3.3

C: Standard solid-chow; F: Standard solid-chow plus a 15% fructose solution *ad libitum*; W: Western solid-chow; W + F: Western solid-chow plus a 15% fructose solution *ad libitum*; VAT: Visceral Adipose Tissue; AUC: Area Under the Curve. Values are expressed as mean ± SD of 5–10 animals, except for Parameters, which were obtained from two cages containing at least 4 animals each.

[a] Parameters which were obtained from two cages containing at least 4 animals each.
# $P < 0.05$ *vs* C.
$P < 0.01$ *vs* C.
& $P = 0.09$ – marginally significant – *vs* W.
* $P < 0.05$ *vs* W.
** $P < 0.01$ *vs* W.

cholesterol synthesis (*hmgcrd*) genes, while in general reducing the mRNA levels of fatty acid oxidation and lipoprotein uptake genes, with the exception of the *cd36* gene, in which mRNA levels were increased by fructose supplementation, especially in the W + F group (×3.09 fold *vs* W). The mRNA level of peroxisome proliferator activated receptor γ (PPARγ), a nuclear receptor involved in the control of CD36 [21], was also significantly increased in livers of W + F rats (×2.95 fold *vs* W).

To find a possible mechanism which explained the increased expression of the *hmgcrd* gene by fructose, we assessed the expression of several micro-RNAs that have been reported to control cholesterol synthesis [22] (Fig. 3). We only detected a clear significant increase in the expression of miR-185-5p and miR-342-5p in the groups of mice receiving a Western-type solid diet. Fructose supplementation in Western diet decreased the expression of miR-122-3p.

3.3. Liquid fructose supplementation in LDLR$^{-/-}$ mice promotes macrophage infiltration in aortic atherosclerosis plaques independently of lipid deposition

To assess the extent of atherosclerosis we determined the lesion percent area in five sequential cross-sections through the heart and aortic origin; quantification of the lesion percent area showed a significant increase in W samples from sections 1 to 5, while Fructose supplementation significantly increased lesion percent area in sections 4 and 5 from W + F animals *versus* W (Fig. 4A). For each of the dietary groups, representative ORO-Hematoxylin-Eosin images of Section 3, corresponding to the aortic valve leaflets, are shown in Fig. 4B; Section 3 was chosen because lesions were visible beneath all three valve leaflets. Quantification of the total lesion area as AUC of lesion areas from section 1 to 5, as shown in Fig. 4C, demonstrated a significant increase in W group (×8.1 fold *vs* C), that was further increased in W + F (×2.6 *vs* W). Total lesion area closely followed the progressive increase in plasma lipids from F through W and W + F mice, presenting a direct and significant correlation with total plasma cholesterol ($r^2 = 0.824$, $P < 0.0001$) and triglycerides ($r^2 = 0.697$, $P < 0.0001$) (Fig. 4D). Nevertheless, the increased atheromatous lesion did not result in stimulation of nuclear factor kappa B (NFκB) mediated inflammation in the vessel wall, as immunodetection of phospho-p65, a marker of NFκB activation, indicated that this parameter was not modified in samples from aortic sections 1 to 5 obtained from the different dietary groups (Fig. 5A).

Again using immunohistochemistry, we detected the presence of macrophages in the lesion area. Macrophage infiltration, detected by specific F4/80 immunohistochemistry (Fig. 5B), did not follow the pattern of increased lesion area, being significantly increased practically in all the aortic sections studied from F group; the W + F group showed a similar pattern, but due to high sample variability statistical significance *vs* W group was only achieved in section 4. A similar behavior

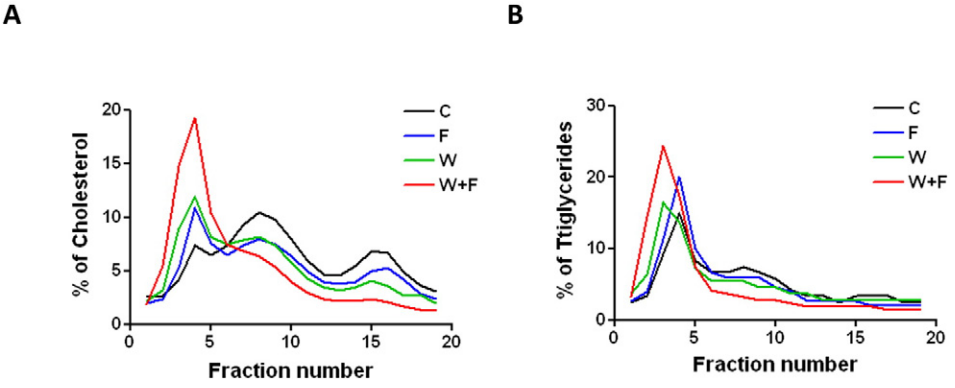


Fig. 1. Percent distribution of total cholesterol (A) and triglycerides (B) among the different FPLC fractions of pooled filtered plasma samples from C (mice fed standard solid-chow), F (mice fed standard solid-chow plus a 15% fructose solution *ad libitum*), W (mice fed Western solid-chow), and W + F (mice fed Western solid-chow plus a 15% fructose solution *ad libitum*). Each fraction was analyzed in duplicate.

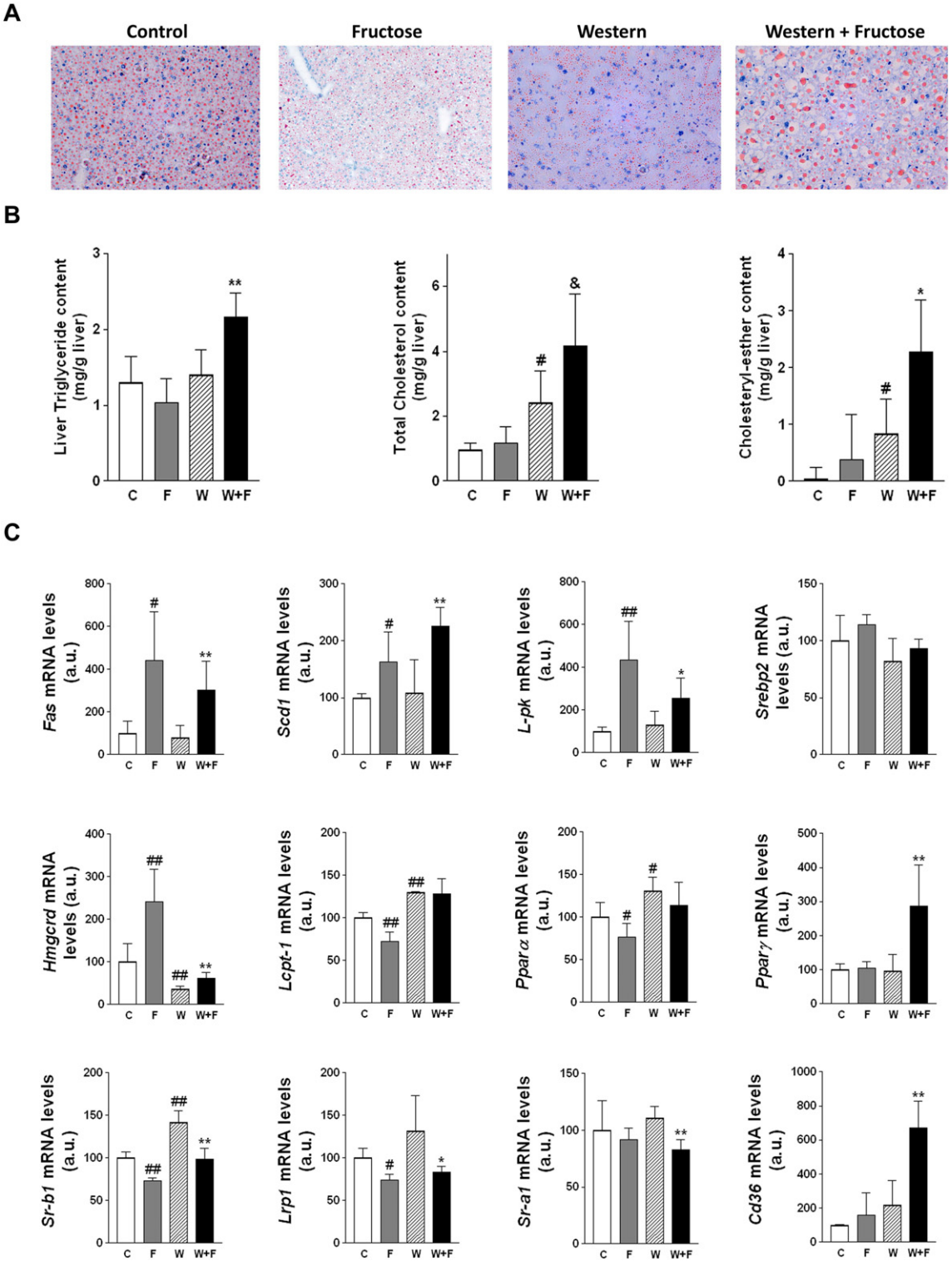


Fig. 2. A. Histological study of liver sections from Control C (mice fed standard solid-chow), Fructose F (mice fed standard solid-chow plus a 15% fructose solution *ad libitum*), Western W (mice fed Western solid-chow), and Western + Fructose W + F (mice fed Western solid-chow plus a 15% fructose solution *ad libitum*). Livers were cryosectioned and processed for histological examination of fat infiltration by Oil-Red O staining. A representative image of liver sections from each experimental group is shown. B. Bar plots showing the liver content of different lipid species from the four experimental dietary groups of mice (mean ± sd of five different liver samples). C. Bar plots showing the relative levels of specific mRNAs from the four experimental dietary groups of mice (mean ± sd of five different liver samples). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* C values; $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* W values.

was observed for the modified lipoprotein receptor oxidized-LDL receptor 1 (LOX1) (Fig. 6A). In the case of another modified receptor studied, the receptor for advanced glycation end products (RAGE), the high dispersion of the immunohistochemical data precluded the validation of any significant change (Fig. 6B).

4. Discussion

A significant relationship between higher sugar consumption and increased risk for cardiovascular disease (CVD) mortality has been found in humans, and the association remains significant after adjusting

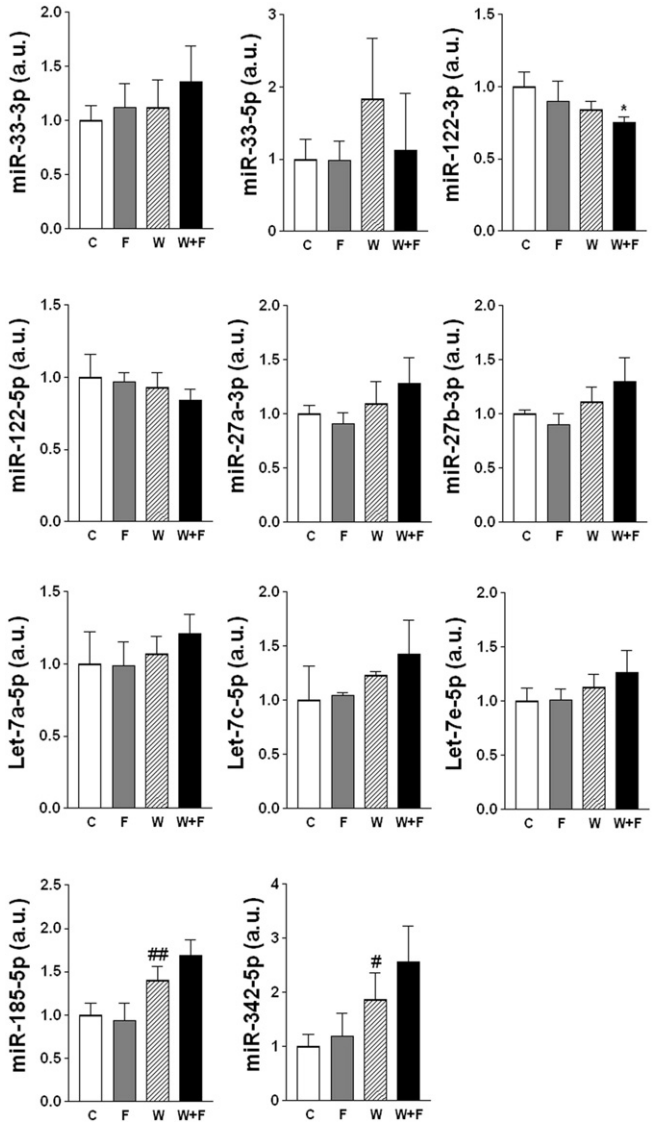


Fig. 3. Bar plots showing the relative levels of specific miRNAs, as mean ± sd, in four different liver samples obtained from C (mice fed standard solid-chow), F (mice fed standard solid-chow plus a 15% fructose solution *ad libitum*), W (mice fed Western solid-chow), and W + F (mice fed Western solid-chow plus a 15% fructose solution *ad libitum*). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* C values; $^{*}P < 0.05$ *vs* W values.

for CVD risk factors [8]. Moreover, although higher sugar intake can be a marker of unhealthy dietary habits, the association is not explained by overall diet quality, and it is also consistently observed in the specific case of sugar-sweetened beverage consumption [8]. As previously reported by Malik et al. [23], one of the proposed causes of this association is fructose, found in similar amounts in sucrose and HFCS. Thus, epidemiological studies indicate that added fructose may increase blood pressure and blood pressure variability, enhance heart rate and myocardial oxygen demand, and contribute to insulin resistance and general metabolic dysfunction in humans (for detailed information see reviews [24,25]). In the present study, we found that at an identical calorie consumption, fructose supplementation in liquid form, besides increasing body weight and visceral adiposity, promoted atherosclerosis in LDL receptor $(LDLR)^{-/-}$ mice fed an unhealthy, Western-type solid chow. This latter effect was directly related to the increase in plasma lipid levels induced by fructose supplementation. Moreover, the addition of liquid fructose to a solid diet significantly increased the expression of a macrophage marker and a modified lipoprotein receptor in atherosclerotic plaques, independently of the extension of the lesion area and the type of solid diet consumed.

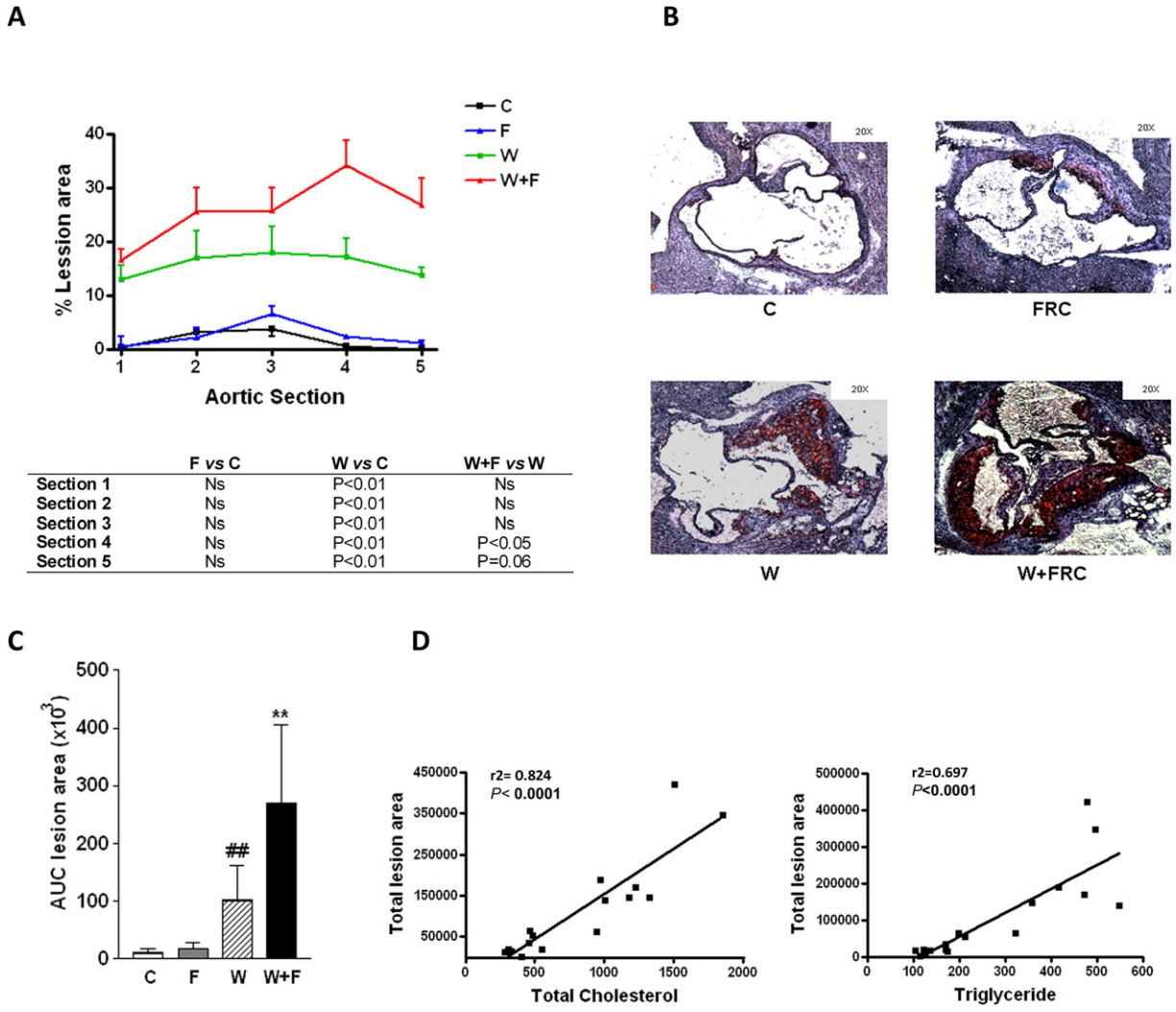
Among the experimental models available for the study of atherosclerosis, non-human primates and rabbits develop atherosclerosis lesions that are more or less similar to those in humans in response to a cholesterol-enriched diet [26]. Besides ethical concerns, non-human primates are both expensive and difficult to house and handle, while rabbits present a disproportionate response to dietary cholesterol. There are few published reports about the effect of fructose supplementation on the development of atherosclerosis in these species. Suzuki et al. [27,28] reported an accumulation of aortic foam cells and infiltrated macrophages after feeding cynomolgus monkeys with a solid diet containing 1% cholesterol and a high proportion of fructose (31%). Akira et al. [29] detected an increase in aortic intimal thickening after feeding a solid diet (1% cholesterol) supplemented with liquid fructose (10% *w*/*v*) to rabbits for 8 weeks. Unfortunately, the effect of the cholesterol-rich solid diet alone was not reported in either case.

Genetically modified rodents are a valid alternative for the study of atherosclerosis [30]. In the two most used models, Apo $E^{-/-}$ and $LDLR^{-/-}$ mice, the former develops spontaneous aortic lesions when fed normal chow, while the latter develops atherosclerosis only when fed a Western-type diet, reproducing the pattern of dietary effects observed in humans. Cannizzo et al. [31] demonstrated enlarged atherosclerotic lesions in aortic and carotid arteries after feeding Apo $E^{-/-}$ mice on normal chow supplemented with liquid fructose (10% *w*/*v*) for 8 weeks. Merat et al. [32] reported an increase in atherosclerosis burden after feeding the more diet-sensitive model, the $LDLR^{-/-}$ mouse, for 5.5 months on a fructose-enriched diet that provided a very unphysiological proportion of energy (61%) from fructose.

To ascertain if the effect of liquid-fructose supplementation on atherosclerosis was different depending on the type of basal dietary substrate, we fed $LDLR^{-/-}$ mice on two different solid diets, a normal, "healthy" mouse chow, and an "unhealthy" mouse diet Western-style, enriched in saturated fat, simple carbohydrates and cholesterol, supplemented or not with a solution of fructose 15% weight/volume. Mice were fed on these diets for twelve weeks, a period sufficient to induce detectable atherosclerosis [15].

4.1. Impact of fructose supplementation on lipid-related parameters

After this feeding period, fructose supplemented mice had consumed an amount of calories from liquid-fructose in the range of the upper quintiles of human consumption (17 and 41% of total energy in W + F and F group, respectively). Nonetheless, the amount of total consumed calories was not significantly increased in both fructose-supplemented groups *versus* their corresponding controls. This finding enabled us to discriminate fructose-derived effects from those driven by a huge increase in the total intake of ingested calories, as in the case between C and W animals (1252 *vs* 840 kcal for W and C, respectively). Of note, while mice on Western diet significantly weighted more than controls (×1.24 fold) at the end of the study, as could be expected from the increase in the total amount of calories ingested (×1.49 fold *vs* C), fructose-supplementation in Western-type fed mice also significantly increased body weight (×1.19 fold *vs* W), despite both groups of mice consuming identical amount of calories. It must be pointed out that animals adjusted their calorie intake through the length of the study spontaneously. Leptin is a key adipocytokine controlling long term energy intake, whose plasma values are directly related to body weight [33]. Plasma leptin concentration and body weight were unchanged after fructose supplementation in LDL receptor $(LDLR)^{-/-}$ mice fed control chow, while they were increased in Western-type fed *vs* control mice. Further, although the difference in plasma leptin between W + F *vs* W values did not reach statistical significance, it increased exactly in the same proportion as the change in final body weight between both groups of animals (×1.2 fold W + F *vs* W, see Table 2). Thus, it can be assumed that, at least under the same solid



	F *vs* C	W *vs* C	W+F *vs* W
Section 1	Ns	P<0.01	Ns
Section 2	Ns	P<0.01	Ns
Section 3	Ns	P<0.01	Ns
Section 4	Ns	P<0.01	P<0.05
Section 5	Ns	P<0.01	P=0.06

Fig. 4. A. Representative plot showing lesion percent area (mean ± sd of 3–5 different heart samples) in five sequential cross-sections through the heart and aortic origin stained with Oil-Red O and Hematoxylin-Eosin of samples obtained from C (mice fed standard solid-chow), F (mice fed standard solid-chow plus a 15% fructose solution *ad libitum*), W (mice fed Western solid-chow), and W + F (mice fed Western solid-chow plus a 15% fructose solution *ad libitum*). The table below Fig. 4A shows the statistical significance for each comparison. For each of the dietary groups studied, representative images corresponding to the aortic valve leaflets (Section 3), are shown (B). C. Bar plot showing the total lesion area (mean ± sd of 4–5 mice) of the AUC from section 1 to 5 from the four experimental dietary groups of mice. D. Correlation plots between total lesion area and either total cholesterol or triglyceride concentrations in plasma samples from the four dietary groups of mice studied. $^{\#\#}P < 0.01$ *vs* C values; $^{**}P < 0.01$ *vs* W values.

food substrate, either control or Western-type, the satiety effect of leptin was preserved after fructose supplementation, thus helping to maintain the total amount of ingested calories to similar values.

Besides the well-known hypertriglyceridemic effect, there was also an increase in plasma cholesterol in the fructose-supplemented animals that reached significance in the W + F group. Although this latter effect could depend on the experimental animal used (LDL-$R^{-/-}$ mice develop hypercholesterolemia under fructose-supplementation (32); rats normally do not), a recent meta-analysis of controlled feeding trials in humans has shown that a high fructose intake increases plasma LDL-cholesterol and total cholesterol concentrations [4]. The increase in plasma cholesterol could not be attributed to a dietary origin, given that the amount of solid chow ingested was similar (W + F *vs* W) or even significantly reduced (F *vs* C) among the dietary groups studied [34]. Our results showing increased liver levels of the specific mRNA for HMGCRD in samples from fructose-supplemented mice, suggests a fructose-driven increase in the endogenous synthesis of cholesterol. This effect can be ascribed to fructose itself, rather than to the increased caloric burden provided by the sugar, as total caloric intake was not different among the "healthy" and "Western" dietary groups, independently of fructose supplementation (see Table 2). The presence of dietary cholesterol in the Western diet could explain the marked reduction in the expression of HMGCRD observed in W and W + F mice. This finding was in agreement with the increased levels of miRNAs, such as miR-342-5p and especially miR-185-5p in the same groups of mice; the increased expression of these miRs has been related to reductions in liver cholesterol synthesis [22] [35]. The progressive reduction in the expression of miR-122-3p, significant in W + F *vs* W mice, in parallel with lipid deposition in liver (see Fig. 2B), would be in agreement with the reported reduction in miR-122 in nonalcoholic fatty liver disease (NAFLD) patients [36]. Unfortunately, none of the changes detected in the studied miRNAs could explain the effect of fructose supplementation on the expression of the *hmgcrd* gene.

The increase in liver fatty acids being incorporated into triglycerides, due to endogenous synthesis (F), diet (W), or both origins (W + F) was probably the main factor responsible for the increase in liver CD36 expression. CD36 in the plasma membrane of hepatocytes facilitates the incorporation of fatty acids and native and modified lipoproteins into liver metabolism [21], and increased liver expression of CD36 has been detected in experimental models of NAFLD and in NAFLD patients [37]. PPARγ, the expression and activity of which has been related to the development of hepatic steatosis [38], controls the expression of CD36.

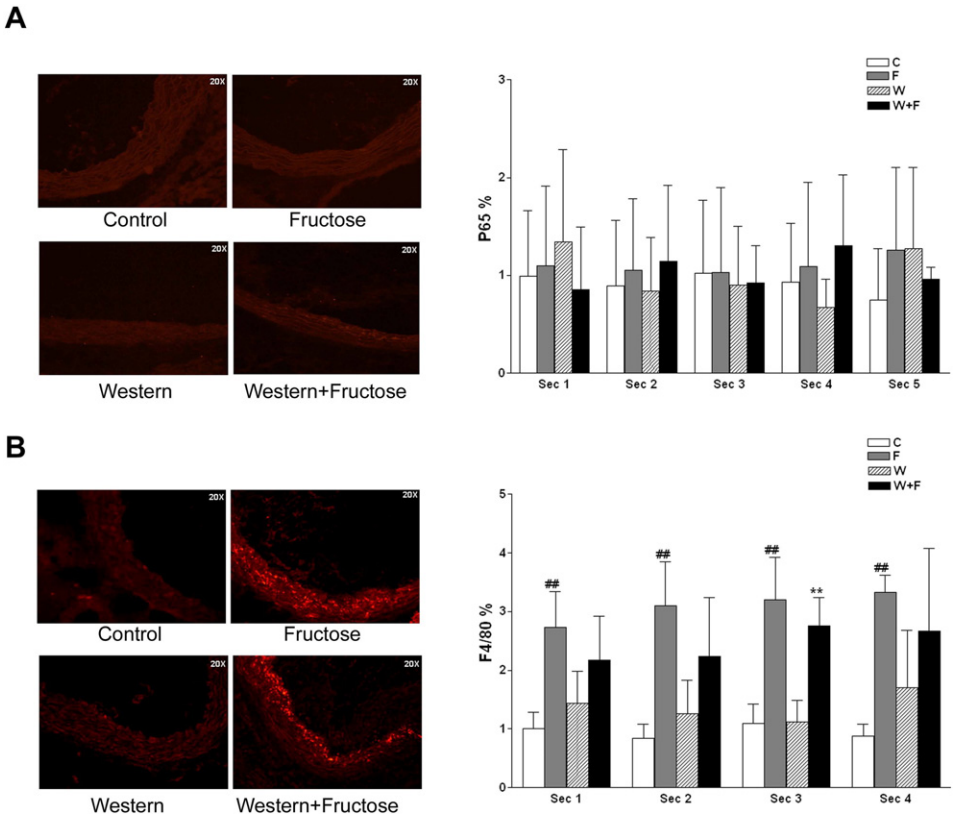


Fig. 5. Representative images of Section 3, corresponding to the aortic valve leaflets, by specific immunohistochemistry for p65 (A) and F4/80 (B) obtained from C (mice fed standard solid-chow), F (mice fed standard solid-chow plus a 15% fructose solution *ad libitum*), W (mice fed Western solid-chow), and W + F (mice fed Western solid-chow plus a 15% fructose solution *ad libitum*). The bar plot on the right-hand of the images shows relative color intensity, as mean ± sd, in 3–5 different images of Section 1 to 4 (F4/80)/5 (p65) obtained from the four experimental dietary groups of mice. $^{\#\#}P < 0.01$ *vs* C values; $^{**}P < 0.01$ *vs* W values.

As fatty acids are endogenous ligands of PPARγ [39], the marked accumulation of lipids in liver, especially in the W + F group, could be responsible for the increased expression of PPARγ and its target gene *cd36*. Based on our data, we cannot rule out the possibility that some of the increase in liver cholesterol concentration could be due to the incorporation of plasma lipoproteins by CD36 binding and internalization.

To summarize, fructose supplementation in liquid form in a background of overweight mice consuming an unhealthy, Western-type solid rodent chow, worsened the metabolic phenotype (visceral obesity and dyslipidemia) of these animals even without increasing their total calorie intake, pointing to a specific, deleterious effect of fructose metabolism. These results are in accordance with recent clinical data showing higher sensibility of already obese subjects to the obesogenic effects of fructose [40].

4.2. Impact of fructose supplementation on atherosclerosis

Lipid deposition and local inflammation in the vessel wall, driven by infiltrated monocyte/macrophages, are accepted causes of atheroma formation [41]. Both factors were markedly altered by fructose supplementation in LDLR$^{-/-}$ mice. As expected, given that plasma lipids – especially cholesterol – are the main recognized risk factors for atherosclerosis [42], there was a direct correlation between plasma lipids and total atherosclerotic lesion area across the four different dietary groups studied. Fructose supplementation significantly increased plasma lipids and total lesion area in mice fed Western-type solid diet and, more important, independently of the total amount of energy consumed. Thus, despite W + F and W mice had exactly the same caloric intake, W + F animals showed increases in plasma cholesterol (×1.73 fold), triglycerides (×1.39 fold) and total lesion area (×2.65 fold) with respect to W values. Thus, fructose had a deleterious effect on atheromatous plaque formation that is due to its particular metabolism; substituting calories provided by the solid Western-diet (221 kcal, see Table 2) with the same amount of calories provided by liquid fructose clearly worsened dyslipidemia and aortic lesion.

The recruitment of blood monocytes to the vessel wall and their transformation into lipid-laden macrophages forming early fatty-streaks constitutes the initial building block of atherosclerosis. Oxidative modification of lipids trapped in the vessel wall is the driving force behind macrophage-lipid engulfment and foam-cell formation [41]. Despite the progressive increase in arterial wall lesion area across the four dietary groups, only the two fructose-supplemented groups showed significant increases in macrophage infiltration, as determined by F4/F80 immunostaining. Macrophage detection paralleled immunodetection of a modified lipid receptor, LOX-1, that is present on the surface of cells involved in the formation of atherosclerosis plaque, including macrophages, and is responsible for the selective uptake of oxidized-LDL [43]. This effect of fructose was also independent of the type of solid food consumed (either standard or Western-type) and of the total energy intake, once again confirming a specific effect of the fructose molecule on plaque formation that was unrelated to the caloric burden provided by its ingestion. Moreover, in this case, fructose supplementation sufficed to induce similar increases in macrophage recruitment and selective LOX-1 detection, irrespective of the lesion area (compare Figs. 4C, 5B, and 6A). The selective increase in F4/F80 and LOX-1 immunostaining in F and W + F groups could be attributed to a specific effect of fructose enhancing the deposition of modified lipoproteins in the vessel wall, which would recruit monocytes through local inflammation and chemoattractant cytokine production [41]. However, we did not detect changes in the immunostaining of phospho-p65, a marker of NFkB activation. NFkB is a pro-inflammatory transcription factor involved in the promotion of inflammation within the arterial wall and atheromatous plaque [44]. The short feeding period (twelve weeks) chosen in our study

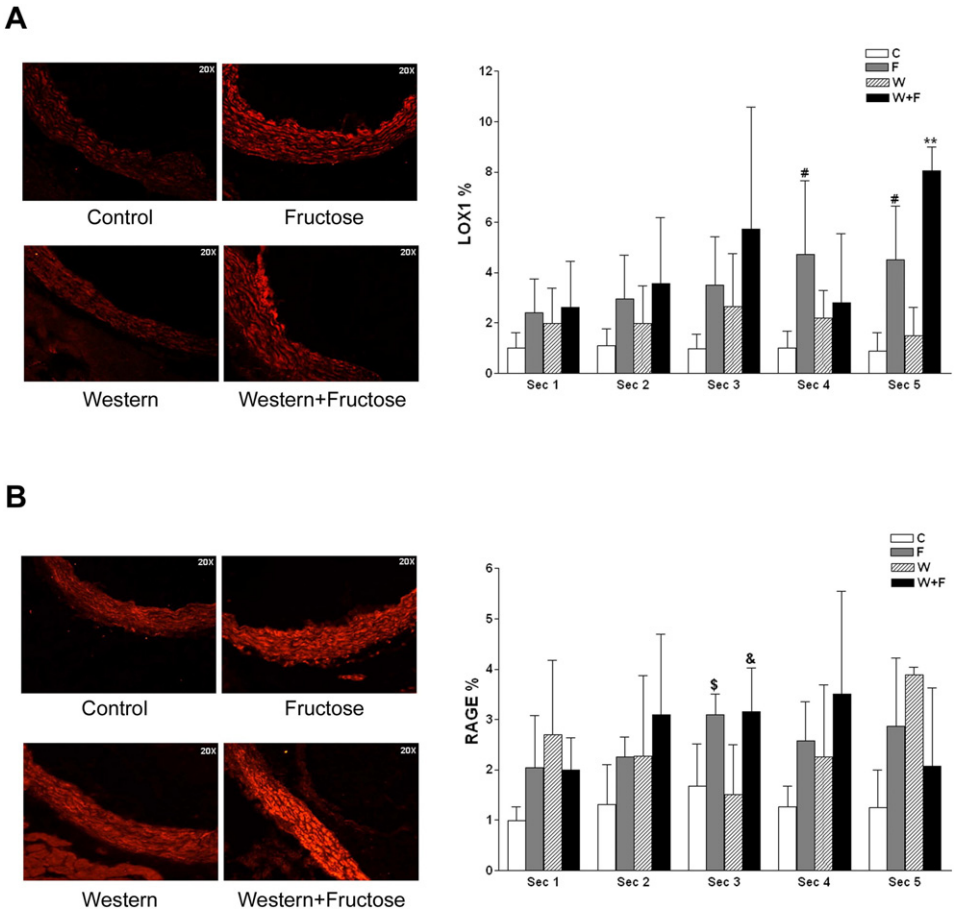


Fig. 6. Representative images of Section 3, corresponding to the aortic valve leaflets, by specific immunohistochemistry for LOX1 (A) and RAGE (B) obtained from C (mice fed standard solid-chow), F (mice fed standard solid-chow plus a 15% fructose solution *ad libitum*), W (mice fed Western solid-chow), and W + F (mice fed Western solid-chow plus a 15% fructose solution *ad libitum*). The bar plot on the right hand of the images shows relative color intensity, as mean ± sd, in 3–4 different images of Section 1 to 5 obtained from the four experimental dietary groups of mice. $^{\#}P < 0.05$ *vs* C values; $^{\$}P = 0.06$ – marginally significant – *vs* C; $^{**}P < 0.01$ *vs* W values; $^{\&}P = 0.09$ – marginally significant – *vs* W.

to induce atherosclerosis plaque formation [15] could imply that there was an insufficient build-up of the inflammatory process for it to be significantly detected by phospho-p65 immunostaining.

4.3. Study limitations

We are well aware of the shortcomings of our experimental model, as regards the direct extrapolation of data obtained from a genetically modified mouse to humans, and the high amount of fructose ingested, which was higher than the average consumption of fructose by humans. Nevertheless, in our animal model fructose was supplemented for a short period of time, while the patterns of human fructose consumption could span many years.

In addition, although the molecular mechanism involved is not clear at this point, our results indicate that fructose is not merely a "calorie provider" in the human-food chain, but also introduces a deleterious effect inherent to its particular metabolism that could accelerate the development of atherosclerosis and cardiovascular disease, especially in overweight people with unhealthy dietary habits.

In conclusion, our results show that fructose consumption in liquid form increases body weight, atherosclerosis and cholesterol burden in $LDLR^{-/-}$ mice independently of energy intake, and significantly in those animals consuming an "unhealthy" solid rodent diet, mimicking a Western-type diet consumed by humans.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijcme.2015.10.002.

Acknowledgments

We would like to thank the University of Barcelona's Language Advisory Service for revising the manuscript.

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