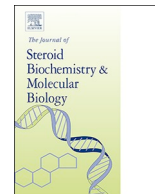




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The deficiency and the supplementation of vitamin D and liver: Lessons of chronic fructose-rich diet in mice

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ABSTRACT

The fructose added to soft drinks and processed food, as well as frequent detection of vitamin D deficiency in the body, are two insults increasingly considered to cause lesions in target organs. We studied the liver after a chronic high-fructose diet deficient and supplemented with vitamin D. Sixty C57BL/6 mature male mice were allocated into six groups (n = 10) for ten weeks: control (C), control deficient in vitamin D (CDD), control supplemented with vitamin D (CDS), fructose (F), fructose deficient in vitamin D (FDD), and fructose supplemented with vitamin D (FDS). The gene expressions of vitamin D receptor and CYP27B1 and 25 hydroxyvitamin D plasma level ensured that the diets caused vitamin D deficiency or supplementation. Body mass did not change, but blood pressure (BP) increased in CDD, F, and FDD, whereas BP was controlled in FDS. Insulinemia, insulin tolerance and resistance were seen in both vitamin D deficiency and fructose groups but improved with vitamin D supplementation. The steatosis and fibrosis were observed in the CDD, F and FDD groups. Also, F and FDD showed activation of stellate cells (HSC). Lipogenesis and inflammation gene expressions were enhanced in the CDD, F and FDD groups, but diminished with vitamin D supplementation. In conclusion, we demonstrated the adverse effects of vitamin D deficiency on metabolism, liver steatosis and, combined with fructose intake, liver interstitial fibrosis with hepatic stellate cell activation, and alteration of the lipogenesis, beta-oxidation, and liver inflammation. All these data improved when vitamin D was supplemented in the animals.

1. Introduction

Fructose, a monosaccharide common in fruits and vegetables, is added to sweeten food and beverages. It is the industrial, not fruit fructose intake that induces metabolic disorders and increased risk of insulin resistance/diabetes [1]. Fructose has a high lipogenic power [2] without significantly affecting adipose tissue mass [3] but enhancing the start and progression of nonalcoholic fatty liver disease (NAFLD) [4] and cardiometabolic alterations [5]. Fructose has become ubiquitous in our food supply, with the highest consumers being teens and young adults [6].

Vitamin D primarily regulates the homeostasis of calcium and phosphorus and is acquired by eating foods such as fatty fish and eggs, and chiefly by cutaneous synthesis through exposure to ultraviolet rays type B. The CYP27B1, the key 1 alpha-hydroxylase that changes 25 hydroxyvitamin D (25 (OH) D) to the hormonal form 125-di-hydroxyvitamin D (125 (OH) 2D), is the ligand for the vitamin D receptor (VDR) [7]. The target genes regulated by vitamin D might control a crowd of biological activities such as the immune system, skin and muscle function, cellular growth, and other biological processes [8].

Although the association between vitamin D deficiency and body mass accumulation is still a matter of debate [9], recent reports re-

Abbreviations: 125 (OH) D, 125-di-hydroxyvitamin D; 25 (OH) D, 25-hydroxyvitamin D; AUC, area under the curve; BM, body mass; BP, blood pressure; Chrebp, carbohydrate-responsive element-binding protein; CYP27B1, 1-alpha-hydroxylase; ELISA, enzyme-linked immunosorbent assay; Fas, fatty acid synthase; FFA, free fatty acids; HE, hematoxylin and eosin; HOMA, homeostatic model assessment; HSC, hepatic stellate cells; IL, interleukin; IR, insulin resistance; NAFLD, nonalcoholic fatty liver disease; Plin, perilipin; Ppar, peroxisome-proliferator-activator-receptor; qPCR, real-time polymerase chain reaction; QUICKI, quantitative insulin-sensitivity check index; Srebp, sterol regulatory element-binding transcription factor; TAG, triacylglycerol; TC, total cholesterol; Tnf, tumor necrosis factor; VDR, vitamin D receptor

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inforce the notion of a definite link of vitamin D deficiency with both general and abdominal obesity [10,11]. Also, studies on the effect of vitamin D deficiency on cardiovascular risk factors and hypertension are conflicting with inconsistent results [12], but recent reports are in favor that vitamin D deficiency raises the risk of developing metabolic dysfunction and increased cardiovascular risk [13–15].

It is noteworthy to say that fructose combined with vitamin D deficiency, may aggravate the harmful effects that each insult already causes [16]. Thus, the current study was planned to examine the impact, mainly in the liver, of vitamin D deficiency and supplementation and the simultaneous consumption of a fructose-rich diet.

2. Materials and methods

2.1. Animals and diets

The study respected the guidelines for the animal ethics committee of the University of the State of Rio de Janeiro, and protocols followed the current standards for animal experimentation (National Institutes of Health Publication No. 85-23, revised 1996). The investigation was submitted and approved by the local ethics committee (protocol number CEUA/36/2016).

Sixty males C57BL/6, 12-weeks-old mice, were individually kept in ventilated cages (NexGen system, Allentown Inc., PA, USA, 12 h/12 h dark/light cycle, $21 \pm 2^\circ\text{C}$ of temperature), and have free access to food and water. They were randomly allocated into six groups ($n = 10$ /group) according to the diet offered (details in Table 1). The formulations followed the AIN93M recommendations for macro- and micro-nutrients [17] and were produced by PragSolucoes (Jau, Sao Paulo, Brazil). The diets were given for ten weeks: C (control diet, including 1000 IU/kg of vitamin D₃); CDD (control diet without vitamin D₃); CDS (control diet supplemented with vitamin D 10,000 IU/kg of vitamin D₃); F (fructose diet, 474.3 g/kg of fructose, including 1000 IU/kg of vitamin D₃); FDD (fructose diet without vitamin D₃); FDS (fructose diet supplemented with vitamin D 10,000 IU/kg of vitamin D₃).

Table 1
Diet composition.

Ingredients (g/kg)	Control			Fructose		
	C	CDD	CDS	F	FDD	FDS
Casein	140.0	140.0	140.0	140.0	140.0	140.0
Cornstarch	620.7	620.7	620.7	146.4	146.4	146.4
Sucrose	100.0	100.0	100.0	100.0	100.0	100.0
Fructose	–	–	–	474.3	474.3	474.3
Soybean oil	40.0	40.0	40.0	40.0	40.0	40.0
Fiber	50.0	50.0	50.0	50.0	50.0	50.0
Vitamin Mix*	10.0	10.0	10.0	10.0	10.0	10.0
Vit D3 (g/kg mix)**	0.25	–	2.50	0.25	–	2.50
Mineral Mix*	35.0	35.0	35.0	35.0	35.0	35.0
Cistin	1.8	1.8	1.8	1.8	1.8	1.8
Colin	2.5	2.5	2.5	2.5	2.5	2.5
Antioxidant	0.008	0.008	0.008	0.008	0.008	0.008
Total	1000	1000	1000	1000	1000	1000
Energy (kcal)	3804	3804	3804	3804	3804	3804
Carbohydrates (%)	76	76	76	76	76	76
Cornstarch (%)	66	66	66	16	16	16
Sucrose (%)	10	10	10	10	10	10
Fructose (%)	0	0	0	50	50	50
Protein (%)	14	14	14	14	14	14
Lipids (%)	10	10	10	10	10	10

Groups: C, control; CDD, control deficient in vitamin D; CDS, control supplemented with vitamin D; F, fructose; FDD, fructose deficient in vitamin D; FDS, fructose supplemented with vitamin D. The vitamin D was added to the Vitamin mix.

* According to the recommendations of the AIN93M [17].

** 400,000 IU/g.

2.2. Intake of food and water, body mass, and blood pressure

The intake of food and water was measured daily (the difference between the quantity offered and the quantity left after 24 h), and body mass (BM, Monday, 11 a.m.) and systolic blood pressure (BP, Friday, 11 a.m.) were assessed weekly. BP was got with tail-cuff plethysmography (Storage Pressure Meter model LE 5002, Panlab Harvard Apparatus, Barcelona, Spain).

2.3. Glucose tolerance, and insulin tolerance, resistance, and sensitivity

One week before euthanasia, glucose was orally given to 6 h fasted animals (glucose 25%, 1 g/kg) to assess glucose tolerance. Three days later, insulin was injected (1 U/kg intraperitoneal) in 4 h fasted animals to estimate insulin tolerance. In both occasions, the blood glucose was measured at zero, 15, 30, 60 and 120 min, and the 'area under the curve' (AUC) was calculated with the trapezoid rule [18] (GraphPad Prism version 8.0 for Windows, GraphPad Software, La Jolla, CA, USA). Also, we estimated the homeostasis model for the evaluation of insulin resistance: HOMA-IR = fasting glycemia (mmol/L) \times fasting serum insulin (IU/mL) / 22.5 [19], and the quantitative insulin-sensitivity check index (QUICKI): $[1/\log(\text{fasting insulin } \mu\text{U/mL}) + \log(\text{fasting glycemia mg/dL})]$ [20].

2.4. Sacrifice and tissue extraction

The animals fasted for six hours, were anesthetized (pentobarbital sodium, 150 mg) and killed by exsanguination (cut of the cervical vessels). Blood was collected and centrifuged (1200g/15 min) at room temperature. The liver was dissected, and fragments of all lobes were taken and prepared for light microscopy ($n = 5$, fixed by immersion in fresh fixative 4% w/v, 0.1 M formaldehyde, pH 7.2 for 48 h, then embedded in Paraplast plus, Sigma-Aldrich Co., St. Louis, MO, USA), or frozen at -80°C for molecular analysis ($n = 5$).

2.5. Biochemistry: liver and plasma

With the use of commercial kits (Bioclin System II, Quibasa, MG, Brazil) we measured fasting glycemia (code K082), hepatic and plasmatic total cholesterol (TC, code K0823), and triacylglycerol (TAG, code K117-2). Also, we used the enzyme-linked immunosorbent assay (ELISA) kits, and measured in duplicate (TP-Reader ELX800, BioTek Instruments Winooski, VT, USA) the plasmatic concentrations of 25 (OH) D (25-Hydroxy Vitamin D EIA, AC-57F1, Immunodiagnostic Systems Limited, Boldon, UK), and with Millipore kits (Darmstadt, Germany): Insulin (#EZRMI-13 K), Adiponectin (#EZMADP-60 K), and Leptin (#EZML-82 K).

2.6. Liver structure

The fixed and embedded fragments were exhaustively sectioned at 5- μm of thickness and stained with hematoxylin and eosin (HE) and Sirius red for light microscopy (Nikon microscope model 80i, and DS-Ri1 digital camera, Nikon Instruments, Inc., New York, USA), or immunofluorescence and confocal scanning laser microscopy.

The sections stained with HE were used to assess the liver steatosis by point-counting. Briefly, the digital images obtained from at least ten nonconsecutive sections per animal were merged with a test-system containing the test-points, and the ratio between the points hitting the fat drops in the hepatocytes and the total points into the frame allow the estimation of the volume density of liver steatosis [21,22]. With the Sirius red stain, we observe the collagen fiber distribution in the liver interstitium (bright field, collagen appeared in red on a pale yellow background) [23].

We performed the antigenic recovery with citrate (pH 6.0), blocking with glycine 2%, followed by blocking with PBS/BSA 5% solution and

incubated with bovine serum albumin in phosphate buffered saline. Then, the antibodies were diluted 1:100 in blocking buffer (PBS/BSA 1%) and incubated overnight at 4 °C. The sections were incubated with anti-perilipin (Plin) 2 (CDSB-PA920084, Cusabio), a cytoplasmic lipid droplet coat protein linked to NAFLD [19], and anti-Reelin (AB78540, Abcam), a large secreted extracellular matrix glycoprotein identified in activated hepatic stellate cells (HSC) [20]. The samples were incubated for one hour with anti-IgG-Alexa 488 action for Plin2, and anti-IgG-Alexa 546 for anti-Reelin (Invitrogen, Molecular Probes, Carlsbad, CA, USA), both diluted 1:100 in PBS/BSA 1%. The slides were assembled with Slow Fade (Invitrogen, Molecular Probes, Carlsbad, CA, USA) to maintain fluorescence. Indirect immunofluorescence images were analyzed by scanning confocal microscope (C2, Nikon Inc., Tokyo, Japan).

2.7. Gene expression (real-time polymerase chain reaction, qPCR)

Total RNA of the liver was extracted using Trizol reagent (Invitrogen, CA, USA). Nanovue spectroscopy (GE Life Sciences) was used to determine RNA amount. Then, 1 µg RNA was treated with DNase I (Invitrogen, CA, USA). Afterward, Oligo (dT) primers for mRNA and Superscript III reverse-transcriptase (both Invitrogen, CA, USA) were applied to the synthesis of first strand cDNA; qPCR used a Biorad CFX96 cyler and the SYBR Green mix (Invitrogen, CA, USA). We used the online software Primer3 to design the primers as described in Table 2. We investigated the genes associated with lipogenesis: *Plin2*; Sterol regulatory element-binding transcription factor 1c, *Srebp1c*; carbohydrate-responsive element-binding protein, *Chrebp*; fatty acid synthase, *Fas*; peroxisome-proliferator-activator-receptor gamma, *Ppar gamma*; beta-oxidation: *Ppar alpha*; inflammation: interleukin, *Il1*; *Il6*; tumor necrosis factor, *Tnf alpha*; vitamin D: receptor, *Vdr* and its activating enzyme, *Cyp27b1*. The endogenous β-actin was used to normalize the expression of the selected genes. After the pre-denaturation and polymerase-activation program (4 min at 95 °C), 44 cycles of 95 °C (10 s) and 60 °C (15 s) were followed by a melting curve program (60–95 °C, a heating rate of 0.1 °C/s). Negative controls consisted of wells in which the cDNA was substituted for deionized water. The relative expression ratio of the mRNA was calculated using the equation $2^{-\Delta\Delta Ct}$, in which $-\Delta Ct$ represents the ratio between the numbers of cycles (CT) of the target genes with the endogenous control.

2.8. Data analysis

The data were shown as the mean and standard deviation (SD). We did not assume equal SD among the groups because of the sample size, and, therefore, used the Brown-Forsythe and Welch analysis of variance (ANOVA), and the Tamhane's T2 multiple comparison tests

(recommended for small samples). Also, a two-way ANOVA was used to assess the contribution and interaction to the results of the two factors, fructose intake, and vitamin D concentration. The *P*-values < 0.05 was consistent with a statistically significant level (GraphPad Prism v. 8.02 for Windows, GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Intake of food and water, body mass, and blood pressure

The animals finished the study without signs of disease (analyzing the hair texture, stool and urine production, and behavior). Diets were isoenergetic, and food intake was not different between the groups (Table 3). There was a higher water intake in the fructose groups compared to the matched control groups C vs. F (+38%) and CDD vs. FDD (+37%) (Fig. 1A). The BM did not differ between the groups (Fig. 1B), but BP was raised in control group with vitamin D deficiency and reduced when vitamin D was supplemented. Also, BP increased in the fructose group compared to the control group but decreased in the fructose group supplemented with vitamin D (Figs. 1C-D).

3.2. Fasting glycemia, glucose tolerance, and insulin tolerance, resistance, and sensitivity, and plasmatic levels of 25 (OH) D and insulin

We checked the metabolism of glucose and insulin. Fasting glycemia increased in the control groups with vitamin D deficiency (Table 3). We did not observe glucose intolerance (Figs. 2A-B), but insulin intolerance (Fig. 2C-D) and insulin resistance (Table 3, HOMA-IR) aggravated by the combination of fructose and vitamin D deficiency and improved by vitamin D supplementation. Vitamin D deficiency diminished insulin sensitivity (Table 3, QUICKI).

Dietary vitamin D restriction lowered plasma levels of 25 (OH) D to an almost undetectable concentration, while dietary vitamin D supplementation was effective in keeping 25 (OH) D levels high (Fig. 3A). The group control supplemented with vitamin D showed low insulin levels. In fructose with vitamin D deficiency group, the insulin concentrations were high compared to its matched control group (Fig. 3B).

3.3. Other biochemical analyses

Liver and plasma TAG, and TC were poorly influenced by fructose and vitamin D in the length of time this study lasted (Table 3). Also, we did not see a difference in the levels of adiponectin (Fig. 3C), but leptin concentrations were lower in the control group with vitamin D deficiency (Fig. 3D).

Table 2
Primers.

Primers	5'-3' - FW	5'-3' - RV
<i>Beta-actin</i>	TGAGACCTTCAACACCCAGCCCA	CGTAGTGGGCACAGTGTGGGTG
<i>Chrebp</i>	CACCTCAGGGAATACAGCCTAC	ATCTTGGTCTTAGGGTCTTCAG
<i>Cyp27b1</i>	CAAATGGCTTTGTCCAGAT	GGTCATGGGCTTGATAGGAA
<i>Fas</i>	TCGAGGAAGGCATACACCT	CACCCACTGGAAGCTGGTAT
<i>Il1</i>	ACGGATTCCATGGTGAAGTC	CTCACAAGCAGAGCACAAGC
<i>Il6</i>	AGTTGCCTTCTTGGGAGTGA	ACAGGTCTGTTGGGAGTGGT
<i>Plin2</i>	AATATGCACAGTGCCAAACCA	CGATGCTCTCTCCACTCC
<i>Ppar alpha</i>	CAAGGCCTCAGGGTACCACTAC	GCCGAATAGTTCGCCGAAA
<i>Ppar gamma</i>	CACAATGCCATCAGGTTTGG	GCTGGTTCGATATCACTGGAGT
<i>Srebp1c</i>	AGCAGCCCCTAGAACAACA	TCTGCCTTGATGAAGTGTGG
<i>Tnf alpha</i>	TCAGCCGATTGCTATCTCA	TGGAAGACTCTCCAGGTA
<i>Vdr</i>	AGCTGATCGAACCCCTCATA	GCAGCACATGTCTTCCTCA

Abbreviations: 25-hydroxyvitamin D-1 alpha hydroxylase (*Cyp27b1*); Carbohydrate-responsive element-binding protein (*Chrebp*); Fatty acid synthase (*Fas*); Interleukin (*Il*).

Perilipin (*Plin*); Peroxisome proliferator activator receptor (*Ppar*); Sterol regulatory element-binding transcription factor (*Srebp*); Tumor necrosis factor (*Tnf*); Vitamin D receptor (*Vdr*).

Table 3
Food intake, biochemistry, insulin resistance and insulin sensitivity.

Data	C	CDD	CDS	F	FDD	FDS
Fasting glycemia (mmol/L)	7.40 ± 0.96	10.53 ± 0.45 [†]	6.28 ± 1.80	9.40 ± 2.86	12.78 ± 2.34	9.00 ± 2.46
Food intake (g/day)	2.67 ± 0.31	2.54 ± 0.15	2.63 ± 0.22	2.71 ± 0.20	2.85 ± 0.19	2.72 ± 0.21
HOMA-IR	8.43 ± 0.75	11.06 ± 0.98	8.23 ± 0.82	9.54 ± 0.57	19.43 ± 2.19 [†]	8.24 ± 0.64 [‡]
Liver TAG (g/L)	1.22 ± 0.20	1.03 ± 0.30	1.24 ± 0.09	2.94 ± 1.29	1.88 ± 0.38	0.93 ± 0.15
Liver TC (mmol/L)	1.55 ± 0.03	1.83 ± 0.14	1.68 ± 0.06	2.30 ± 0.08 [‡]	1.78 ± 0.09 [‡]	1.61 ± 0.05 [‡]
Plasma TAG (g/L)	0.79 ± 0.09	0.83 ± 0.01	0.78 ± 0.06	0.88 ± 0.12	1.05 ± 0.13	0.87 ± 0.08
Plasma TC (mmol/L)	2.79 ± 0.58	3.69 ± 0.09	3.39 ± 0.24	4.43 ± 0.24 [‡]	4.40 ± 0.56	3.66 ± 0.14 [‡]
QUICKi	0.293 ± 0.006	0.270 ± 0.008 [‡]	0.294 ± 0.016	0.271 ± 0.019	0.252 ± 0.007 [†]	0.290 ± 0.006

Groups: C, control; CDD, control deficient in vitamin D; CDS, control supplemented with vitamin D; F, fructose; FDD, fructose deficient in vitamin D; FDS, fructose supplemented with vitamin D.

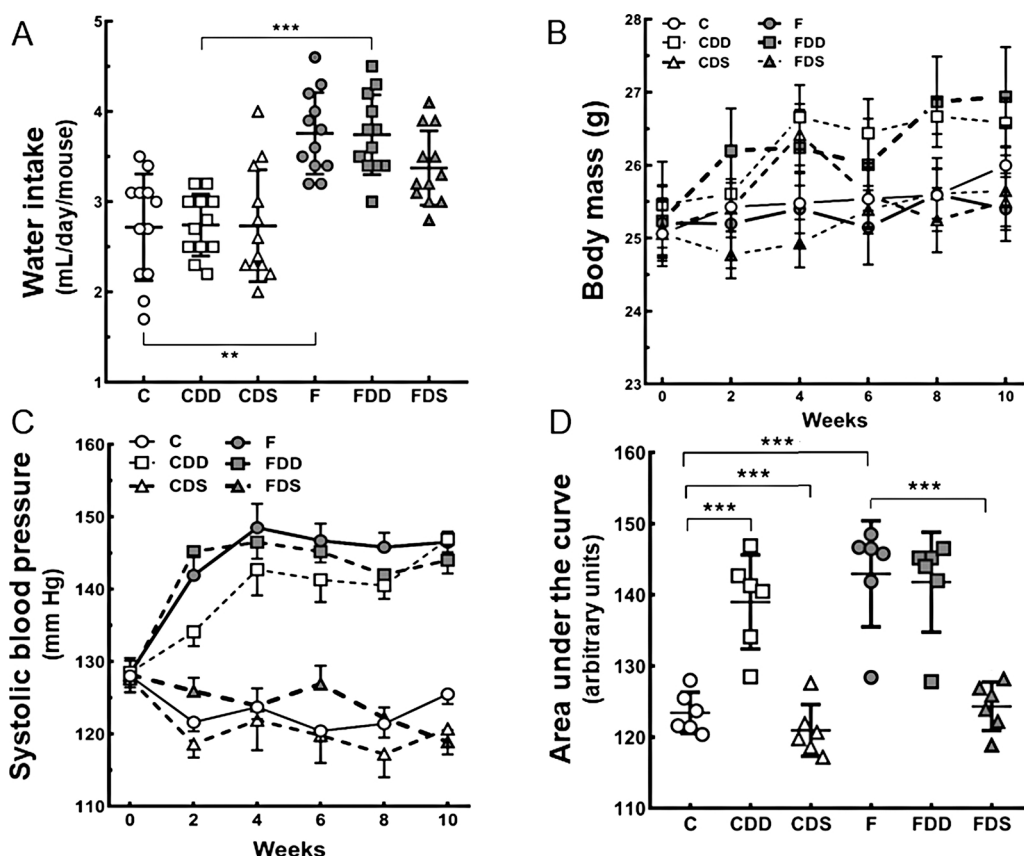
Abbreviations: HOMA-IR, homeostasis model for the evaluation of insulin resistance; QUICKi, quantitative insulin-sensitivity check index; TAG, triacylglycerol; TC, total cholesterol.

Values with mean ± SD (n = at least 5/group), Brown-Forsythe and Welch ANOVA and the Tamhane's T2 multiple comparisons test: $P < 0.05$ when:

* ≠ C.

† ≠ CDD.

‡ ≠ F.



3.4. Liver structure

The vitamin D deficiency and chronic intake of fructose are two additive insults capable of modifying the liver structure. Interstitial fibrosis was intense and widespread in the group control with vitamin D deficiency, the fructose group, and the fructose group with vitamin D deficiency. Though, vitamin D supplementation was beneficial in restoring the liver structure, even with the persistence of the fructose intake (Fig. 4). The HSC activation (identified by the expression of Reelin, Fig. 5) was seen in the fructose group and the fructose group with vitamin D deficiency and might be a reasonable explanation for observed interstitial fibrosis.

The hepatic steatosis was quantified (Fig. 6A) and illustrated

(Fig. 6B). The control group with vitamin D deficiency, fructose group, and fructose group with vitamin D deficiency showed significant steatosis. However, vitamin D supplementation was proved beneficial in reducing steatosis. We were tempted to associate this outcome on steatosis with Plin2 expression in hepatocytes, knowing that Plin2 is involved in lipid droplet formation in the liver and peripheral tissues. We detected an increased Plin2 expression in the control group with vitamin D deficiency, fructose group, and fructose group with vitamin D deficiency. Once more, vitamin D supplementation reduced Plin2 expression in the control group and the fructose group (Figs. 7A-B).

¹ Webpage: www.lmmc.uerj.br.

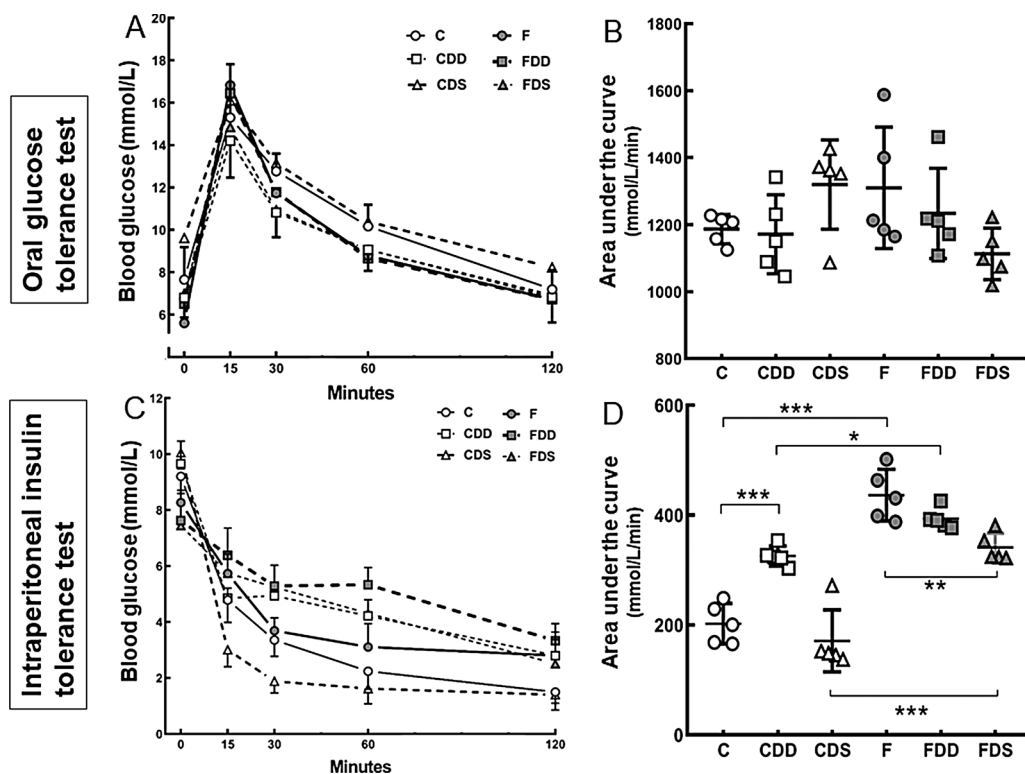


Fig. 2. Glucose and insulin. (A) Oral glucose tolerance test, and (B) Area under the curves of oral glucose tolerance test; (C) Intra-peritoneal insulin tolerance test, and (D) Area under the curves of intra-peritoneal insulin tolerance test with statistical significance. Values are the mean \pm SD (n = 5/group), Brown-Forsythe and Welch ANOVA and the Tamhane's T2 multiple comparison tests: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. **Groups:** C, control; DD, vitamin D deficiency; DS, vitamin D supplementation; F, fructose.

3.5. Gene expression (qPCR)

a) **Lipogenesis and beta-oxidation** - We analyzed the expression of genes related to lipogenesis in the liver (Fig. 8). In general, both

vitamin D deficiency and fructose intake are insults that elevate the expression of these genes. However, the supplementation of Vitamin D might diminish Srebp1c, Fas, and Ppar gamma, except Chrebp (which is more influenced by fructose intake). The gene expression

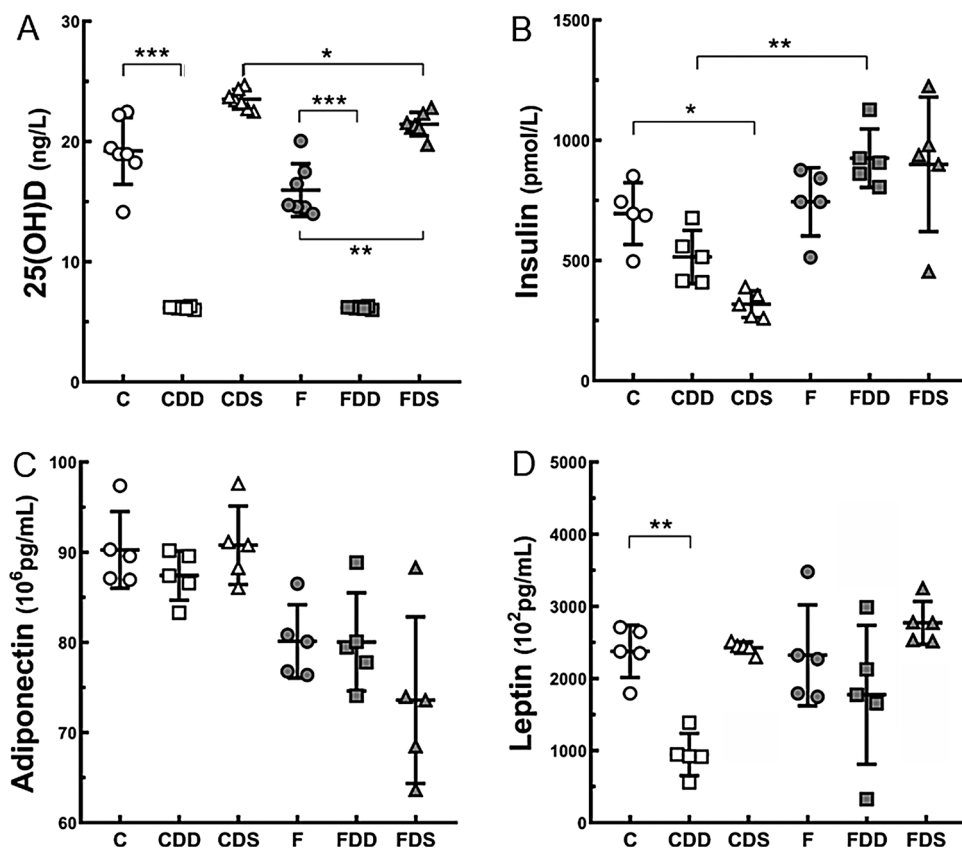


Fig. 3. Plasmatic analyses. (A) 25 (OH) D, 25 hydroxyvitamin D; (B) Insulin; (C) Adiponectin; (D) Leptin. Values are the mean \pm SD (n = 5/group), Brown-Forsythe and Welch ANOVA and the Tamhane's T2 multiple comparison tests: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. **Groups:** C, control; DD, vitamin D deficiency; DS, vitamin D supplementation; F, fructose.

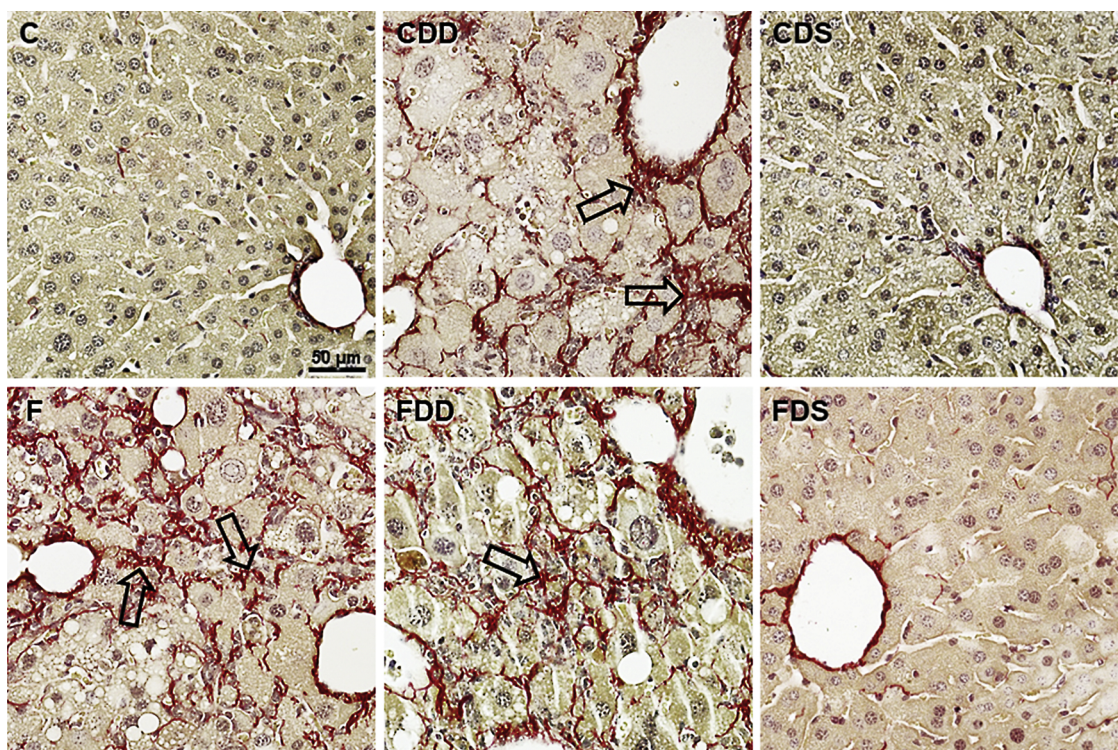


Fig. 4. Liver fibrosis. Representative photomicrographs of the liver tissue stained by Sirius red (same magnification in all photomicrographs). The identification of the groups is indicated in the left upper corner of the photomicrograph. There is interstitial fibrosis (red, open arrows) in CDD and more pronounced in F and FDD. The supplementation of vitamin D restored the liver structure (CDS and FDS). Groups: C, control; DD, vitamin D deficiency; DS, vitamin D supplementation; F, fructose.

of Ppar alpha is linked with beta-oxidation (Fig. 9A), and like lipogenesis, it was increased by vitamin D deficiency and fructose, but decrease by vitamin D supplementation.

- b) **Inflammation** - Some proinflammatory genes were more expressed because of vitamin D deficiency and fructose intake (Il1 and Il6, Figs. 9B-C), whereas Tnf alpha was notably increased due to the ingestion of fructose (Fig. 9D). In all cases, vitamin D supplementation had a beneficial effect in reducing the expression of these genes.
- c) **Vitamin D** - We confirmed the activation state of vitamin D by measuring the gene expression of *Vdr* and *Cyp27b1*. As expected, vitamin D deficiency increased *Vdr* expression (mainly in the control

groups, Fig. 10A), but reduced in vitamin D supplementation. *Cyp27b1*, a ligand for *Vdr* transforming 25 (OH) D in the hormonal form 125 (OH) 2D, was enhanced in the groups submitted to vitamin D deficiency (Fig. 10B).

3.6. Contribution and interaction of the fructose intake and vitamin D: two-way ANOVA

The analysis is summarized in Table 4. We studied three sets of data linked to biometry (seven analyzes), biochemistry (eight analyzes), and gene expression (eleven genes).

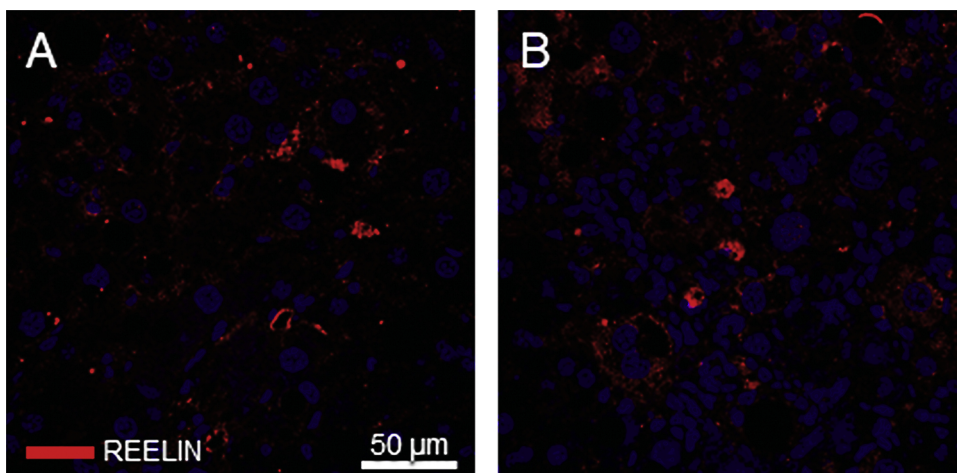


Fig. 5. Activated hepatic stellate cells. Representative sections of the liver tissue prepared for immunofluorescence with the anti-Reelin antibody (red, confocal scanning laser microscopy to identify the hepatic stellate cells, same magnification in all photomicrographs). (A) Fructose (group F); (B) Fructose with vitamin D deficiency (group FDD). The other groups were not shown because of the lack of anti-Reelin staining (indicating that the hepatic stellate cells were not activated in these groups).

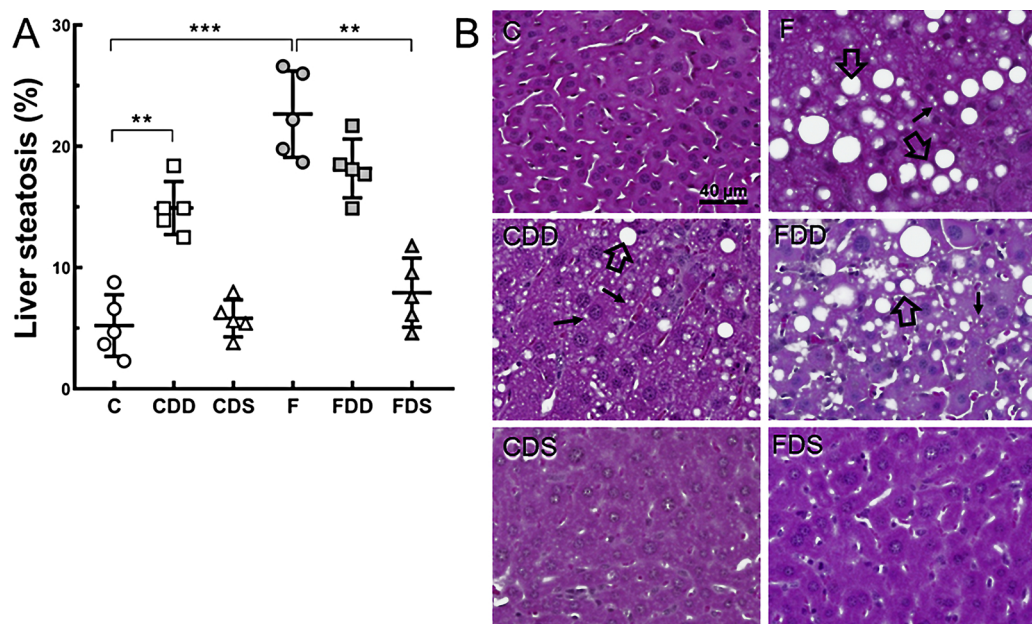


Fig. 6. Liver steatosis. (A) Volume density of liver steatosis quantified with stereology; (B) representative sections of the liver stained with HE (same magnification in all photomicrographs). The identification of the groups is indicated in the left upper corner. Micro- (arrow) and macro-vesicular (open arrow) steatosis were identified in CDD, and the liver structure was restored in F and FDD. Marked steatosis was seen in F and FDD, and the liver structure was renovated in FDS. Groups: C, control; DD, vitamin D deficiency; DS, vitamin D supplementation; F, fructose.

- a) **Biometry** - vitamin D was significant in modifying six of the seven items studied (except water intake) as well as fructose (except BM), corresponding to 86% of the analyses. Fructose and vitamin D interacted in five of the seven data (71%), indicating a strong influence of these two factors in the results.
- b) **Biochemistry** - fructose affected all biochemical data analyzed (100%), and vitamin D changed seven data (88%). Fructose and vitamin D interacted in five of the eight data (63%), also indicating a strong influence of these two factors on the results.

4. Gene expression

Fructose was responsible for altering eight genes (73%), while vitamin D changed all genes analyzed (100%). Fructose and vitamin D interacted modifying eight genes (73%), again reinforcing the interpretation that these two factors might affect the gene expression in this

model.

5. Discussion

Vitamin D is rapidly depleted in rodents after some weeks of dietary restriction, also affected when animals are fed with a high-fat diet [24] or a fructose-rich diet [25]. The murine model has been used to study the effects of vitamin D deficiency [26] and supplementation [27], which could be considered translational for humans [28]. Also, liver and adipose tissue are essentially sites that concentrate vitamin D more than other tissues in the body [29]. Also, as recently demonstrated, adipose tissue is the most critical storage site for vitamin D in the body [30]. In the current study, after ten weeks of dietary restriction, the circulating levels of 25 (OH) D were significantly decreased in comparison with animals did not submit to dietary restriction in vitamin D.

The animals have no significant difference in their BM agreeing with

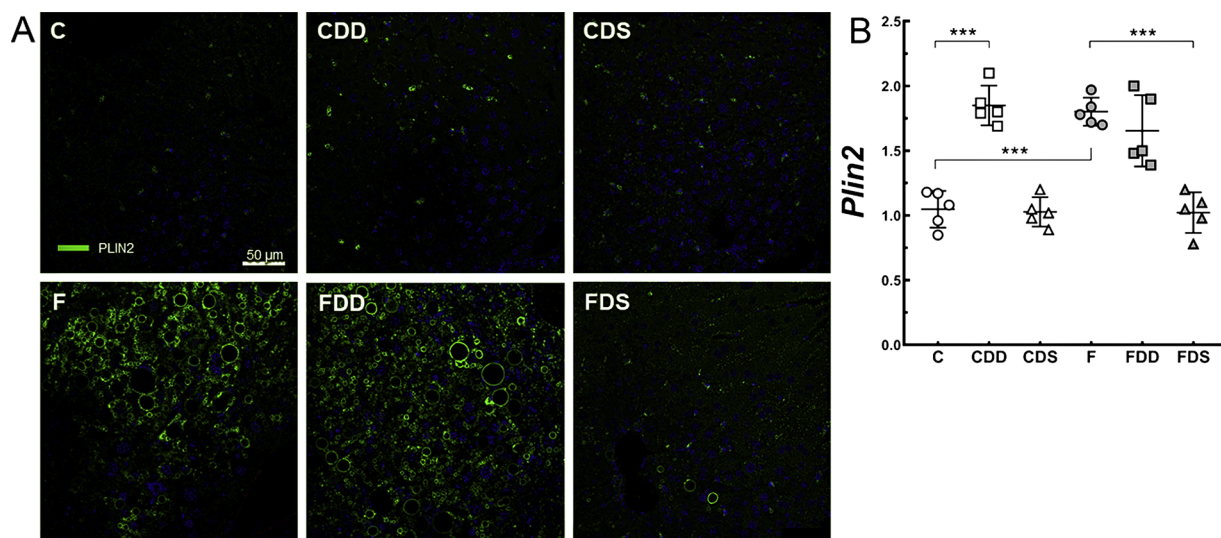


Fig. 7. Perilipin 2 (Plin2). (A) Representative sections of the liver prepared for immunofluorescence with anti-Plin2 (green, confocal scanning laser microscopy to identify a constitutively associated cytoplasmic lipid droplet coat protein, same magnification in all photomicrographs). The identification of the groups is indicated in the left upper corner. There was increased Plin2 stain in CDD, and still more pronounced in F and FDD. In CDS and FDS the liver structure was restored; (B) hepatic gene expression of Plin2. Values are the mean \pm SD (n = 5/group), Brown-Forsythe and Welch ANOVA and the Tamhane's T2 multiple comparison tests: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Groups: C, control; DD, vitamin D deficiency; DS, vitamin D supplementation; F, fructose.

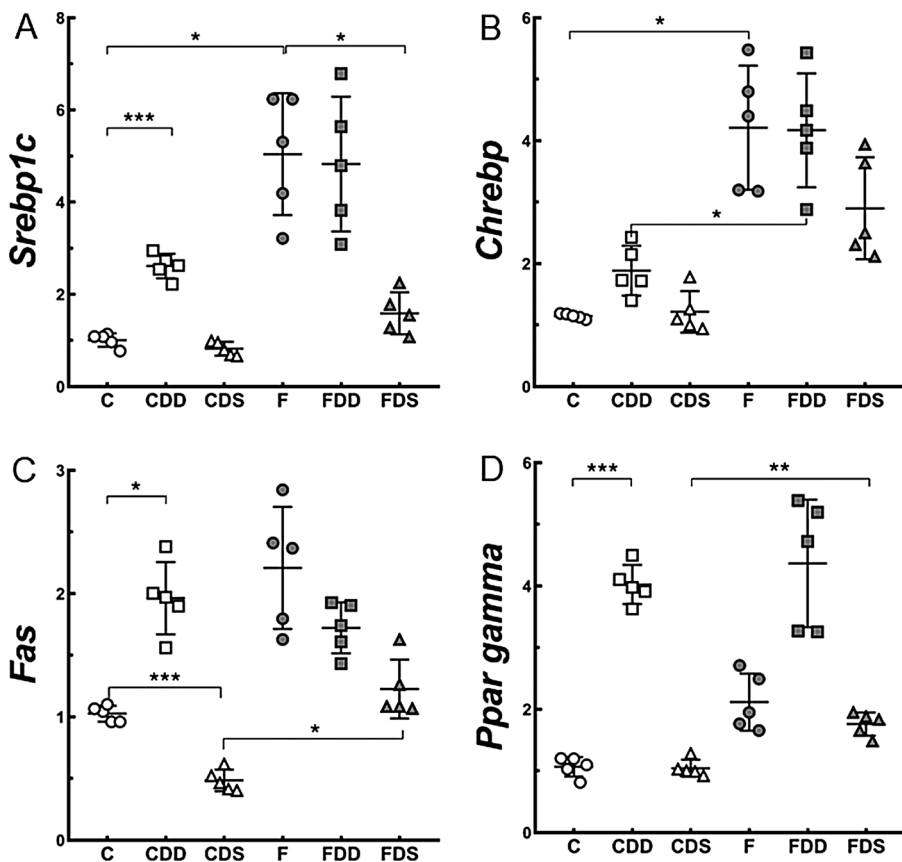


Fig. 8. Hepatic gene expression. (A) *Srebp1c*, sterol regulatory element-binding transcription factor 1c; (B) *Chrebp*, carbohydrate-responsive element-binding protein; (C) *Fas*, fatty acid synthase; (D) *Ppar gamma*, peroxisome-proliferator-activator-receptor gamma. Values are the mean \pm SD (n = 5/group), Brown-Forsythe and Welch ANOVA and the Tamhane's T2 multiple comparison tests: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Groups: C, control; DD, vitamin D deficiency; DS, vitamin D supplementation; F, fructose.

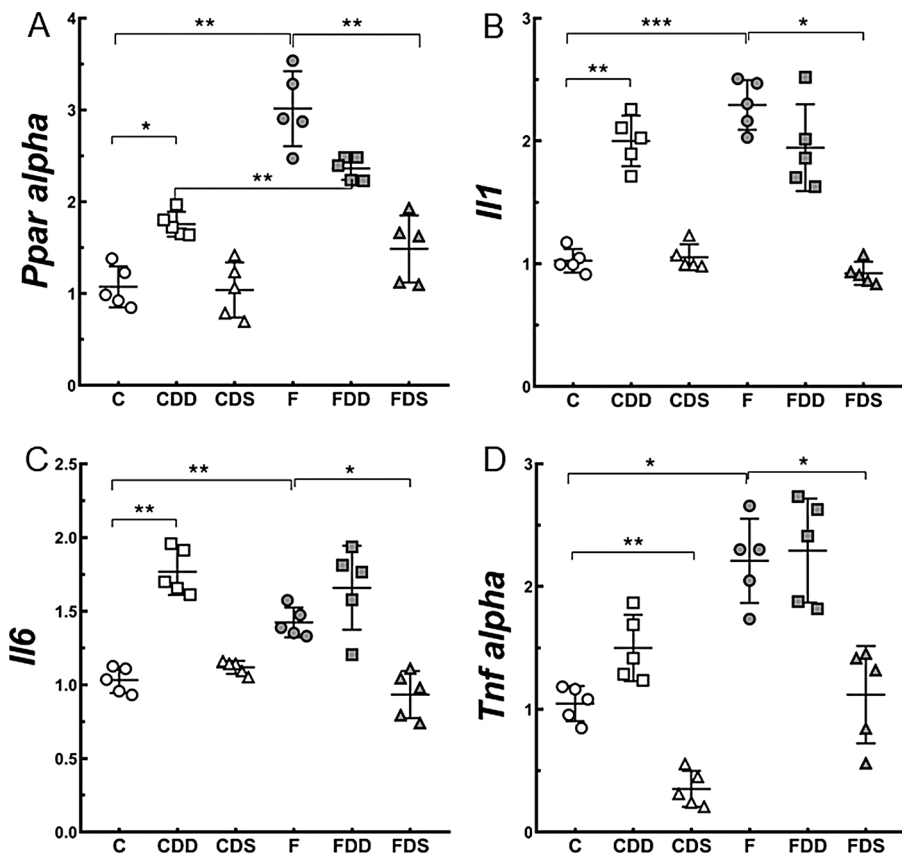


Fig. 9. Hepatic gene expression (continuation). (A) *Ppar alpha*, peroxisome-proliferator-activator-receptor alpha; (B) *Il1*, interleukin 1; (C) *Il6*, interleukin 6; (D) *Tnf alpha*, tumor necrosis factor alpha. Values are the mean \pm SD (n = 5/group), Brown-Forsythe and Welch ANOVA and the Tamhane's T2 multiple comparison tests: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Groups: C, control; DD, vitamin D deficiency; DS, vitamin D supplementation; F, fructose.

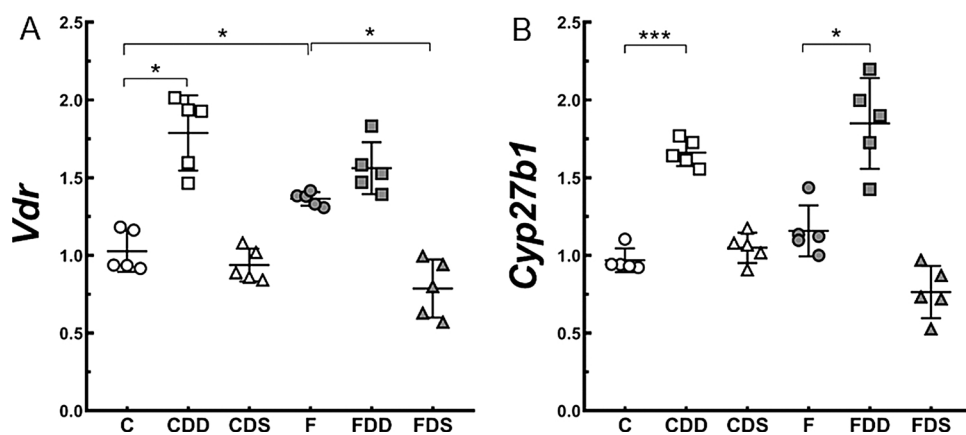


Fig. 10. Hepatic gene expression (continuation). (A) *Vdr*, vitamin D receptor; (B) *Cyp27b1*, *Vdr* activating enzyme. Values are the mean \pm SD ($n = 5/\text{group}$), Brown-Forsythe and Welch ANOVA and the Tamhane's T2 multiple comparison tests: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Groups: C, control; DD, vitamin D deficiency; DS, vitamin D supplementation; F, fructose.

Table 4
Two-way ANOVA: fructose intake vs. vitamin D concentration.

Two-way ANOVA	% of the variation and significance test					
	Interaction		Fructose		Vitamin D	
	%	<i>P</i>	%	<i>P</i>	%	<i>P</i>
Biometry						
Blood pressure	17.40	< 0.0001	13.65	< 0.0001	50.06	< 0.0001
Body mass	3.10	ns	2.17	ns	57.60	< 0.0001
HOMA-IR	12.41	0.0096	13.78	0.0016	47.56	< 0.0001
Intake of food	5.91	0.0043	61.87	< 0.0001	21.96	< 0.0001
Intake of water	1.96	ns	48.90	< 0.0001	1.79	ns
QUICKi	33.14	< 0.0001	10.47	0.0020	35.47	< 0.0001
Steatosis	13.57	0.0484	20.32	0.0037	18.84	0.0179
Biochemistry						
25 (OH) D	0.94	ns	1.74	0.0033	93.4	< 0.0001
Adiponectin	12.02	ns	31.16	0.0009	4.14	ns
Insulin	36.63	< 0.0001	21.87	< 0.0001	41.4	< 0.0001
Leptin	25.2	0.0017	16.68	0.0028	22.02	0.0033
TAG liver	23.03	0.0023	19.04	0.0014	22.85	0.0024
TAG plasma	6.52	ns	28.46	0.0010	16.69	0.0284
TC liver	54.85	< 0.0001	16.96	< 0.0001	19.86	< 0.0001
TC plasma	18.93	0.0010	44.63	< 0.0001	12.02	0.0082
Gene expressions						
<i>Chrebp</i>	4.70	ns	65.37	< 0.0001	6.86	0.0440
<i>Cyp27b1</i>	7.31	0.0044	0.14	ns	79.76	< 0.0001
<i>Fas</i>	21.85	< 0.0001	19.48	< 0.0001	44.13	< 0.0001
<i>Il1</i>	30.72	< 0.0001	9.74	< 0.0001	50.11	< 0.0001
<i>Il6</i>	13.65	0.0008	0.22	ns	68.32	< 0.0001
<i>Plin 2</i>	26.37	< 0.0001	5.57	0.0012	58.09	< 0.0001
<i>Ppar alpha</i>	19.91	< 0.0001	44.32	< 0.0001	24.53	< 0.0001
<i>Ppar gamma</i>	1.06	ns	6.28	0.0007	82.70	< 0.0001
<i>Srebp1c</i>	12.73	0.0009	39.01	< 0.0001	32.21	< 0.0001
<i>Tnf alpha</i>	1.52	ns	38.44	< 0.0001	45.88	< 0.0001
<i>Vdr</i>	10.62	0.0011	0.03	ns	75.55	< 0.0001

Abbreviations: 25 (OH) D, 25-Hydroxyvitamin D; *Cyp27b1*, 1- α -hydroxylase; *Fas*, Fatty acid synthase; HOMA-IR, homeostasis model for the evaluation of insulin resistance; *Il*, Interleukin; *Plin*, Perilipin; *Ppar*, Peroxisome proliferator activator receptor; QUICKi, quantitative verification of the insulin sensitivity index; *Srebp*, Sterol regulatory element-binding transcription factor; TAG, triacylglycerol; TC, Total cholesterol; *Tnf*, tumor necrosis factor; *Vdr*, Vitamin D receptor.

the literature about vitamin D deficiency, supplementation [31], and fructose intake [3,32], although the water intake was higher in the groups fed the fructose diet, which may be linked with the high BP detected in these animals. Besides, both insults fructose and vitamin D deficiency related to an increase of BP because of a direct effect on the renin-angiotensin-aldosterone axis [33,34].

In the current study, vitamin D deficiency affected insulin tolerance and sensitivity in agreement with a recent report (vitamin D

supplementation might restore the impaired muscle insulin signaling) [31] and our previous study of obese animals [24]. However, others did not see an effect of vitamin D supplementation on fasting glucose, insulin, HOMA-IR, plasma lipids, liver enzymes, and BM [35]. Our animals did not show a difference in their adiponectin concentration, which is compatible with the regular BM we have seen, while leptin levels were changed with vitamin D deficiency.

The intake of fructose causes a decrease in insulin sensitivity and promotes dyslipidemia [36], increases *de novo* lipogenesis, reduces the antioxidant defenses, and diminishes mitochondrial biogenesis [37]. Fructose may upregulate *Chrebp* by growing glycolytic flux, affecting glucose homeostasis [38], and lead to insulin resistance and hyperinsulinemia, since insulin activates transcription and proteolytic activation of *Srebp1c* [39] and *Chrebp* [38]. In our findings, vitamin D supplementation could reduce *Srebp1c*, *Fas*, and *Ppar gamma*, while *Chrebp* was connected to fructose. Also, *Ppar alpha* increased in animals deficient in vitamin D and fed a fructose-rich diet and diminished with the supplementation of vitamin D. In another study of our group, the combination of vitamin D deficiency and high-fat diet caused an additive effect on the liver, with increased lipogenesis and reduced beta-oxidation [26]. However, vitamin D supplementation in rodents may attenuate the hepatic steatosis by modulation of the lipid metabolism through the negative regulation of *Srebp1c* and its target genes [40].

The association between vitamin D levels and NAFLD has been increasingly recognized [41]. Here, we demonstrated that the control group with vitamin D deficiency, the fructose group and the fructose group with vitamin D deficiency had improved steatosis in the liver. *Plin2* is a candidate for a molecular marker of prolonged lipid accumulation since it is a vital protein associated with lipid droplets in normal and steatotic liver [42]. *Plin2* was increased in the control group with vitamin D deficiency, and also in the fructose group. Recent literature reported enhancement of the accumulation of lipid droplets in muscle fibers because of the vitamin D deficiency, while its supplementation prevents lipid accumulation and regulating the *Plin2* expression *in vivo* and *in vitro* [43].

Moreover, vitamin D deficiency may increase the expression of proinflammatory cytokines, while its supplementation may attenuate the adverse effects of diet-induced obesity and accompanying inflammation [44,45]. Besides, combined with the postmenopausal insult, vitamin D deficiency is an inductor of inflammatory cytokines in the liver [26]. In the current study, although the expression of the *Il1* and *Il6* genes increased in vitamin D deficient groups, and the *Tnf alpha* gene expression was more affected by the ingestion of fructose, all groups had beneficial effects in these gene expressions when the vitamin D was supplemented.

Liver fibrosis frequently follows all types of chronic liver diseases because of the substantial increase in extracellular matrix synthesis by HSC exceeding the capacity of liver degradation [46]. HSC, liver cells positive for Reelin [47], are the primary source of extracellular proteins

during fibrogenesis [48], and play critical roles in hepatic development with regeneration, hepatic immune regulation, and fibrogenesis in response to liver injury [49]. When hepatic fibrosis occurs, HSC in their resting state is activated [50]. Fructose may induce liver steatosis and then liver fibrosis in *Macaca fascicularis* [51]. Also, liver fibrosis may be modulated by vitamin D (125-dihydroxyvitamin D) through binding to its receptor (Vdr), disrupting many fibrotic pathways [52]. Our observation of the activation of HSC, which occurred mainly in the fructose group and the fructose group with vitamin D deficiency, is an essential original result of this study, mainly because of vitamin D supplementation showed benefits by decreasing interstitial fibrosis in the liver.

6. Conclusion

In conclusion, our study showed that vitamin D supplemented animals have controlled their blood pressure, and improved insulin resistance. Also, our experimental observation demonstrated the adverse effects of vitamin D deficiency on metabolism, liver steatosis and, combined with fructose intake, liver interstitial fibrosis with hepatic stellate cell activation, and alteration of the lipogenesis, beta-oxidation, and liver inflammation. All these data improved when vitamin D was supplemented in the animals.

Disclosure statement

The authors have nothing to disclose.

Declarations of interest:

None

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