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Basic nutritional investigation

Beneficial effects of intermittent fasting on steatosis and inflammation of the liver in mice fed a high-fat or a high-fructose diet

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ABSTRACT

Objective: Intermittent fasting (IF) is a nutritional intervention with significant metabolic effects on the liver that are not yet fully understood. The aim of this study was to investigate the effects of IF on body mass, lipid profile, glucose metabolism, liver lipogenesis, β-oxidation, and inflammation.
Methods: We used cellular and molecular techniques to investigate the effects of IF on 3-mo-old male C57 BL/6 mice that were fed control (10% kcal fat), high-fat (HF; 50% kcal fat), or high-fructose (HFr; 50% kcal fructose) diets for 8 wk. Half of the animals were submitted to IF (1 d fed, 1 d fast) for an additional 4 wk.
Results: Although food intake on the fed day did not differ between the groups, mice in the HF and HFr groups showed diminished body mass, total cholesterol, and triacylglycerol levels. Also, plasma adiponectin increased in the HFr group and leptin decreased in the HF mice. Oral glucose tolerance test and insulin were ameliorated by IF, regardless of the diet consumed (HF or HFr), and decreased hepatic lipogenesis and increased β-oxidation markers, resulting in a reduction of the hepatic steatosis and inflammation.
Conclusions: There were beneficial effects of IF even with the continuity of the obesogenic diet and proinflammatory diet in mice. It is recommended that based on the beneficial effects of IF on glucose and liver metabolism and inflammation that IF be a coadjutant factor in the treatment of hepatic metabolic issues and steatosis.

Introduction

Intermittent fasting (IF) is characterized by periods of voluntary abstinence from food and drink, associated or not to religious and other practices [1]. IF improved insulin sensitivity, with effects on blood glucose, adiposity, and type 2 diabetes mellitus (T2DM) in rodents [2] and humans [3], making it an option to alleviate the deleterious effects of unhealthy diets. Fasting organisms enter into alternative metabolic stages, which rely less on glucose and more on ketone body-like carbon sources [4], reducing body mass (BM) [5] and enhancing cognitive functions [6]. The mechanisms involved with IF are related to adaptive cellular stress response signaling pathways, improving mitochondrial health, DNA repair, autophagy, promotion of stem cell-based regeneration, and long-lasting metabolic effects [7].

The high-fat diet (Western or "cafeteria" diet) is a primary factor associated with obesity and its comorbidities, even in rodents [8,9]. The consumption of a high-fat diet (HFD) causes adipose tissue expansion, and triacylglycerol (TG) stored in this tissue can be hydrolyzed to release glycerol and free fatty acids (FFA). The FFA generated by lipolysis are conducted to the liver and are used to synthesize TG, which accumulate in the liver, favoring the development of non-alcoholic fatty liver disease (NAFLD) [10,11].

Also, the fructose component of sugar may be particularly harmful because of its unique metabolic properties, and fructose-rich diets can rapidly produce all the key features of the metabolic syndrome [12], without a significant increase in BM [13]. Excess fructose leads to an increase in the production of acetyl-coenzyme A (acetyl-CoA), which is not metabolized by the tricarboxylic cycle,

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accumulating in the cytoplasm. Excess acetyl-CoA will serve as a substrate for de novo lipogenesis, being converted into malonyl-CoA, which will inhibit mitochondrial β-oxidation [14]. Therefore, a high-fructose diet may generate NAFLD, but the mechanisms involved are uncertain.

Therefore, the present study aimed to investigate an animal model that presented metabolic disorders when stressed by a HFD or high-fructose (HFr) diet, to observe the effect of IF, especially in the hepatic lipogenesis, β-oxidation, and inflammation pathways.

Materials and methods

Animals and diets

The experiment was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of the State of Rio de Janeiro.

The terminology of genes and proteins was standardized [15]: Gene symbols were italicized, with only the first letter in uppercase; protein symbols were the same as the gene symbol, but all were uppercase and not italicized.

Three groups (n = 40 each) of 12-wk-old male C57 BL/6 mice were kept for 8 wk with free access to food and water (initially five animals per ventilated cages under controlled conditions, NexGen system, Allentown Inc., PA, USA; 20 ± 2° C and 12 h/12 h dark/light cycle): conrol group (C; 10% kcal lipids, 14% kcal proteins, 76% kcal carbohydrates, 380 kcal /100 g); HF (50% kcal lipids, 14% kcal proteins, 36% kcal carbohydrates, 500 kcal /100 g); HFr (10% kcal lipids, 14% kcal proteins, 76% kcal carbohydrates, 50% kcal of carbohydrates from fructose, 380 kcal/100 g). Pellets were produced by PragSolucoes (Jau, SP, Brazil) based on the AIN93 recommendations for rodents [16] (Supplementary Table 1).

After week 8, the animals were housed individually, half of each group continued to consume its respective diets (n = 20), and the other half underwent IF (n = 20) for an additional period of 4 wk. The IF protocol alternated 24-h feeding and 24-h fasting periods, and the new groups were labeled as C (control group), C-IF, HF, HF-IF, HFr, and HFr-IF.

Oral glucose tolerance test and homeostasis model assessment for insulin resistance

The oral glucose tolerance test was analyzed before (week 8) and after IF (week 12). The 6-h fasting animals (1300 h–1900 h), received glucose 1 g/kg (25% in sterile saline) by orogastric gavage. Blood samples were collected at 0, 15, 30, 60, and 120 min after glucose administration, and the area under the curve was calculated (GraphPad Prism v. 7.04 for Windows, La Jolla, CA, USA). Also, the homeostasis model assessment for insulin resistance (HOMA-IR), to evaluate insulin resistance, was calculated as (fasting insulin × glucose)/22.5 [17].

Body mass and food intake

BM was measured every Friday morning (1000 h), and food intake (FI, the difference between the food provided and the amount of food left in the cage after 24 h) was determined daily (0700 h). The cumulative FI was established including all periods (days of fasting and days on feed). Energy intake was the product of food consumption by the energy content of the diet.

Sacrifice

Food-deprived animals (from 1300 h–0700 h) were anesthetized (sodium pentobarbital, 150 mg/kg intraperitoneal), then blood was collected, centrifuged (1200 *g* for 15 min), and analyzed. The liver was dissected, weighed, and fragments of all lobes were fixed for light microscopy (formaldehyde 4% w/v, 0.1 M phosphate buffer pH 7. 2), or frozen at −80°C.

We carefully dissected and weighed the fat pads epididymal (located in the lower part of the abdomen and connected to the epididymis), retroperitoneal (attached to the posterior abdominal wall near the kidneys), and inguinal (subcutaneous adipose tissue between the lower part of the rib cage and the thigh). The adiposity index was estimated as the ratio between the sum of epididymal, retroperitoneal, and inguinal fat pads and the total BM [18].

Plasma

We measured total cholesterol (TC), TG, aspartate aminotransferase (AST), and alanine aminotransferase (ALT; commercial kits, Bioclin System II, Quibasa, MG, Brazil). Insulin, leptin, and adiponectin were measured in duplicate (TP-Reader ELX800, BioTek Instruments Winooski, VT, USA). The enzyme-linked immunosorbent assay (ELISA) kits (Millipore, Merck, Temecula, CA, USA) were insulin (EZRMI-13 K), leptin (EZML-82 K), and adiponectin (EZMADP-60 K).

Liver

Fixed fragments embedded in Paraplast plus (Sigma-Aldrich Co., St. Louis, MO, USA) were sectioned (5 μm) and stained (hematoxylin and eosin). Digital images were obtained (BX51 microscope, DP71 digital camera, Olympus Co., Tokyo, Japan) and the volume density of steatosis in the liver (Vv [steatosis, liver]) was assessed by point counting (≥10 random fields per animal), as described [19]. Briefly, a system made up of test points was superimposed with images. Vv (steatosis, liver) was the ratio between the points hiting the fat drops (P_P [steatosis, liver]) and the total points of the system (P_T): Vv [steatosis, liver] = P_P [steatosis, liver] / P_T [20]. Also, hepatic TC and TG were measured (ultrasonicated 50 mg of liver tissue processed with isopropanol). The homogenate was centrifuged (1200*g* for 15 min), and the supernatant was used for the analyses (Bioclin System II, Quibasa, MG, Brazil).

Confocal laser scanning microscopy

In the slices, antigenic recovery used citrate buffer (pH 6.0) for 20 min and blocks with ammonium chloride and 2% glycine and then incubated with the primary antibodies. The secondary antibodies (anti-PLIN 2, Perilipin, CSB-PA920084 - Cusabio, College Park, MD, USA) were conjugated to the Alexa fluorophore, and the sections were mounted with slow-fade (Invitrogen, Thermo Fisher, Waltham, MA, USA). The slices were observed with a confocal laser scanning microscope (Model C2; Nikon Instruments, Inc., New York, NY, USA).

Gene expressions (quantitative polymerase chain reaction)

The total RNA was isolated with Trizol (Invitrogen, Thermo Fisher, Waltham, MA, USA) and mRNA was quantified (GE Healthcare Life Sciences, Piscataway, NJ, USA) with 1 μg of RNA treated with DNAse I (Invitrogen). The cDNA was synthesized from the mRNA of the samples and cDNA was mixed with the primer of the gene of interest and Sybergreen Mix and amplified. Primers were designed using the Primer3 software (Supplementary Table 2); quantitative polymerase chain reaction was performed with the CFX96 cycler (Bio-Rad, Hercules, CA, USA). The expression of each target gene was standardized using β-actin, and the ratio of relative mRNA expression was made with the equation 2-ΔΔCT -ΔCT expressed as the difference between the number of cycles (CT) of the target gene and the endogenous control.

Protein expressions (Western blot)

- Total protein was extracted from liver fragments in homogenizing buffer containing protease inhibitors. Equivalent quantities of total protein resuspended in sodium dodecyl sulfate (SDS)-containing sample buffer were heated for 5 min at 100°C and separated by SDS-polyacrylmide gel electrophoresis (PAGE). After electrophoresis, the proteins were electroblotted onto polyvinyl difluoride transfer membranes (Amersham Biosciences, Piscataway, NJ, USA). The blockade of the membrane and homogenates were incubated with the primary antibodies:
- Lipogenesis: sterol regulatory element-binding protein (SREBP1)-c (68 kDa; SC367), carbohydrate-responsive element-binding protein (ChREBP; 93 kDa; SC33764), fatty acid syntase (FAS; 270 kDa; SC20140), peroxisome proliferator-activated receptor (PPAR) γ (67 kDa; SC7273)
- β-oxidation: PPAR α (55 kDa; SC9000), acyl-CoA oxidase (ACOX1; 4 kDa, SC7476)
- Inflammation: interleukin (IL)1 β (17 kDa, SC52012); IL6 (21 kDa, ab7737); nuclear factor (NF)κB (65 kDa, SC109); tumor necrosis factor (TNF)α (26 kDa, SC1350) (β-actin 43 kDa; SC81178 served as a loading control)

We used electron chemiluminescence for protein expression detection system and the molecular imaging ChemiDoc XRS Systems (Bio-Rad, Hercules, CA, USA). The chemiluminescence intensity of the bands was quantified using the ImageJ software (v.1.51, imagej.nih.gov/ij, USA).

Statistical analysis

The data were presented as the mean and SD after testing for normal distribution and homogeneity of variances (Brown-Forsythe test). Before IF, the differences among the groups were analyzed by one-way analysis of variance (ANOVA) and the post hoc test of Holm-Šídák. The BM evolution analysis was made at each time point (a cross-sectional study). After IF, we performed a two-way ANOVA with the post hoc test of Holm-Šídák to assess the interactions between diet and IF (GraphPad Prism v. 7.04 for Windows, La Jolla, CA, USA). In all cases, $P < 0.05$ was considered statistically significant.

Results

The percent variation in the data was calculated as the ratio between the group variation we wish to know (e.g., HF group) relative to another group (e.g., control group) minus 1 (the unity), and the result is presented as a percentage.

IF affects BM, fasting glucose, and adiposity index, but not food intake

The BM in the HF group showed a difference from the control group beginning in week 6 until the end of the study. When IF started progressively, BM diminished, ending significantly different from the mice that did not fast (Fig. 1).

In week 8, BM increased 12% in the HF group compared with the control arm; there was no difference in FI between the groups; FG increased 19% in the HF group compared with the control arm and 32% in the HFr group compared with control (Table 1, Fig. 2).

In week 12, BM was 15% greater in the HF arm than in the C but diminished because of IF. C-IF versus C (−12%), HF-IF versus HF (−11%), HFr-IF versus HFr (−16%) were associated with a lower cumulative FI: C-IF versus C (−51%), HF-IF versus HF (−53%), and HFr-IF versus HFr (−56%). Accordingly, FG was higher in HF versus C (+35%) and HFr versus C (+43%), and IF did not change it (Table 1, Fig. 2).

The adiposity index was 105% greater in the HF group than in the C group but diminished because of IF. C-IF versus C (−26%), HF-IF versus HF (−47%), HFr-IF versus HFr (−45%). The HF-IF group remained bigger than the C-IF group (+47%; Table 1).

IF may affect plasmatic concentrations of TG, TC, adiponectin, insulin, and leptin

TG and TC varied similarly in plasma: HF versus C (TAG +43%, TC +31%), HFr versus C (TAG +63%, TC +55%), HF-IF versus HF (TAG −22%, TC −20%), HFr-IF versus HFr (TAG −7%, TC −20%).

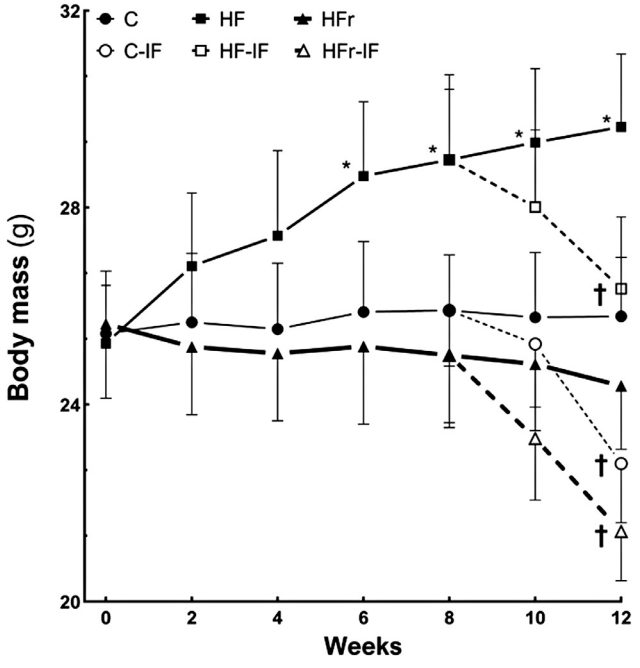

Fig. 1. Body mass evolution (mean and SD). Statistical significance ($P < 0.05$):* ≠ control. † ≠ matched group without IF. C, control; HF, high fat; HFr, high-fructose; IF, intermittent fasting.

Likewise, changes in adiponectin, leptin, and insulin followed similarly, although not entirely: adiponectin, HF versus C −56%; HFr versus C, −58%; HFr-IF versus HFr, +71%; leptin, HF versus C, +141%; HFr versus C, +35%; HF-IF versus HF, −29%; insulin, HF versus C, +35%; HFr versus C, +35%; HF-IF versus HF, −27%; HFr-IF versus HFr, −31% (Table 1).

It was frequent that diet and IF alone or in combination affect these data, but TG and adiponectin showed no interaction between diet and IF (Table 2).

IF decreases HOMA-IR, attenuating insulin resistance

HOMA-IR was higher in the HF group (+81%) and the HFr group (+91%) compared with C. The HF-IF group had a lower HOMA-IR than the HF group (−31%), and the HFr-IF group showed lower HOMA-IR than the HFr group (−38%; Table 1).

IF lessens markers of injury and treats steatosis in the liver

Elevated levels of ALT and AST observed in HF and HFr animals were controlled by IF, mainly ALT: HF versus C (ALT +119%, AST +93%), HFr versus C (ALT +53%, AST +99%), HF-IF versus HF (ALT −31%, AST −27%), HFr-IF versus HFr (ALT −29%; Table 1). ALT and AST were affected by both the diet and IF. In the AST result, diet and IF showed and interaction. (Table 2).

The liver parenchyma showed abundant steatosis in the groups HF (HF versus C, +468%) and HFr (HFr versus C, +516%), which was partially controlled by IF (HF-IF versus HF, −25%; HFr-IF versus HFr, −37%; Fig. 3A,B).

IF acts on PLIN2 marker and lessens the formation of lipid droplets in the liver

PLIN2 was increased in the HF and HFr groups (HF versus C, +386%; HFr versus C, +461%) and IF significantly reduced PLIN2 (HF-IF versus HF, −62%; HFr-IF versus HFr −50%; Fig. 4A). The immunofluorescence for PLIN2 in the groups observed under the confocal microscope is very expressive (Fig. 4B).

IF efficiently reduces the lipogenesis in the liver

Gene and protein expressions of hepatic lipogenesis markers showed the same result: an increase in HF and HFr groups and a reduction in IF (Figs. 5A–F, 6A,B; only PPAR γ had no reduction in the HFr-IF group compared with the HFr group; Fig. 6C).

We studied nine markers of hepatic lipogenesis and all were affected by diet and IF as independent factors. Only the protein expression of PPAR γ showed no effect of the interaction between diet and IF (Table 2).

IF is relevant for improving the hepatic β-oxidation

With the markers of β-oxidation in the liver (gene and protein), the results were somewhat different from those observed in lipogenesis. β-oxidation was decreased in the HF and HFr groups compared with C, but here IF was beneficial only to the HFr group (not in the HF group; Fig. 6D–F), except for PPAR α (Fig. 7A,B).

All five hepatic β-oxidation markers were affected by diet or IF as an independent factor, except PPAR α (protein expression), which was not affected by IF. Diet and IF did not interact with the results of ACOX and PPAR α (gene and protein; Table 2).

Table 1
Feeding behavior and biochemistry (mean ± SD)

Data	C		HF		HFr	
Before IF – week 8						
Body mass (g)	25.9 ± 1.7		29.1 ± 2.3†		25.0 ± 1.6	
Fasting glucose (mmol/L)	5.3 ± 0.4		6.3 ± 0.5†		7.0 ± 0.6*	
	C	C-IF	HF	HF-IF	HFr	HFr-IF
After IF – week 12						
Adiponectin (10^6 pg/mL)	11.7 ± 2.6	11.7 ± 2.3	5.1 ± 0.4*	6.5 ± 0.8	4.9 ± 0.9†	8.4 ± 1†
Adiposity index (%)	2.69 ± 0.4	1.98 ± 0.1†	5.53 ± 0.8†	2.92 ± 0.4†	2.81 ± 0.6	1.55 ± 0.6†
Body mass (g)	25.8 ± 2.2	22.8 ± 2‡	29.6 ± 2.6*	26.4 ± 2†	25.4 ± 1.6	21.4 ± 2.3†
Fasting glucose (mmol/L)	5.4 ± 0.2	5.5 ± 0.2	7.3 ± 0.5*	7 ± 0.4	7.7 ± 0.3†	6.8 ± 0.8
Food intake (g/mouse/fasting day)	2.3 ± 0.1	-	2.2 ± 0.2	-	2.3 ± 0.06	-
Food intake (g/mouse/fed day)	2.3 ± 0.1	2.3 ± 0.2	2.2 ± 0.2	2.1 ± 0.1	2.3 ± 0.06	2.3 ± 0.2
Cumulative food intake (g/mouse/mo)	69.5 ± 4.6	34 ± 3.43†	65.0 ± 8	30.7 ± 6.8†	70.4 ± 1.3	30.8 ± 5.7†
HOMA-IR	2.05 ± 0.3	2.00 ± 0.3	3.71 ± 0.3*	2.59 ± 0.6†	3.92 ± 0.1*	2.41 ± 0.7†
Insulin (IU/L)	8.5 ± 1.2	8.2 ± 1.2	11.5 ± 0.3*	8.4 ± 1.8†	11.5 ± 0.3*	7.9 ± 2.1†
Leptin (10^2 pg/mL)	15 ± 2.2	14.3 ± 3.2	36.1 ± 5.2*	25.7 ± 2.4†	20.2 ± 2.5*	19.4 ± 4.2
Liver mass (g)	1 ± 0.02	0.8 ± 0.1	1.3 ± 0.1*	1 ± 0.2	1.3 ± 0.2*	1.0 ± 0.1
Liver TG (mg·dL·g^{-1})	1.6 ± 0.2	1.4 ± 0.3	2.5 ± 0.3*	1.8 ± 0.13†	2.2 ± 0.4*	1.6 ± 0.2†
Liver TC (mg·dL·g^{-1})	0.8 ± 0.04	0.8 ± 0.04	1 ± 0.09*	0.9 ± 0.05	1.2 ± 0.1†	0.9 ± 0.06†
Plasma ALT (U/L)	52.2 ± 6.7	36 ± 10.1	114.4 ± 16.8*	78.4 ± 6.8†	80 ± 11.1†	56.5 ± 10.1†
Plasma AST (U/L)	67.6 ± 8.2	69.6 ± 5.4	130.2 ± 16.6*	94.8 ± 9.3†	134.4 ± 27.9*	106.8 ± 15.8
Plasma TG (mg/dL)	63.6 ± 12.3	64.5 ± 9.3	91 ± 8.1*	71.2 ± 6.9†	103.6 ± 3.4*	85.8 ± 6.7†
Plasma TC (mg/dL)	118.9 ± 15.0	112.8 ± 13.4	156 ± 18.6*	124.6 ± 11.8†	183.9 ± 20.2*	147.4 ± 12.6†

ALT, alanine aminotransferase; AST, aspartate aminotransferase; C, control; C-IF, contol-intermittent fasting; HF, high fat; HF-IF, high fat-intermittent fasting; HFr, high fructose; HFr-IF, high fructose-intermittent fasting; HOMA-IR, homeostasis model assessment for insulin resistance; TC, total cholesterol; TG, triacylglycerol
*$P < 0.05$ when compared with control.
†$P < 0.05$ compared with matched group without IF.

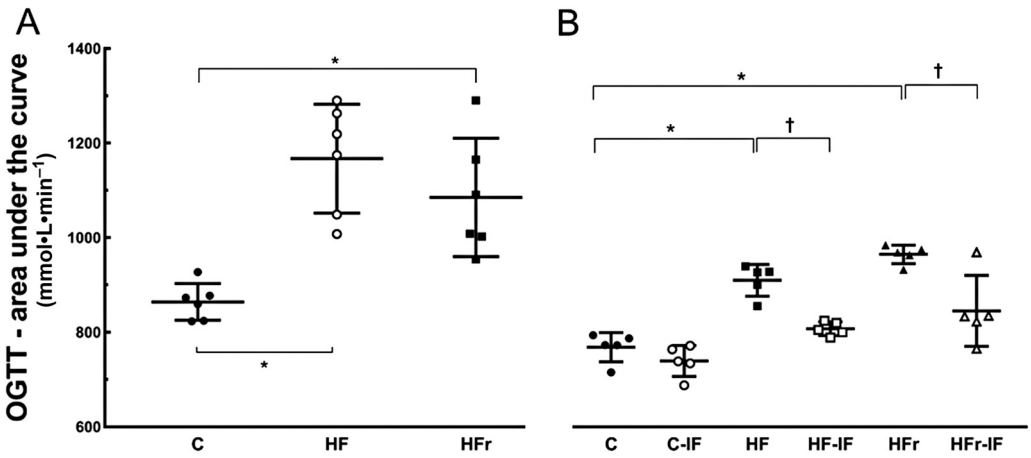

Fig. 2. OGTT (based on the area under the curve). (**A**) Before intermittent fasting. (**B**) After intermittent fasting (mean and SD). Statistical significance: *$P < 0.0001$ and †$P < 0.001$ (mean and SD). C, control; HF, high fat; HFr, high-fructose; IF, intermittent fasting; OGTT, oral glucose tolerance test.

IF has effects in diminishing the inflammation in the liver

A constant finding was that the HF and HFr groups had higher inflammatory markers (gene and protein) than C (Figs. 7C–F, 8A–C), and IF reduced inflammation in the HF and HFr groups, except for TNF-α, which was reduced by the IF only in the HFr group (Fig. 8A,B).

All seven markers of hepatic inflammation were affected by the diet or by IF as an independent factor, which also showed interaction in the observed results (Table 2).

Discussion

We investigated the effects of IF in a model that mimics the human metabolic syndrome when nutritionally stressed [21]. We observed that the chronic consumption of the diets resulted, as expected, in high BM and adiposity index (HF only), glucose intolerance, hyperinsulinemia, hyperleptinemia, and increased lipid profile, but decreased adiponectin associated with liver steatosis. Also, there were enhanced lipogenesis and decreased β-oxidation in the liver, accompanied by increased proinflammatory cytokines. Contrary to the HF animals, HFr did not show a significant change in BM and adiposity index, so the damages in the HFr group seems to be independent of BM gain. After IF, the liver steatosis and biochemical and molecular data were usually recovered.

In addition to the well-known effects provoked by the HF diet (rich in saturated fat) in the obesity epidemic [22], the reported increase of worldwide consumption of fructose induces adverse effects in the population [23]. When fructose was consumed in food isoenergetic with controls, no difference in BM was found [13], but fructose given in drinking water might increase BM because of the high energy supplied by the sweetened drink [24].

Table 2
Two-way analysis of variance

Data	% of variation and significance test					
	Diet effect		IF effect		Diet vs IF	
	%	*P*-value	%	*P*-value	%	*P*-value
Biochemistry						
Adiponectin	68.7	<0.001	6.6	0.009	5.2	ns
ALT	62.8	<0.001	22.0	<0.001	2.3	ns
AST	58.8	<0.001	11.6	0.0017	7.3	0.033
Insulin	12.8	0.03	36.5	<0.001	13.9	0.021
Leptin	70.9	<0.001	6.2	0.005	7.9	0.006
OGTT	54.1	<0.001	23.7	<0.001	5.2	0.04
TG (liver)	38.9	<0.001	31.6	<0.001	5.1	ns
TG (plasma)	58.8	<0.001	14.0	<0.001	8.1	0.02
TC (liver)	33.4	<0.001	33.4	<0.001	18.5	0.002
TC (plasma)	51.4	<0.001	18.9	<0.001	5.5	ns
Lipogenesis						
Cd 36	40.7	<0.001	19.6	<0.001	10.5	0.02
Chrebp	45.1	<0.001	32.3	<0.001	45.1	<0.001
ChREBP	30.3	<0.001	33.0	<0.001	17.4	<0.001
Fas	38.8	<0.001	31.8	<0.001	19.0	<0.001
FAS	54.5	<0.001	9.6	0.004	12.4	0.006
Ppar γ	41.5	<0.001	21.7	0.001	12.0	0.009
PPAR γ	46.6	<0.001	20.3	<0.001	4.1	ns
Srebp1-c	76.9	<0.001	11.1	<0.001	6.8	<0.001
SREBP1-c	55.1	<0.001	15.2	<0.001	10.0	0.007
Lipid droplet						
PLIN2	50.9	<0.001	26.1	<0.001	15.8	<0.001
β-oxidation						
ACOX	45.1	<0.001	11.6	0.01	7.1	ns
Cpt1 α	53.9	<0.001	9.1	0.008	10.6	0.02
Pgc1 α	83.2	<0.001	5.1	<0.001	5.1	0.001
Ppar α	59.8	<0.001	18.6	<0.001	2.0	ns
PPAR α	53.8	<0.001	5.2	ns	8.5	ns
Inflammation						
Il1 β	64.9	<0.001	12.5	<0.001	6.5	0.02
IL1 β	62.4	<0.001	15.0	<0.001	9.5	0.002
Il6	64.5	<0.001	13.7	<0.001	7.0	0.01
IL6	50.1	<0.001	17.4	<0.001	7.7	0.04
NF-κB	22.6	<0.001	34.5	<0.001	16.7	0.003
Tnf α	56.8	<0.001	14.5	<0.001	13.8	<0.001
TNF-α	43.2	<0.001	21.8	<0.001	12.7	0.005

ACOX, acyl-coenzyme A oxidase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BM, body mass; ChREBP, carbohydrate-responsive element-binding protein; CPT, carnitine palmitoyl transferase; CD, cluster of differentiation; FAS, fatty acid synthase; IF, intermittent fasting; IL, interleukin; NF, nuclear factor; ns, not significant; OGTT, oral glucose tolerance test; PGC, peroxisome proliferator-activated receptor γ coactivator; PLIN, perilipin; PPAR, peroxisome proliferator-activated receptor; SREBP, sterol regulatory element-binding protein; TC, total cholesterol; TG, triacylglycerol; TNF, tumor necrosis factor

We note here that fructose in the food that was isoenergetic with the diet of the C, which might explain why the HFr diet did not alter BM and adiposity of the animals, thus significantly indicating that there was no expansion of the adipose tissue in this model. This result agrees with a previous study investigating the fructose-rich diet in rodents [13]. The best way to assess adiposity uses dual-energy x-ray absorptiometry (DXA) of the whole body (directly evaluates all body compartments: bone mass, BM, water, fat mass) without inferring data from the measurement of only one compartment [25]. In this study, we estimated the adiposity of the animals indirectly, a piece of useful but less accurate information than what would be obtained with DXA.

IF has been suggested as a nutritional intervention for the treatment of obesity. Different mechanisms may explain the effects of IF on metabolism. The organism uses fat pad depots for energy during IF, decreasing adipose tissue and softening the inflammatory profile [26]. Also, caloric restriction positively affects metabolism, helping especially prediabetic and insulin-resistant patients without any pharmacologic approach [27], possibly because of Forkhead Box A genes [28].

The literature has sometimes conflicting reports about IF action on metabolism. IF can enhance cellular resistance to disease using different signaling pathways and circulating ketones during fasting, depleting glycogen from hepatocytes while fasting results in lipolysis and formation of ketone bodies [29]. However, meal patterns seem to be only transiently altered during IF in obese male mice [30]. In continued HF intake, IF could restore autophagic flux in islets by improving glucose tolerance, β cell survival, and nuclear expression of a marker of pancreatic regeneration [31]. Thus, IF decreased insulin resistance with the HF diet, possibly by attenuating hepatic insulin signaling and lowering glycogen phosphorylase expression despite decreased fat mass in young male rats [32].

IF might prevent (or diminish) diabetes by increasing the sensitivity of insulin receptor signaling such that insulin more readily stimulates glucose uptake by muscle and liver cells [33]. Herein, IF showed improved effects on fasting glucose, glucose tolerance, and insulin resistance, which is in agreement with previous reports [2,34].

Contrarily, female rats submitted to a short period of IF alters the expression of SREBP1-c in fat pads and SREBP2 in the hypothalamus, causing overeating after fasting and increasing risks for T2DM and obesity after IF [35].

In our study with two different nutritional insults, IF beneficial effects were seen even with the continuity of the diet consumed; IF regularizes the levels of insulin and glucose tolerance [2], in association with an increased sensitivity of insulin receptor signaling and a more significant stimulation of glucose uptake by muscle and liver cells by insulin [36]. Also, IF might improve the lipid profile, diminishing plasma levels of TG and TC, the accumulation of hepatic TG, and liver steatosis independent of the diet, agreeing with a recent report [37]. Moreover, IF attenuated the hepatic enzymes, mainly in HF-IF, probably because of a diet-dependent action for AST and ALT.

A recent study demonstrated that the small intestine shields the liver from otherwise toxic fructose exposure. The clearance demands the fructose-phosphorylating enzyme ketohexokinase. Low doses of fructose are ~90% cleared by the intestine, whereas high doses of fructose overwhelm intestinal fructose absorption and clearance, resulting in fructose reaching both the liver and colonic microbiota [38]. After being consumed, fructose is rapidly phosphorylated at carbon 1 (mediated by fructose-1-phosphate, or carbon 6 [mediated by a hexokinase] to form fructose-6-phosphate) [39]. The phosphorylation of fructose in fructose-1-phosphate is capable of activating SREBP1-c via PGC1 α [40]. Moreover, by conversion into fructose-6-phosphate, there is the activation of ChREBP. Both SREBP1-c and ChREBP are transcription factors that activate lipogenic enzymes, such as acetyl-CoA carboxylase and FAS [41]. As a consequence, the concentration of malonyl-CoA is increased within the hepatocyte, inhibiting CPT1, PPAR α, and β-oxidation [42,43].

PPAR γ is a transcription factor mediating the development of NAFLD in the mouse, increasing the transcription of genes involved in the hepatic FAS and fatty acid uptake (including FAT/CD36) [44]. Such pathways may explain the increase of lipogenesis with β-oxidation impairment, resulting in steatosis in HFr animals. Likewise, we observed the same alterations in gene and protein expressions in HFr and HF animals, suggesting that these diets influence the same signaling pathways in the liver.

Herein, higher gene expression of SREBP1-c was observed in HF and HFr animals. Also, both diets diminished PGC1 α in the liver that was not reversed by IF. This is relevant because PGC1 α increases both mitochondrial biogenesis and respiration rate and directly coactivates multiple transcription factors such as PPAR α [45]. Furthermore, PPAR α activation did not mediate the

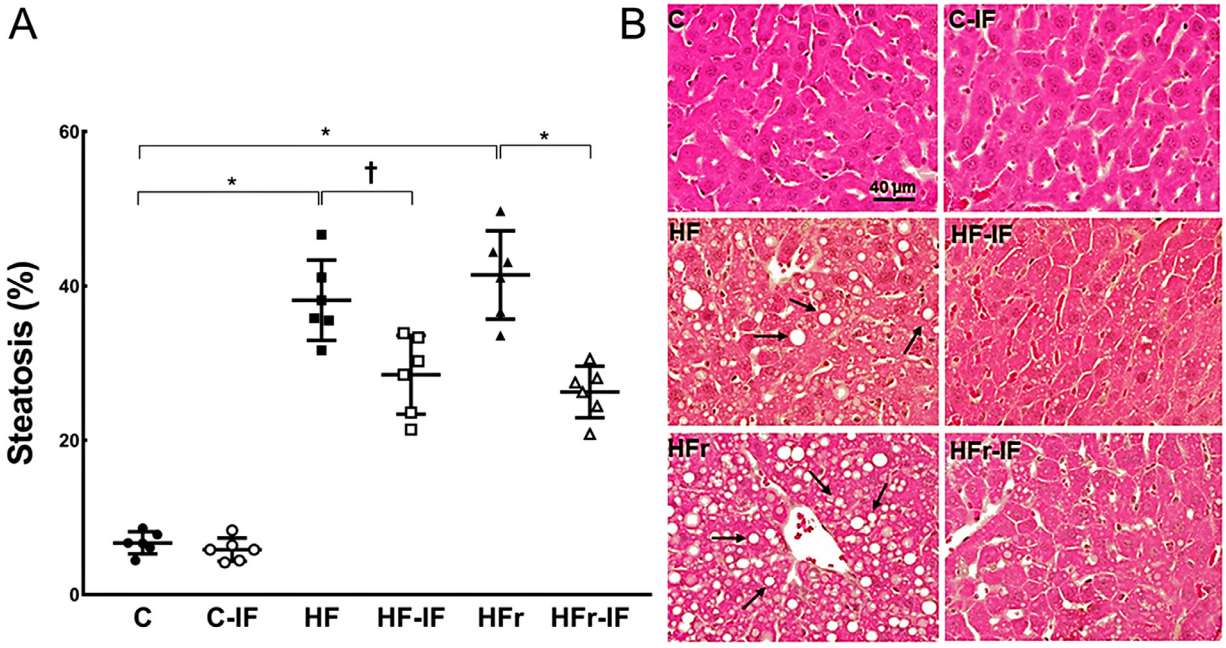

Fig. 3. Liver steatosis. (**A**) Vv (steatosis, liver) (mean and SD). Statistical significance: $^{*}P < 0.05$; (**B**) Photomicrographs of the liver tissue. C, control; HF, high fat; HFr, high-fructose; IF, intermittent fasting.

metabolic adaptation to fasting, at least in preventing acute fasting-induced steatosis [46]. Our results demonstrated a reduced gene expression and immunostaining of *PLIN2* in the IF groups, regardless of diet, which is relevant because *PLIN2* is linked to cytoplasmic lipid droplet coat protein found in hepatocytes of humans and rodents [47,48].

Adiponectin is a potent anti-inflammatory agent, a protein hormone secreted from adipose tissue that modulates several metabolic processes, including glucose regulation and fatty acid oxidation and is an independent risk factor for metabolic syndrome [49,50]. The levels of adiponectin were low in HF and HFr animals and IF increased adiponectin corroborated with another report [51].

Hyperleptinemia might be connected to insulin resistance and inflammation [52,53], and a significant decrease of leptin was seen in the HF-IF animals, probably linked with the improvement of carbohydrate metabolism and expression of inflammatory markers in this group.

The enhanced formation of inflammatory markers, such as IL-6 and TNF-α, is associated with uric acid (an additional product of fructose, not measured here), which may decrease adiponectin [54]. The effect of IF decreasing hepatocellular damage may be due to the suppression of the toll-like receptor 4 and NF-κB protein in the liver and inhibition of the inflammatory pathway genes *IL1* β and *TNF-*α [55]. These adipokines are controlled by NF-κB, resulting in further cycles of inflammation [56]. In our study, IF was not able to decrease TNFα in the HF group but had a role in dropping all other markers of liver inflammation in both HF and HFr animals.

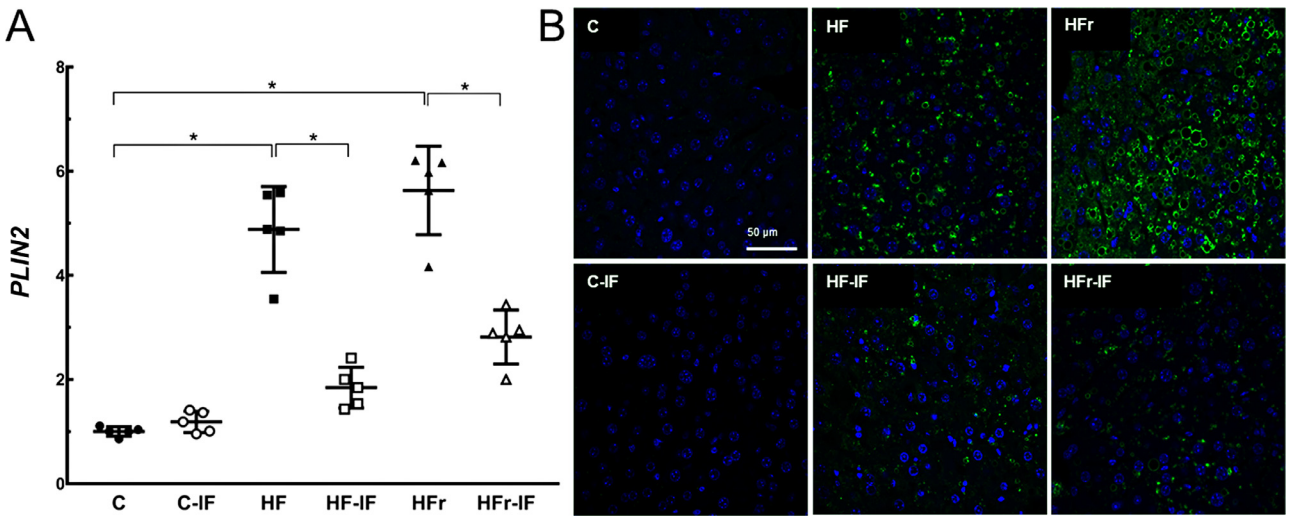

Fig. 4. Perilipin 2 () (PLIN 2) expression and confocal microscopy in the liver. (**A**) Protein expression (mean and SD). Statistical significance: $^{*}P < 0.0001$; (**B**) Confocal laser scanning microscopy (blue: Cell nuclei, DAPI; green: PLIN2, Alexa 488). C, control; HF, high fat; HFr, high-fructose; IF, intermittent fasting.

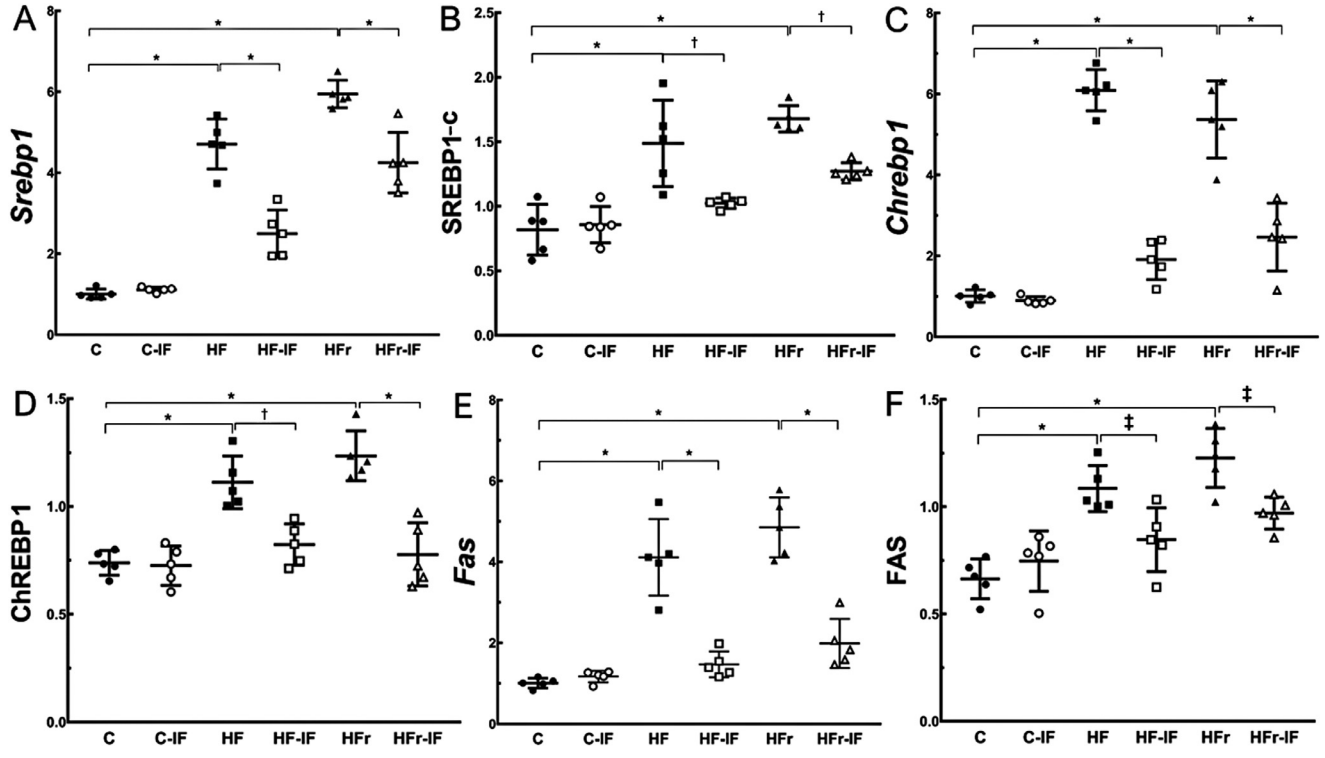

Fig. 5. Lipogenesis pathways in the liver. (**A**) mRNA expression and (**B**) protein expression of sterol regulatory element-binding protein 1 c; (**C**) mRNA expression and (**D**) protein expression of carbohydrate-responsive element-binding protein; (**E**) mRNA expression and (**F**) protein expression of fatty acid synthase. Statistical significance: $^{*}P < 0.0001$, $^{\dagger}P < 0.001$, and $^{\ddagger}P < 0.05$ (mean and SD). C, control; HF, high fat; HFr, high-fructose; IF, intermittent fasting.

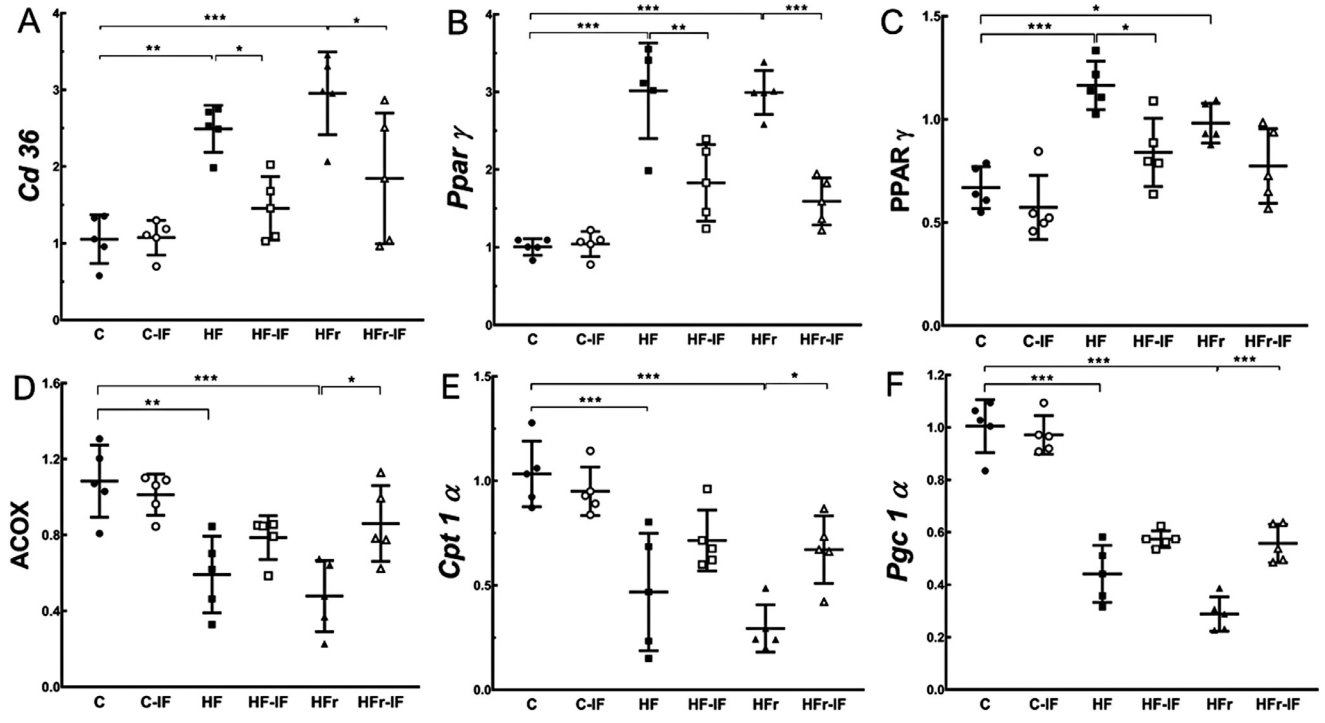

Fig. 6. Lipogenesis and β-oxidation pathways in the liver. (**A**) mRNA expressions of the cluster of differentiation (Cd) 36; (**B**) mRNA expression and (**C**) protein expression of peroxisome proliferator-activated receptor gamma); (**D**) protein expression of acyl-coenzyme A oxidase (ACOX) 1; (**E**) mRNA expression of carnitine palmitoyltransferase (Cpt) 1 alpha; (F) mRNA expression of peroxisome proliferator-activated receptor γ coactivator (Pgc) 1 α (mean and SD). Statistical significance: $^{*}P < 0.0001$, $^{\dagger}P < 0.05$, and $^{\ddagger}P < 0.001$ (mean and SD). C, control; HF, high fat; HFr, high-fructose; IF, intermittent fasting.

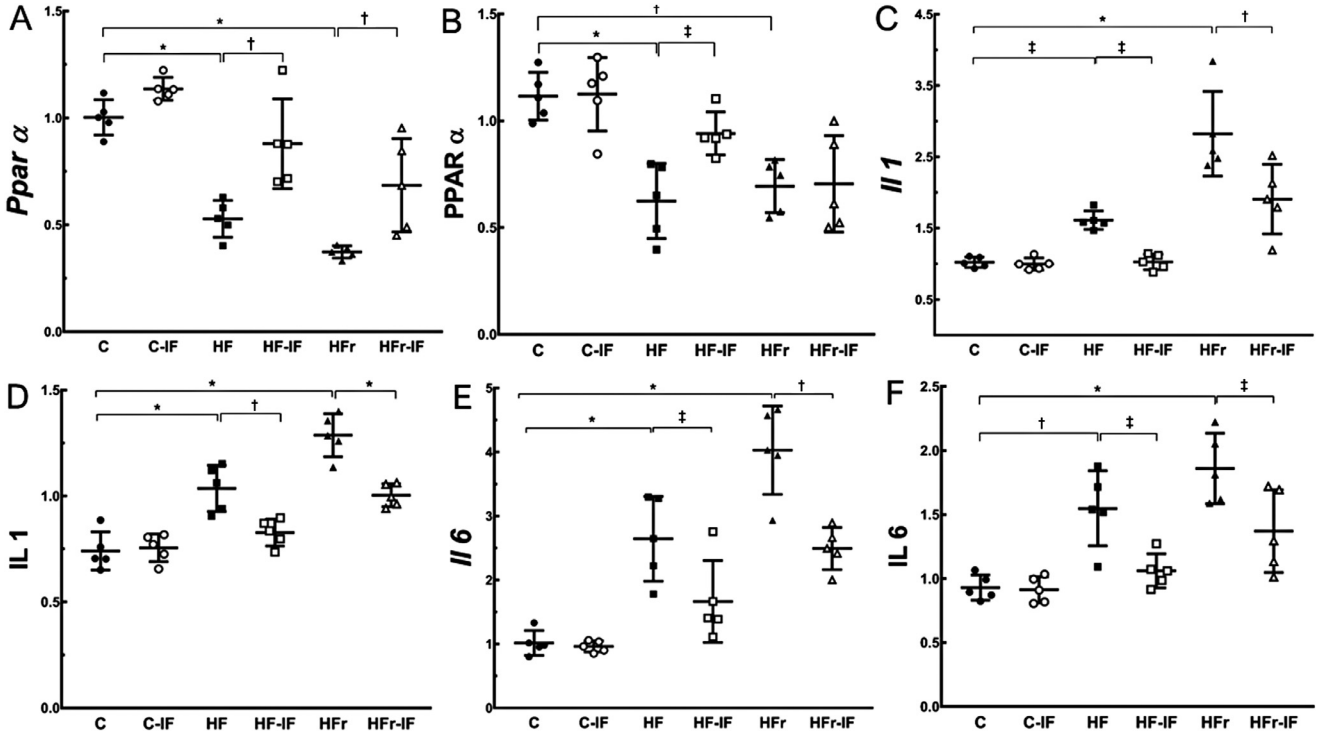

Fig. 7. β-oxidation and inflammation pathways in the liver. (**A**) mRNA expression and (**B**) protein expression of peroxisome proliferator-activated receptor (PPAR)α; (**C**) mRNA expression and (**D**) protein expression of IL1 β; (**E**) mRNA expression and (**F**) protein expression of IL6 (mean and SD). Statistical significance: $^{*}P < 0.0001$, $^{\dagger}P < 0.001$, and $^{\ddagger}P < 0.05$. C, control; HF, high fat; HFr, high-fructose; IF, intermittent fasting; IL, interleukin.

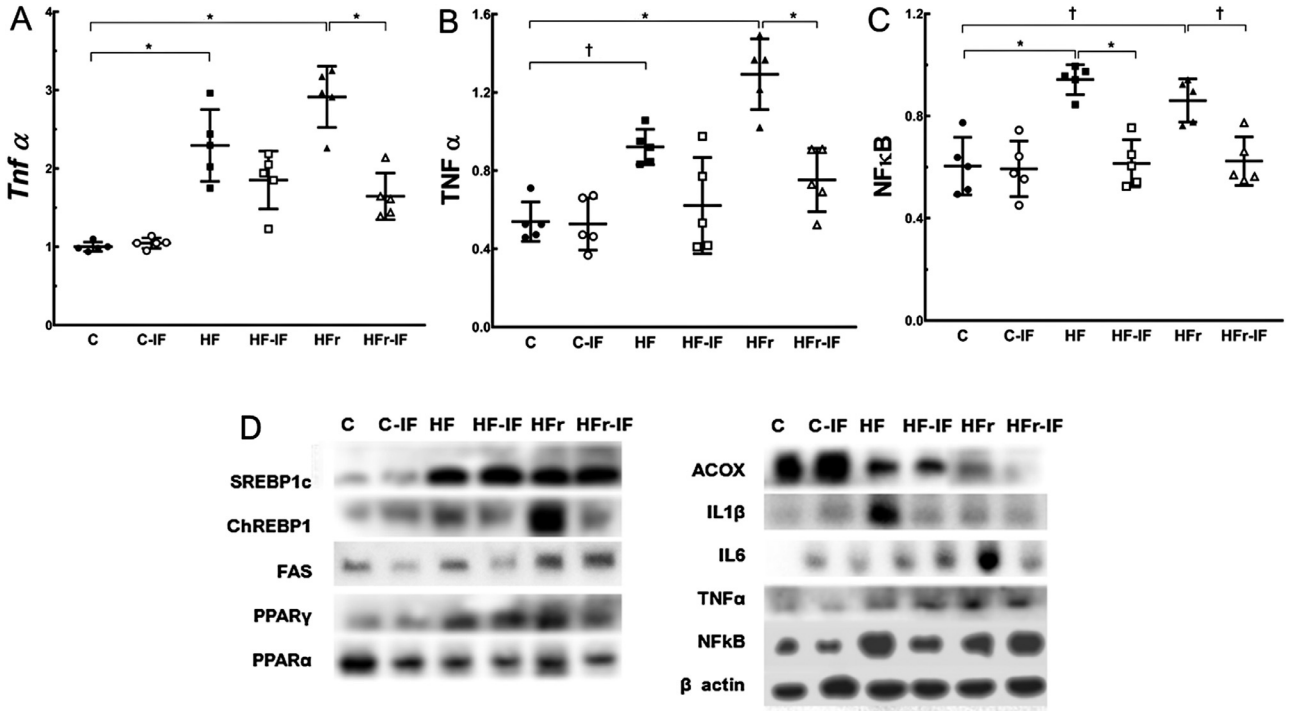

Fig. 8. Inflammation pathways in the liver and representative bands of Western blot. (**A**) mRNA expression and (**B**) protein expression of tumor necrosis factor (TNF) α; (**C**) protein expression of nuclear factor kappa (NF) κB; (**D, E**) representative immunoblotting with bands. Statistical significance: $^{*}P < 0.05$, $^{**}P < 0.001$, and $^{***}P < 0.0001$ (mean and SD). C, control; HF, high fat; HFr, high-fructose; IF, intermittent fasting.

Conclusion

The findings from the present study lead us to conclude that IF has beneficial effects apart from BM control, even with the continuity of the obesogenic diet and proinflammatory diet in mice. The beneficial effects of IF on glucose metabolism and the liver metabolism and inflammation recommend it as coadjutant factor treating hepatic metabolic troubles and steatosis.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at https://doi.org/10.1016/j.nut.2019.02.020.

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