The Journal of Nutrition
Nutrition and Disease

Rosa Mosqueta Oil Prevents Oxidative Stress and Inflammation through the Upregulation of PPAR-α and NRF2 in C57BL/6J Mice Fed a High-Fat Diet[1–3]

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Abstract

Background: Rosa mosqueta (RM) oil is characterized by high concentrations of antioxidants and α-linolenic acid (ALA; 18:3n–3). We have previously demonstrated in male C57BL/6J mice that RM decreases hepatic steatosis, a condition strongly associated with oxidative stress and inflammation.

Objective: We studied the molecular mechanisms that underlie the role of RM in preventing high-fat diet (HFD)–induced oxidative stress and inflammation.

Methods: Male C57BL/6J mice aged 28 d and weighing 12–14 g were divided into the following groups and fed for 12 wk: control diet (CD; 10% fat, 20% protein, and 70% carbohydrates); CD + RM (1.94 mg ALA · g body weight^{-1} · d^{-1} administered by oral gavage); HFD (60% fat, 20% protein, and 20% carbohydrates); and HFD + RM. General parameters (body weight, visceral fat, and histology); glucose metabolism [homeostasis model assessment and blood glucose area under the curve (AUC)]; oxidative stress [hepatic nuclear factor (erythroid-derived 2)-like-2 (NRF2) and heme oxygenase 1 (HO-1) concentrations]; and inflammation [hepatic peroxisome proliferator-activated receptor α (PPAR-α) and acyl-coenzyme A oxidase 1 (ACOX1) concentrations, blood tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β) concentrations, and *Tnfa* and *Il1b* mRNA expression in liver and visceral adipose tissue] were evaluated.

Results: In the HFD + RM mice, the final body weight (24.8 ± 1.1 g) was 19% lower than in the HFD mice (30.6 ± 2.8 g) ($P < 0.05$). Visceral fat was 34% lower in the HFD + RM mice than in the HFD mice ($P < 0.05$). The blood glucose AUC was 29% lower and *Tnfa* and *Il1b* expression levels were 47% and 59% lower, respectively, in the HFD + RM mice than in the HFD mice ($P < 0.05$). HFD + RM mice had 40% less hepatic steatosis ($P < 0.05$) and lower upregulation of PPAR-α (33%), ACOX1 (50%), NRF2 (39%), and HO-1 (68%) protein concentrations than did the HFD mice ($P < 0.05$).

Conclusions: Our findings suggest that RM supplementation prevents the obese phenotype observed in HFD-fed mice by downregulating inflammatory cytokine expression and secretion and stimulating hepatic antioxidant and fatty acid oxidation markers. *J Nutr* 2017;147:579–88.

Keywords: rosa mosqueta, inflammation, oxidative stress, PPAR-α, NRF2

Introduction

Nonalcoholic fatty liver disease (NAFLD)[6] is the hepatic manifestation of metabolic syndrome and is characterized by excessive hepatocyte lipid accumulation (>5% of total hepatocyte weight) (1). The severity of the disease ranges from steatosis (excessive TG accumulation in the liver) to acute steatohepatitis and fibrosis. Mitochondrial dysfunction, oxidative stress, and inflammation are considered crucial factors in the pathogenesis that drives the progression of NAFLD to more advanced stages (2).

[1] Supported by National Fund for Scientific and Technological Development grant 1140547.
[2] Author disclosures: D González-Mañán, A D'Espessailles, CG Dossi, M San Martín, RA Mancilla, and GS Tapia, no conflicts of interest.
[3] Supplemental Tables 1 and 2 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.

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[6] Abbreviations used: AA, arachidonic acid; ACOX1, acyl-coenzyme A oxidase 1; ALA, α-linolenic acid; CD, control diet; HFD, high-fat diet; KEAP1, Kelch-like ECH-associated protein 1; LCPUFA, long-chain PUFA; NAFLD, nonalcoholic fatty liver disease; NRF2, nuclear factor (erythroid-derived 2)-like-2; RM, rosa mosqueta; ROS, reactive oxygen species.

Manuscript received October 20, 2016. Initial review completed November 25, 2016. Revision accepted February 9, 2017.
First published online March 15, 2017; doi:10.3945/jn.116.243261.

Several reports have suggested that dietary FA composition is a crucial contributing factor in metabolic disorders (3, 4). The n–3 long-chain PUFAs (LCPUFAs) found in fish oil [EPA (20:5n–3) and DHA (22:6n–3)] are the most studied dietary FAs because they are involved in many biological processes. EPA and DHA seem to provide substantial protection against the adverse effects of NAFLD (5, 6). These FAs participate in the regulation of hepatic lipid metabolism, decreasing de novo lipogenesis by reducing the transcription of lipogenic genes and inducing the expression of FA oxidation gene components (7). n–3 LCPUFAs are ligands of PPAR-α, a key transcription factor in the β-oxidation of lipids, that upregulate carnitine acyl transferase-1 (CAT-1) and acyl-coenzyme A oxidase (ACOX1) gene expression. In addition, spontaneous EPA and DHA lipid peroxidation generates cyclopentenone-containing J-ring isoprostanes. These molecules in turn activate nuclear factor (erythroid-derived 2)-like 2 (NRF2) (8), which controls the expression of antioxidant enzymes and other cytoprotective proteins such as heme oxygenase 1 (HO-1) (9).

Despite the evidence supporting the positive effects of dietary n–3 LCPUFAs (10), the consumption among NAFLD patients is very low, making it necessary to seek alternatives (11). One alternative is essential n–3 α-linolenic acid (18:3n–3) (ALA), which is found mainly in vegetable oils. ALA is a precursor of n–3 LCPUFAs, which can be converted to EPA and DHA. Although ALA-rich oils such as chia, flaxseed, and rosa mosqueta (RM) have potentially beneficial metabolic actions, these sources have received less attention than fish oil.

RM (*Rosa rubiginosa*) is a wild shrub that is widely distributed throughout the Andean region of Chile. RM oil contains a high ratio of ALA (representing ~30% of its total FA content), making it an excellent nutritional source of this essential FA. ALA consumed in the diet can undergo subsequent hepatic bioconversion to EPA and DHA (12). It has been reported that oral RM oil administration in lean rats substantially increases hepatic concentrations of ALA, EPA, and DHA and decreases the n–6:n–3 FA ratio (13); in this model, RM oil supplementation activated PPAR-α. Previous work further demonstrated that dietary RM oil supplementation prevents the metabolic disruptions induced by a high-fat diet (HFD) in mice (14). RM oil protects against hepatic steatosis, hepatic oxidative stress, insulin resistance, and blood TG elevations as measured after 12 wk of HFD.

Because RM oil may provide considerable protection against diet-induced hepatic steatosis and metabolic syndrome, it is important to understand its mechanisms of action. The aim of this study, therefore, was to investigate the mechanisms that underlie the role of dietary RM oil in preventing HFD-induced hepatic steatosis. Parameters related to insulin resistance (glucose tolerance, HOMA), systemic and local inflammation (blood, hepatic, and adipose inflammatory cytokine concentrations), oxidative stress (hepatic NRF2 and HO-1), and lipid metabolism (hepatic PPAR-α and ACOX1 concentrations) were assessed.

Methods

Ethics statement. Experimental animal protocols and procedures complied with the *Guide for the Care and Use of Laboratory Animals* and were approved by the Bioethics Committee for Research in Animals, Faculty of Medicine, University of Chile.

Animal preparation and supplementation with RM oil. Weaned male C57BL/6J mice weighing 12–14 g were obtained from the University of Chile. The room was kept at 21°C on a 12-h light-dark cycle. At 28 d of age, the mice were randomly divided into 4 groups and fed for 12 wk.: control diet (CD) containing 10% fat, 20% protein, and 70% carbohydrates (D12450B; Research Diets); CD + RM in an oral dose of 1.94 mg ALA/g animal body weight/d (Coesam); HFD containing 60% fat, 20% protein, and 20% carbohydrates (D12492; Research Diets); and HFD + RM.

Supplemental Table 1 indicates the nutritional composition of the CD and HFD whose complete dietary components and vitamin and mineral mixture compositions have also been previously described (15). RM oil was administrated to C57BL/6J mice by gavage because it is the best manner to ensure the correct and complete RM oil dose administration to experimental mice and because it avoids PUFA lipoperoxidation from RM oil. As a way of controlling the effects of mice manipulation, nonsupplemented groups were given isovolumetric amounts of saline solution. After 12 wk, the mice were deprived of food for 6–8 h and then intraperitoneally anesthetized with 20–40 mg Zoletil/kg (Tiletamine hydrochloride and Zolazepam hydrochloride). Weekly body weight and dietary intake measurements were performed throughout the study period. **Table 1** shows the FA composition of the RM oil used. The RM oil contained 0% EPA and 0% DHA and had an n–6:n–3 FA ratio of 1.3.

Tissue and blood samples. Visceral adipose tissue was extracted from the epididymal area. Liver and visceral adipose tissue samples were weighed, frozen in liquid nitrogen, and stored at −80°C or fixed in phosphate-buffered formalin, embedded in paraffin, sectioned by a microtome, and stained with hematoxylin and eosin. Blood samples were taken by cardiac puncture and then centrifuged (600 × *g*; 10 min; 4°C), and serum was stored at −20°C.

Parameters for hepatic steatosis: histology and TG content. Liver and adipose tissue slides were stained with hematoxylin and eosin and assessed with the use of optical microscopy (Olympus CX31) for morphology analysis in a blind fashion. Both steatosis and inflammation were graded as absent, mild, moderate, or severe (16). Liver TG content was assessed with the use of an ab65336 TG quantification kit (Abcam) according to the manufacturer's instructions.

Liver n–3 LCPUFA profile. Total fat was extracted from whole-liver homogenates with the use of a modified Bligh and Dyer extraction procedure (17). FAMEs were separated and identified by comparing retention times with those for individual purified standards, and values were expressed as g/100 g FAMEs (13).

Glucose tolerance test and biochemical measurements (blood glucose and insulin). Glucose tolerance tests were performed at 6 and 12 wk of treatment. Mice were deprived of food for 6 h, and blood glucose was measured at baseline with the use of a Johnson & Johnson

TABLE 1 FA profile of rosa mosqueta oil

FAs	g/100 g FAMEs
Total SFAs	6.3
Palmitic acid (16:0)	3.5
Stearic acid (18:0)	1.8
Eicosanoic acid (20:0)	0.75
Docosanoic acid (22:0)	0.16
Tetracosanoic acid (24:0)	0.067
Total MUFAs	15
Palmitoleic acid (16:1)	0.12
Oleic acid (18:1)	14
Eicosenoic acid (20:1)	0.35
Total PUFAs	77
Linoleic acid (18:2n–6)	43
α-linolenic acid (18:3n–3)	34
n–6:n–3 FA ratio	1.3

OneTouch glucometer according to the manufacturer's instructions. Glucose (1.5 mg body weight/g) was injected intraperitoneally. Blood glucose concentrations were measured at 15, 30, 60, 90, and 120 min after glucose intake. Blood samples were obtained by performing a small cut at the end of the mouse tail. One blood drop (≤10 μL) per experimental time point in a total of ≤60 μL for each mouse was collected. The data obtained were analyzed and plotted, and the AUC was calculated. Plasma insulin concentration was determined with the use of a commercially available immunoassay specific for mice (Mercodia). Insulin resistance was estimated with the use of the HOMA method [fasting insulin × fasting glucose/405] (18).

Immunohistochemistry studies. Immunostaining for PPAR-α and NRF2 was performed after deparaffination, rehydration, and antigen retrieval with EDTA. Endogen peroxidase activity was then blocked, and nonspecific binding was blocked with normal horse serum. Incubation with primary antibodies (Abcam) was performed according to the manufacturer's instructions. Secondary antibodies were revealed with the use of the ImmPACT DAB kit (Vector), and Mayer's hematoxylin was used for the nuclear contrast (Lillie's Modification; ScyTek Laboratories). Ten adjacent regions per slide were analyzed by blinded raters with the use of light microscopy (400×) in a blind fashion, and the percentage of positive nuclei per slide was calculated.

Western blot analysis of ACOX1 and HO-1. Liver samples (100–500 mg) were homogenized and suspended in a buffer solution (pH 7.9) (5). Soluble protein fractions were separated with the use of SDS-PAGE (12% gel), transferred to nitrocellulose membranes (19), and blocked with Tris-buffered saline containing 5% BSA at room temperature. After washing, specific primary antibodies were added (ACOX1; Santa Cruz Biotechnology Inc.) (HO-1; Abcam). β-Actin (ACTN 05; Thermo Fisher Scientific) was used as an internal control for all measurements. Antigen-antibody complexes were detected with the use of a chemiluminescence detection system (Super Signal West Pico; Pierce). Bands were quantified by densitometry with the use of a gel documentation system (C-DiGit Blot Scanner; LI-COR). Results were expressed as relative units (individual protein/β-actin).

Quantitative PCR assay of Tnfa and Il1b mRNA expression. *Tnfa* and *Il1b* mRNA expression was performed with the use of real-time quantitative PCR (20). Nucleotide sequences for sense and antisense primers used in this study were as follows: 5′-TATGGCTCAGGGTCCAACTC-′3 and 3′-GCTCCAGTGAATTCGGAAAG-′5 for *Tnfa*; 5′-GGGAAACAACAGTGGTCAGG-′3 and 3′-GAGCTGTCTGCTCATTCACG-′5 for *Il1b*; 5′-AGCCATGTACGTAGCCATCC-′3 and 3′-CTCTCAGCTGTGGTGGTGAA-′5 for *β-actin* as housekeeping. Data were calculated as relative expression levels with the use of the comparative computed tomography method.

Blood inflammatory cytokines. The cytokine blood levels for TNF-α and IL-1β were estimated with the use of a Multiplex ELISA kit (ProcartaPlex Cytokine & Chemokine Panel; eBioscience) according to the manufacturer's instructions.

Statistical analyses. All data are presented as means ± SEMs. Statistical analyses were performed with GraphPad Prism version 5.0 (GraphPad Software Inc.). Two-factor ANOVAs and Bonferroni post-tests were used to assess the statistical significance of differences between mean values among the groups, with $P < 0.05$ considered significant.

Results

Body and visceral adipose tissue

Initial body weight did not vary significantly among the 4 groups. After 12 wk of treatment, HFD + RM significantly ($P < 0.05$) prevented the increase in body weight observed in the HFD group. The HFD + RM mice also had a 34% lower adipose tissue:body weight ratio ($P < 0.05$) than the HFD mice, although this adipose tissue:body weight ratio in the HFD + RM group did not normalize to CD and CD + RM values. The HFD + RM group did not differ significantly from the CD and CD + RM groups in terms of final body weight, as observed in **Table 2**. The changes were induced despite the fact that CD groups (CD and CD + RM) or HFD groups (HFD and HFD + RM) consumed equivalent amounts of calories either at the beginning or end of the treatment (**Supplemental Table 2**).

Hepatic steatosis parameters

CD and CD + RM mice did not show evidence of hepatic steatosis, as reflected by the absence of lipid vesicles. HFD mice showed micro- and macrovesicular steatosis, whereas the HFD + RM group had significantly lower lipid infiltration into hepatocytes ($P < 0.05$) than the HFD group, normalizing it to fat content similar to CD and CD + RM groups. The liver histology for all groups showed no evidence of inflammation, necrosis, or fibrosis and exhibited preserved liver histoarchitecture (**Figure 1**A). The results were confirmed by TG measurements that indicated a 184% greater ($P < 0.05$) accumulation for the HFD than in the CD groups. The HFD + RM group had significantly lower TG accumulation than did the HFD group ($P < 0.05$), although RM supplementation did not normalize TG content to CD levels (Figure 1B).

Hepatic n–3 LCPUFA content

The CD + RM group increased ALA accumulation by 64% ($P < 0.05$) compared with the CD group. ALA concentrations did not differ

TABLE 2 General parameters in male mice fed CD or HFD with or without RM oil supplementation for 12 wk[1]

					P^2		
Parameter	CD ($n = 5$)	CD + RM ($n = 5$)	HFD ($n = 9$)	HFD + RM ($n = 9$)	Diet	Oil	Interaction
Initial body weight, g	15.7 ± 0.3	14.6 ± 0.6	14.0 ± 1.1	15.0 ± 0.6	0.17	0.91	0.051
Final body weight, g	25.3 ± 1.8^b	20.9 ± 0.4^b	30.6 ± 2.8^a	24.8 ± 1.1^b	0.0078	0.0017	0.61
Adipose tissue:body weight ratio × 100	1.0 ± 0.1^c	1.3 ± 0.1^c	5.3 ± 0.7^a	3.5 ± 0.5^b	<0.0001	0.029	0.0028
Blood glucose, mg/dL	144 ± 7^b	136 ± 4^b	178 ± 10^a	173 ± 7^a	<0.0001	0.43	0.61
Insulin, μUI/mL	19 ± 1^b	18 ± 3^b	25 ± 2^a	19 ± 2^b	0.0078	0.0047	0.23
HOMA, μUI/mL × mg/dL	6.5 ± 0.3^b	5.8 ± 0.9^b	11 ± 1^a	8.2 ± 1.1^b	<0.0001	0.016	0.13
α-Linolenic acid, g/100 g FAMEs	0.25 ± 0.02^b	0.41 ± 0.05^a	0.30 ± 0.03^{a,b}	0.30 ± 0.01^{a,b}	0.088	0.0003	0.0026
EPA, g/100 g FAMEs	0.30 ± 0.06^b	0.44 ± 0.02^a	0.23 ± 0.01^b	0.21 ± 0.06^b	0.0045	<0.0001	0.045
DHA, g/100 g FAMEs	3.3 ± 0.1^{a,b}	3.9 ± 0.2^a	1.6 ± 0.2^c	3.1 ± 0.2^b	<0.0001	<0.0001	<0.0001

[1] Values are means ± SEMs. Means without a common superscript letter differ, $P < 0.05$. CD, control diet; HFD, high-fat diet; RM, rosa mosqueta.
[2] Derived with the use of 2-factor ANOVA followed by a Bonferroni posttest.

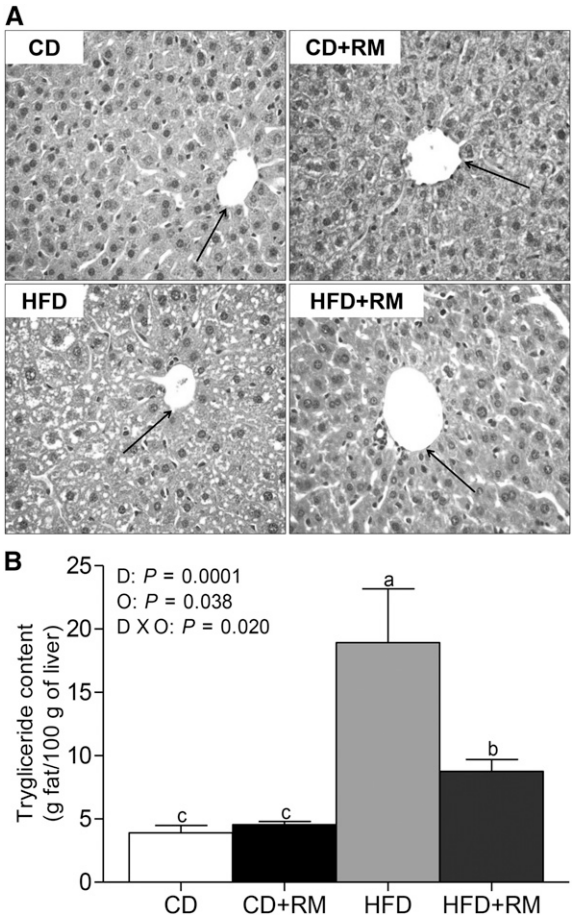

FIGURE 1 Effect of RM oil supplementation on hepatic steatosis induced in male mice fed CD or HFD with or without RM oil supplementation for 12 wk. (A) Liver histology; representative images are at 400× magnification. (B) Quantification of total liver fat content. Values are means ± SEMs, $n = 5$ (CD groups) or $n = 9$ (HFD groups). Means without a common letter differ, $P < 0.05$. Black arrows point to the central vein. CD, control diet; D, diet effect; HFD, high-fat diet; O, RM oil effect; RM, rosa mosqueta.

significantly among the CD, HFD, and HFD + RM groups (Table 2). The HFD + RM group showed EPA concentrations that were comparable to the HFD and CD groups, whereas the CD + RM mice had significantly higher ($P < 0.05$) EPA content than the CD, HFD, and HFD + RM mice had. The HFD + RM mice showed significantly enhanced ($P < 0.05$) DHA concentrations compared with the HFD group and did not differ significantly from the CD and CD + RM groups (Table 2).

Blood glucose and insulin, HOMA index, and glucose tolerance test results

The HFD group showed 23% higher ($P < 0.05$) glucose concentrations than did the CD group. The HFD + RM mice had 3% lower glucose concentrations than the HFD group, although this difference was not statistically significant (Table 2). Blood insulin concentrations were 29% higher ($P < 0.05$) in the HFD group than in the CD group, whereas concentrations for the HFD + RM group were 23% lower than in the HFD group ($P < 0.05$), with HFD + RM values normalized to CD and CD + RM levels. Results for the HOMA index were similar, with insulin concentrations in the HFD + RM group normalized to the CD and CD + RM groups ($P < 0.05$). RM oil supplementation did not affect glucose, insulin, or HOMA values in the CD groups (Table 2). Glucose tolerance test results revealed a similar pattern. At 6 wk of treatment, the HFD + RM group showed significantly lower blood glucose concentrations than those in HFD mice, with an 18% decrease ($P < 0.05$) in the AUC (**Figure 2**A, B). Results at the end of the treatment (12 wk) were also similar. At this time point, HFD + RM glucose concentrations did not differ significantly from the CD groups at any of the times evaluated (15, 30, 60, 90, and 120 min). The AUC for the HFD + RM mice was 29% smaller ($P < 0.05$) than for the HFD group (Figure 2C, D).

Systemic inflammation

The CD + RM mice showed similar blood TNF-α and IL-1β concentrations to the CD mice. However, RM supplementation prevented the increased cytokine concentrations found in the HFD group: TNF-α and IL-1β concentrations were 66% and 90% lower in the HFD + RM group than in the HFD group, respectively ($P < 0.05$) (**Figure 3**A, B).

Adipocyte size and adipose tissue inflammation

Epididymal adipocyte size was lower in both RM groups (CD + RM and HFD + RM) than in the respective non-supplemented groups ($P < 0.05$) (**Figure 4**A, B). CD + RM treatment decreased adipocyte size by 20% ($P < 0.05$) compared with the CD group, whereas adipocyte size was 53% smaller ($P < 0.05$) in the HFD + RM than in the HFD group. The HFD + RM group did not differ significantly from the HFD group in terms of the histologic characteristics of the adipose tissue.

Regarding inflammatory cytokine expression in the adipose tissue, *Tnfa* mRNA was 47% lower ($P < 0.05$) in the HFD + RM group than in the HFD mice. The HFD + RM group showed *Tnfa* expression similar to the CD groups. A similar pattern was observed for *Il1b* expression. The HFD + RM group showed 59% lower ($P < 0.05$) *Il1b* mRNA levels than shown in the HFD mice. The HFD + RM group showed *Il1b* mRNA expression that was comparable to that in the CD groups (Figure 4C, D).

Hepatic metabolism

Hepatic inflammation. HFD-fed mice showed 51% higher *Tnfa* mRNA levels ($P < 0.05$) than in the HFD + RM group. *Tnfa* expression was similar in the HFD + RM and CD mice (**Figure 5**A). There were no significant differences in *Il1b* mRNA levels among the experimental groups (Figure 5B).

Hepatic PPAR-α and ACOX1. HFD downregulated PPAR-α concentrations by 46% compared with CD mice. Nuclear PPAR-α concentrations were normalized to CD values in the HFD + RM mice, with RM supplementation preventing PPAR-α depletion by 33% (**Figure 6**A, B). Regarding protein levels for ACOX1 (Figure 6C), RM supplementation prevented the ACOX1 downregulation observed in the HFD group: ACOX1 protein levels were 50% higher ($P < 0.05$) in the HFD + RM group than in the HFD group, with HFD + RM values normalized to CD levels.

Hepatic NRF2 and HO-1. HFD decreased the presence of NRF2 by 38% ($P < 0.05$) compared with CD mice. The HFD + RM group increased NRF2 by 39% ($P < 0.05$) compared with HFD mice and normalized the percentage of NRF2-positive nuclei to control values (**Figure 7**A, B). Similar to NRF2, the HFD group showed 35% lower HO-1 protein levels than in the CD mice ($P < 0.05$), whereas the HFD + RM

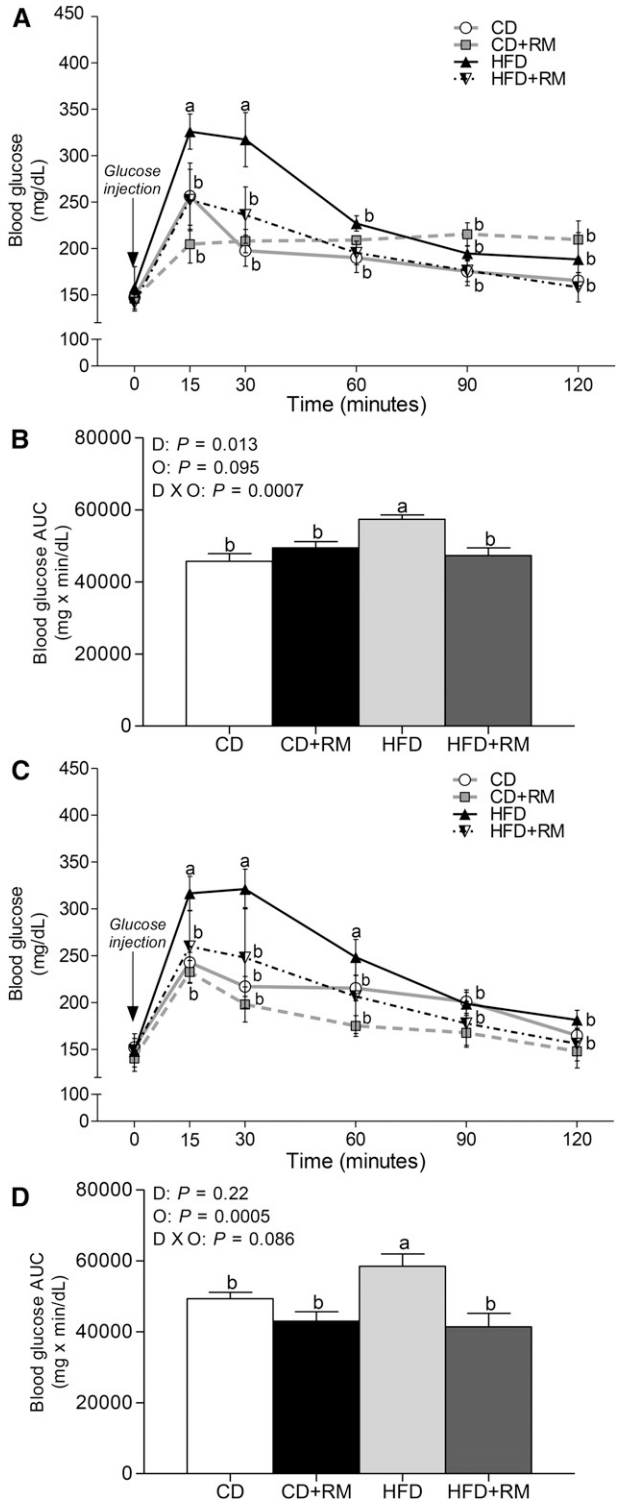

FIGURE 2 Effect of RM oil supplementation on glucose homeostasis in male mice fed CD or HFD with or without RM oil supplementation for 12 wk. (A) Glucose tolerance test results and (B) their respective blood glucose AUCs at 6 wk of treatment. (C) Glucose tolerance test results and (D) their respective blood glucose AUCs at 12 wk of treatment. Values are means ± SEMs, $n = 5$ (CD groups) or $n = 9$ (HFD groups). Means without a common letter differ, $P < 0.05$. CD, control diet; D, diet effect; HFD, high-fat diet; O, RM oil effect; RM, rosa mosqueta.

group increased HO-1 by 68% ($P < 0.05$) compared with the HFD mice, with HFD + RM protein levels normalized to CD values (Figure 7C).

Discussion

We have previously demonstrated that 12 wk of oral RM oil administration to HFD-fed mice decreases the incidence of hepatic steatosis and ameliorates the pro-oxidative state associated with the bioconversion of ALA to EPA and DHA (14). In this study, we investigated the molecular mechanisms that underlie this hepatoprotective effect of RM oil and focused on the factors involved in metabolic syndrome, namely glucose metabolism, local and systemic inflammation, oxidative stress, and the β-oxidation of FAs. The molecular mechanisms that underlie the effects of RM oil in preventing hepatic lipid accumulation, chronic inflammation, and oxidative stress may be associated with the bioconversion of ALA to EPA and DHA (13, 14). Several studies have supported a positive association between fish oil intake and augmented nuclear concentrations of PPAR-α (5, 21), suggesting a potential role for EPA and DHA in preventing the oxidative stress and inflammation observed in NAFLD mice.

HFD administration in mice triggers alterations in glucose metabolism (22). We observed that mice fed an HFD for 12 wk developed glucose intolerance and insulin resistance (23). It has been well documented that long-term exposure to an HFD has major consequences for metabolism, triggering *1*) chronic oxidative stress with an increased production of reactive oxygen species (ROS) derived from the peroxidation of free SFAs along with lower antioxidant enzyme activity (24, 25) and *2*) chronic low-grade inflammation as a consequence of white adipose tissue expansion,

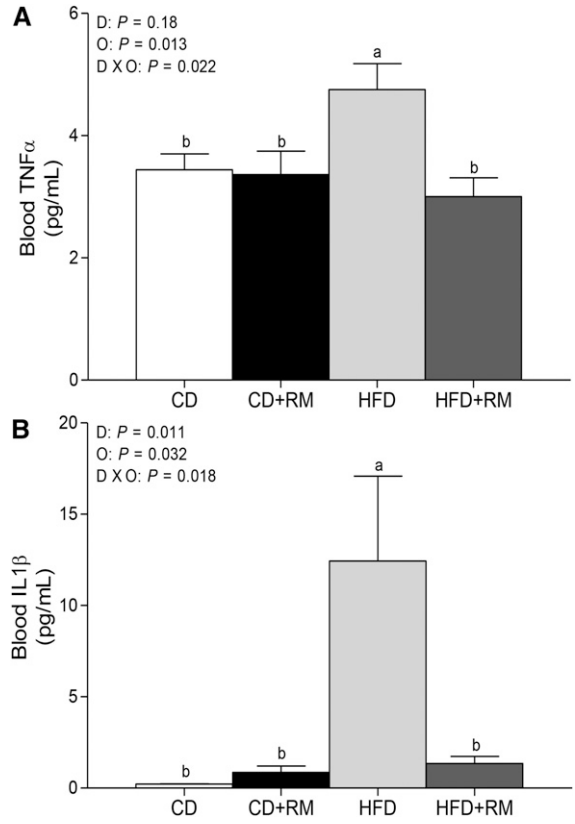

FIGURE 3 Effect of RM oil supplementation on systemic inflammation in male mice fed CD or HFD with or without RM oil supplementation for 12 wk. Plasma concentrations of (A) TNF-α and (B) IL-1β. Values are means ± SEMs, $n = 5$ (CD groups) or $n = 9$ (HFD groups). Means without a common letter differ, $P < 0.05$. CD, control diet; D, diet effect; HFD, high-fat diet; O, RM oil effect; RM, rosa mosqueta.

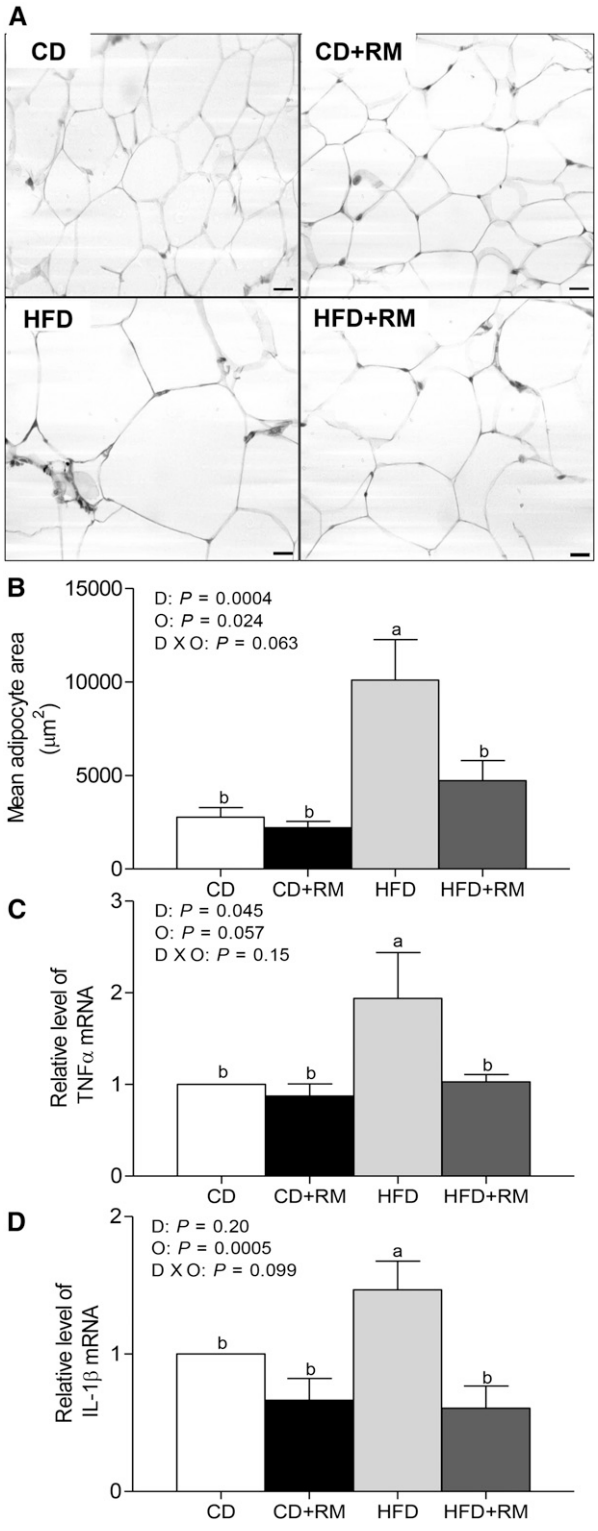

FIGURE 4 Effect of RM oil supplementation on visceral adipose tissue histology (A) and adipocyte area and adipose tissue mRNA (B) for TNF-α (C) and IL-1β (D) in male mice fed CD or HFD with or without RM oil supplementation for 12 wk. Representative images shown in panel A are at 400× magnification. Values are means ± SEMs, $n = 5$ (CD groups) or $n = 9$ (HFD groups). Means without a common letter differ, $P < 0.05$. The scale bars indicate 25 μm. CD, control diet; D, diet effect; HFD, high-fat diet; O, RM oil effect; RM, rosa mosqueta.

macrophage infiltration, and the enhanced production of proinflammatory cytokines and other adipokines (26–28). The overconsumption of a lipid-enriched diet also stimulates enhanced lipolysis and the transport of palmitate to the liver, where it is used as substrate for the de novo synthesis of ceramides (29). These physiologic alterations can impair insulin signaling by increasing serine and threonine protein kinase activity, which in turn decreases insulin receptor substrate 1 and phosphatidylinositol 3-kinase and protein kinase B activity (30, 31). One potential mechanism for the RM oil-dependent normalization of glucose homeostasis and insulin resistance in HFD-fed mice (Figure 2) is the increased incorporation of n–3 LCPUFAs into phospholipids, increasing membrane fluidity and improving the behavior of membrane-bound receptors associated with insulin signaling (32). In fact, it has been demonstrated that the administration of ALA-rich sources increases the incorporation of ALA, EPA, and DHA into phospholipids in liver, muscle, and adipose tissue (33, 34). Moreover, n–3 LCPUFAs and the phytochemical components of RM oil may improve insulin sensitivity by enhancing the antioxidant potential of liver and adipose tissue, acting through direct (ROS scavenging) and/or indirect (NRF2 activation) mechanisms to increase the phosphorylation of insulin target enzymes in tyrosine residues (5, 35).

In our model, mice subjected to an HFD showed hypertrophic adipocytes, whereas the administration of RM oil in HFD-fed mice considerably abated this adipocyte enlargement. It has been reported that HFD-induced central adiposity is an important risk marker because of its direct relation with the development of metabolic syndrome and associated comorbidities such as insulin resistance, systemic inflammation, and dyslipidemia (36, 37). The PUFA composition of adipocytes also has an important impact on adipocyte size. It has been demonstrated

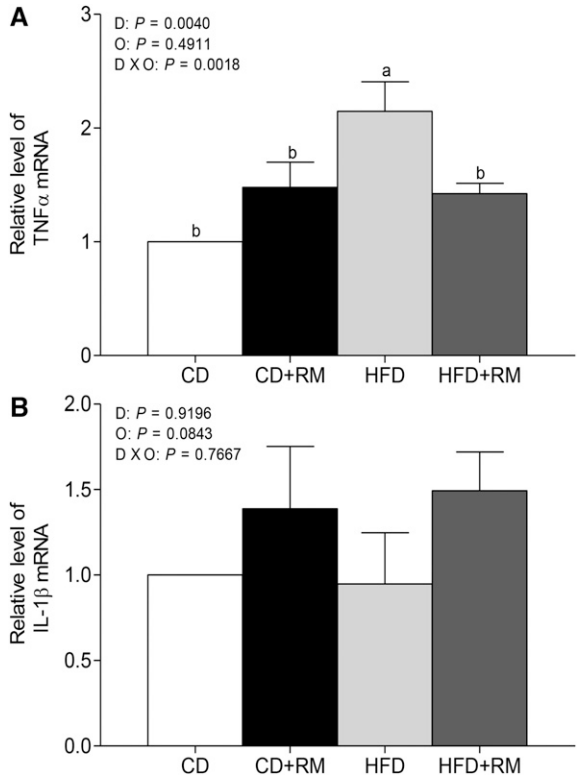

FIGURE 5 Effect of RM oil supplementation on hepatic TNF-α (A) and IL-1β (B) mRNA expression in male mice fed CD or HFD with or without RM oil supplementation for 12 wk. Values are means ± SEMs, $n = 5$ (CD groups) or $n = 9$ (HFD groups). Labeled means without a common letter differ, $P < 0.05$. CD, control diet; D, diet effect; HFD, high-fat diet; O, RM oil effect; RM, rosa mosqueta.

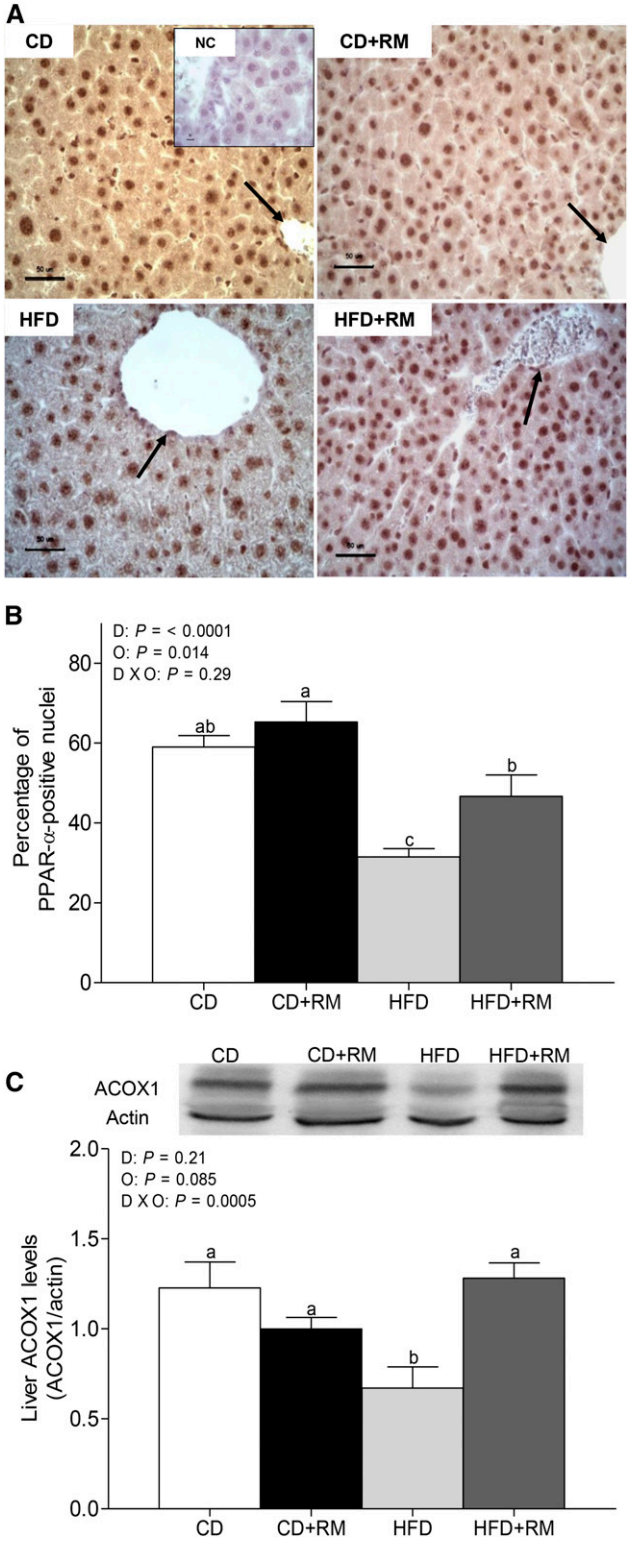

FIGURE 6 Effect of RM oil supplementation on determining nuclei positive for liver PPAR-α (1800–2000 nuclei per sample) (A and B) and hepatic concentrations of ACOX1 (C) in male mice fed CD or HFD with or without RM oil supplementation for 12 wk. Values are means ± SEMs, n = 5 (CD groups) or n = 9 (HFD groups). Means without a common letter differ, $P < 0.05$. The scale bars indicate 50 μm, and the black arrows point to the central vein. The insert in panel A is the NC in the absence of the primary antibody. ACOX1, acyl-coenzyme A oxidase 1; CD, control diet; D, diet effect; HFD, high-fat diet; NC, negative control; O, RM oil effect; RM, rosa mosqueta.

that EPA and DHA can decrease lipid accumulation in adipocytes by upregulating mitochondrial biogenesis and FA β-oxidation (38). Likewise, it has been reported that high ALA intake alone can decrease visceral adiposity enlargement in dyslipidemic rats (39) and school-aged children (40). This phenomenon may be explained by the capacity of ALA to activate AMP–activated protein kinase and increase its target protein levels [PPAR-γ coactivator 1 α (PGC1α), uncoupling protein 2 (UCP2), carnitine palmitoyltransferase (CPT-1)] in epididymal fat (41). ALA can also modulate hyperplasia by steeply decreasing arachidonic acid (20:4n–6) (AA) concentrations; AA stimulates preadipocyte maturation through enhanced prostaglandin I_2 synthesis, the activation of the protein kinase A pathway, and the upregulation of CCAAT/enhancer-binding proteins β and δ (42). This finding is consistent with our previous studies that showed that administering RM oil to normocaloric-fed mice reduced plasma, hepatic, and adipose tissue AA concentrations (13). The amelioration of adipocyte hypertrophy through RM oil administration in HFD-fed mice was also associated with reduced inflammation in liver and adipose tissue, as reflected by the decreased blood concentrations and mRNA expression of the proinflammatory cytokines *Tnfa* and *Il1b* (Figure 4C, D). It is worth mentioning that *Il1b* mRNA expression in the liver and adipose tissue is not necessarily correlated with local or systemic inflammation because IL-1β is synthesized as a proprotein that must be cleaved before its release into the circulatory system (43). Nonetheless, we observed a substantial reduction in blood IL-1β as a consequence of RM oil administration in HFD-fed mice, suggesting a possible inhibition of NLR family pyrin domain-containing 3 inflammasome assembly and caspase-1 activation through the direct action of n–3 LCPUFAs as ligands (44).

In addition to the antioxidant effects of the EPA and DHA converted from ALA, RM oil contains high concentrations of α- and γ-tocopherol that increase the antioxidant capacity of this oil (45). The HFD mice that received supplementary RM oil exhibited enhanced hepatic NRF2 activation compared with the HFD-only group. NRF2 is a transcription factor associated with a crucial step in the cellular antioxidant response to ROS. Under baseline conditions, NRF2 is bound to Kelch-like ECH-associated protein 1 (KEAP1), which inhibits NRF2 signaling by stimulating NRF2 degradation through the proteasomal pathway (46). In addition, RM oil increased HO-1 protein levels. This phase II detoxification enzyme is involved in defending against increased oxidative stress (47, 48). NRF2 stimulation also inhibits the expression of proinflammatory molecules such as cytokines, cell-adhesion molecules, cyclooxygenase-2, and inducible NO synthase by inhibiting the NF-κB pathway. Therefore, NRF2 is also implicated in modulating the inflammatory cascade (47). There are several mechanisms through which the components of RM oil could induce NRF2 activation. The spontaneous nonenzymatic lipid peroxidation of EPA and DHA generates cyclopentenone-containing J_3-isoprostanes, which are electrophilic compounds that can induce NRF2 activation by binding covalently to sulfhydryl groups of KEAP1 and, consequently, induce NRF2-KEAP1 dissociation and NRF2 translocation (8, 49). The γ-tocopherol present in RM oil is capable of promoting NRF2 activity, although to our knowledge the specific mechanism remains unknown (46, 50). However, it is possible that other phytochemicals (such as tocopherols, phytosterols, and carotenoids) present in RM oil may be able to abate the redox imbalance alone, independent of NRF2 signaling (45, 51).

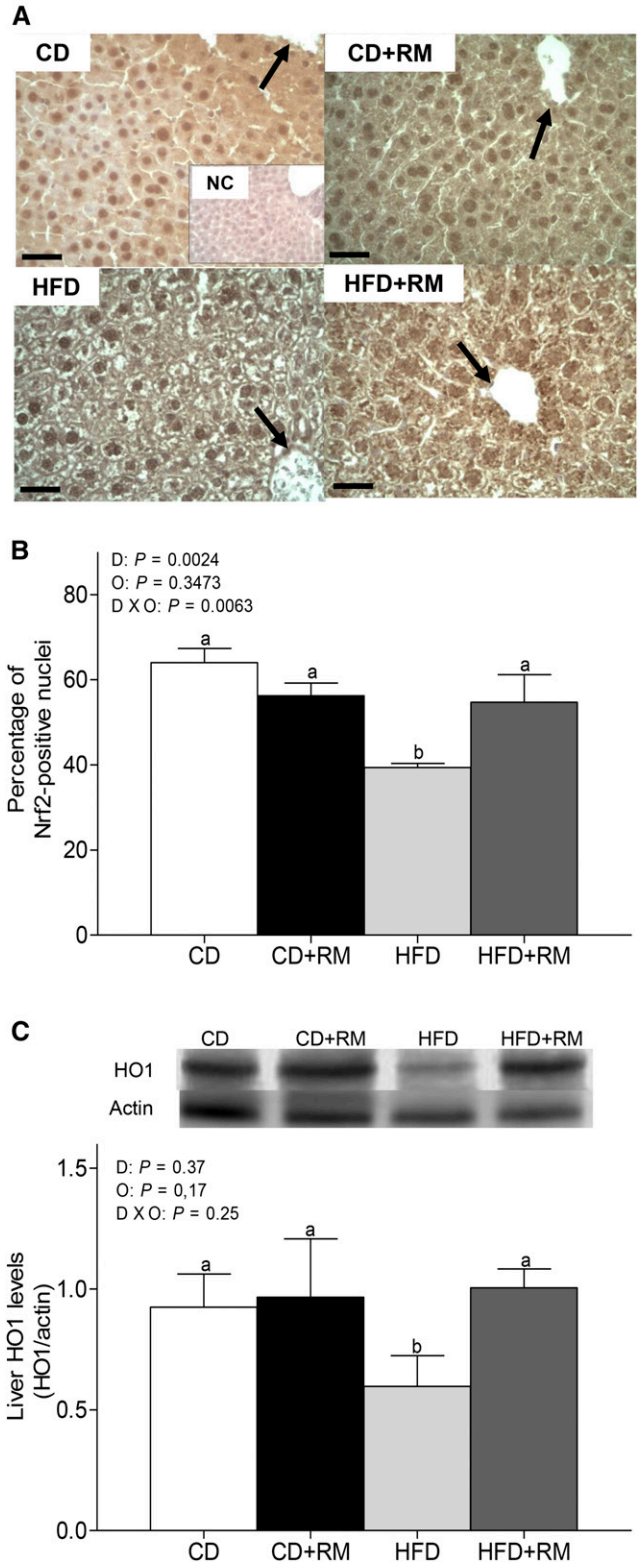

FIGURE 7 Effect of RM oil supplementation on determining nuclei positive for liver NRF2 (1800–2000 nuclei per sample) (A and B) and hepatic concentrations of HO-1 (C) in male mice fed CD or HFD with or without RM oil supplementation for 12 wk. Values are means ± SEMs, $n = 5$ (CD groups) or $n = 9$ (HFD groups). Means without a common letter differ, $P < 0.05$. The scale bars indicate 50 μm, and the black arrows point to the central vein. The insert in panel A is the NC in the absence of the primary antibody. CD, control diet; D, diet effect; HFD, high-fat diet; HO-1, heme oxygenase 1; NRF2, nuclear factor (erythroid-derived 2)-like 2; O, RM oil effect; RM, rosa mosqueta.

Our data demonstrate that RM oil supplementation normalizes hepatic PPAR-α activation and ACOX1 concentrations in mice subjected to an HFD, which would account for the antisteatotic and anti-inflammatory effects attributed to RM oil. The down-regulation of liver PPAR-α in a metabolic syndrome model is triggered by both decreased PPAR-α expression and n–3 long-chain PUFA depletion (25, 52), which in turn decreases the expression of lipolytic enzymes such as ACOX1, a key protein that initiates peroxisomal FA β-oxidation (53). A possible mechanism that underlies the ability of RM oil to ameliorate hepatic mRNA expression of *Tnfa* (Figure 5A) could also implicate PPAR-α signaling: n–3 LCPUFA-dependent PPAR-α activation promotes its interaction with the p65 subunit of NF-κB to form the PPAR-α/NF-κB complex, which leads to the decreased NF-κB–stimulated mRNA expression of proinflammatory genes (54). Finally, mice belonging to RM oil groups were supplemented with 1.94 mg ALA · g body weight^{-1} · d^{-1} in this study. Considering the concept of body surface area, which accounts for differences in the metabolic rate among different species (55), we determined that the human equivalent dose for dietary RM oil supplementation is 0.16 g ALA · kg body weight^{-1} · d^{-1}, which is ~40 mL RM oil/d for humans weighing ~70 kg. The higher dose of RM oil used in mice is needed to achieve comparable results considering that HFD is given in a comparatively shorter period and the metabolic rate of mice is higher than in humans (13).

Taken together, these data emphasize the effectiveness of RM oil in preventing HFD-induced insulin resistance and metabolic alterations in a fashion similar to dietary EPA and DHA (5, 25), validating the use of this oil as a nutritional source of ALA for the synthesis of n–3 LCPUFAs and phytochemicals with a high antioxidant potential. These data also make it possible to establish that dietary RM oil supplementation in a mouse model of metabolic alteration caused by obesity prevents insulin sensitivity and glucose homeostasis alteration, ameliorates the systemic and local proinflammatory state, and upregulates hepatic NRF2 and PPAR-α, all of which may be associated with the antioxidant, anti-inflammatory, and antisteatotic properties ascribed to RM oil. The data presented herein support the potential use of dietary RM oil in preventing comorbidities induced by nutritional factors.

Acknowledgments

DG-M, CGD, and MSM conducted the research; DG-M, AD, CGD, and GST analyzed the data and wrote the paper; DG-M, RAM, and GST provided the essential reagents and materials; and GST had primary responsibility for the final content. All authors conceived and designed the research and read and approved the final manuscript.

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