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Olive oil-derived nitro-fatty acids: protection of mitochondrial function in non-alcoholic fatty liver disease

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is characterized by excessive liver fat deposition in the absence of significant alcohol intake. Since extra virgin olive oil (EVOO) reduces fat accumulation, we analyzed the involvement of nitro-fatty acids (NO_2-FA) on the beneficial effects of EVOO consumption on NAFLD. Nitro-fatty acids formation was observed during digestion in mice supplemented with EVOO and nitrite. Mice fed with a high-fat diet (HF) presented lower plasma NO_2-FA levels than normal chow, and circulating concentrations recovered when the HF diet was supplemented with 10% EVOO plus nitrite. Under NO_2-FA formation conditions, liver hemoxygenase-1 expression significantly increased while decreased body weight and fat liver accumulation. Mitochondrial dysfunction plays a central role in the pathogenesis of NAFLD while NO_2-FA has been shown to protect from mitochondrial oxidative damage. Accordingly, an improvement of respiratory indexes was observed when mice were supplemented with both EVOO plus nitrite. Liver mitochondrial complexes II and V activities were greater in mice with EVOO supplementation and further improved in the presence of nitrite. Overall, our results strongly suggest a positive correlation between NO_2-OA formation from EVOO and the observed

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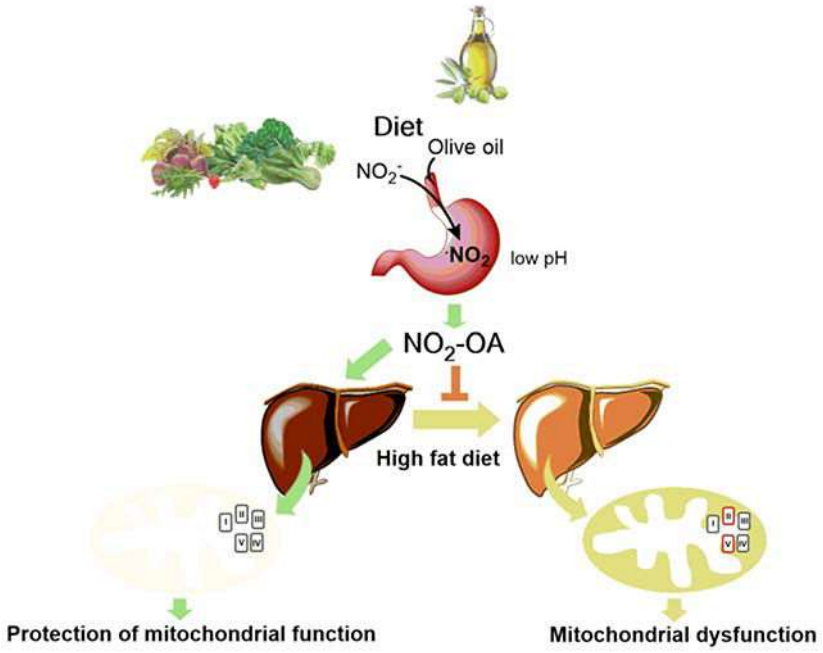
Author contributions:

BSC designed and performed experiments, analyzed data, and drafted the manuscript; AC designed, performed, and analyzed mitochondrial studies; MM performed and analyzed mass spectrometry experiments; MS supervised and executed the animal protocols; ET analyzed microscopy studies; EEK made manuscript revision; HR discussed data and made manuscript revisions; AT conceived and supervised the study, as well as made manuscript revisions and final format approval.

improvement of mitochondrial function in NAFLD. The formation of NO_2-FA can account for the health benefits associated with EVOO consumption.

Graphical Abstract



Keywords

Cell signaling; mitochondria; nonalcoholic fatty liver disease; nutrition; liver; nitroalkenes; fresh biopsies; High fat diet; Mediterranean diet

1. INTRODUCTION

Non-Alcoholic fatty liver disease (NAFLD) is characterized by excessive fat deposition in hepatocytes in the absence of significant alcohol intake [1]. NAFLD encompasses a wide spectrum of conditions ranging from simple steatosis, predominantly as large droplets or macrovesicular fat, to a more severe clinical status by the appearance of inflammation, termed nonalcoholic steatohepatitis [2,3]. The latter may progress to cirrhosis, hepatocellular carcinoma, and liver failure [2,3]. During the development of NAFLD, hepatic mitochondria adapt to hepatocytic fat accumulation. Lipotoxicity promotes the increase in reactive oxygen species generation, with the consequent damage to hepatocytes and induction of inflammation. Indeed, hepatic mitochondrial dysfunction plays a critical role in the development and the pathogenesis of NAFLD by still undefined mechanisms [4–8]

Reports from the literature show a decrease in hepatic lipid accumulation by extra virgin olive oil (EVOO) [9–11]. Most of the studies on EVOO have been focused on the beneficial effects of its consumption, being EVOO the main source of beneficial lipids in the Mediterranean diet. Olive oil is considered a functional food with high levels of

monosaturated fatty acids that also contains multiple minor components with biological activities [12]. As well as for the liver lipid accumulation, the mechanisms for the observed EVOO effects remain undetermined.

Nitro-fatty acids (NO_2-FA) are formed at the gastric compartment after fat intake. In addition to diet-derived nitrite (NO_2^-), the acidic milieu of the gastric compartment promotes a nitrative environment that, in turn, can mediate fatty acid nitration [13–18]. NO_2-FA are formed through reactions of nitric oxide-derived oxidant species, e.g. nitrogen dioxide ($^{\bullet}NO_2$), with either free or esterified unsaturated fatty acids [19–23]. Previous work from our group showed that EVOO contains low levels of nitro-conjugated linoleic acid (NO_2-cLA) [15]. Under gastric conditions, the formation of nitro-oleic acid (NO_2-OA) significantly increased [15]. NO_2-FA exerts cytoprotective and anti-inflammatory signaling responses at different cell types and physiopathological conditions [19,24–27]. NO_2-OA, the most studied NO_2-FA, presents potent anti-inflammatory and antioxidant properties in preclinical studies [23]. The protective role of NO_2-OA has been shown in diverse animal models of ischemic cardiac injury [28], pulmonary arterial hypertension [29], renal disease [30,31], hepatic injury [5,32,33] among others [26,34]. No data has been reported about the effect that EVOO can produce in high-fat diet (HF) fed mice, a condition that stimulates NAFLD, when fatty acid nitration is stimulated by the concomitant addition of NO_2^-. In the present study, we assessed the capacity of EVOO-associated NO_2-FA generation in HF mice to protect key parameters associated with NAFLD focusing on the protection of liver mitochondria function.

2. MATERIALS AND METHODS

2.1. Materials.

Labeled nitro-oleic acid ($[^{13}C]_{18}NO_2$-OA) was provided by Professor Bruce Freeman from the University of Pittsburgh. EVOO was from the Frantoio variety and obtained from a first press extraction. EVOO was provided by Dr. Juan B. Barroso from Universidad de Jaén, Spain. Antibody to HO-1 was from StressGen Biotech and antibody to GAPDH was from Sigma Co (Saint Louis, MO). All solvents were of HPLC grade from Pharmco (Brookfield, CT). All other reagents were from Sigma Chemical Co (Saint Louis, MO) unless otherwise specified.

2.2. Animal model

Female C57Bl/6J mice, at 6–8 weeks of age, were from the Dirección de Laboratorios Veterinarios (DILAVE, Uruguay) and housed at the Bioterio of Facultad de Medicina, Universidad de la República, Uruguay (UdelaR). Animal care and procedures followed the Guide for the Care and Use of Laboratory Animals, approved by the Comisión Honoraria de Experimentación Animal (CHEA, Protocol number N° 0011/11). The animals were treated for 12 weeks while randomly assigned to one of the 8 groups, with ten animals each, according to their feeding conditions as shown in Scheme 1. Four groups were fed with a normal chow diet with 10% of the adjusted calories derived from fat (ND), with the following experimental conditions: ND; ND plus water with 150 μM NO_2^-; ND supplemented with 10% (w/w) EVOO; ND plus water with 150 μM NO_2^- and 10% (w/w)

EVOO. NAFLD was induced by feeding the other four groups with a high-fat diet (HF) prepared as previously [35]. The composition of the HF was analyzed by Laboratorio de Nutrición Animal y Evaluación de Alimentos, Instituto Nacional de Investigaciones Agropecuarias (INIA) and had 60% of the adjusted calories derived from fat (fat source, beef tallow). HF groups were like those with ND (Scheme 1). In all cases, EVOO was from the Frantoio variety and animals had access to food and water ad libitum. Weight gain, food, and water intake were monitored every week during the period of study. Both ND and HF control groups containing 150 μM NO_2^- showed no differences in all the evaluated parameters compared to the same groups in the absence of NO_2^- (data not shown). Total water consumption and food intake did not present any differences between the groups. Thus, the results shown correspond to six groups in total with ND and HF named as the control groups. At the end of the experiment, animals were slaughtered under anesthesia with isoflurane

2.3. Plasma nitro-fatty acid quantitation

Analysis of plasma NO_2-FA was done as reported [36–38]. Plasma from heparinized blood was obtained after centrifugation at 1800 rpm for 10 min. The plasma was separated and incubated with 10 nM $[^{13}C]_{18}NO_2$-OA for 5 min at 4°C. Then, lipids were extracted with cold acetonitrile (1:4, v/v) and centrifuged 10 min at 1800 rpm and 4°C. After extraction, qualitative and quantitative analysis of NO_2-FA by LC-ESI MS/MS was performed using a hybrid triple-quadrupole linear ion trap mass spectrometer (QTRAP4500, ABSciex, Framingham, MA) coupled to a quaternary pump HPLC (Infinity 1260, Agilent). Reverse-phase HPLC was performed using a C18 column (Luna 100×2 mm, 2 μM, Phenomenex), and NO_2-FA were separated and eluted from the column using a gradient solvent system consisting of A (H_2O containing 0.05% acetic acid) and B ($CNCH_3$ containing 0.05% acetic acid) at 700 μl/min under the following conditions: 0 min 30% B, then 30–100% B in 10 min; 10–11 min 100% B and 100–0% B in 0.1 min and maintained for 4 min. Quantitative analysis was performed in the multiple reaction monitoring (MRM) scan mode. The transitions used were as follows: NO_2-OA, m/z 326.2/46.1; $[13C]_{18}NO_2$-OA, m/z 344.2/46.1 [36,37].

2.4. Histology

Formalin-fixed liver samples were cryoprotected in 30% sucrose at 4°C overnight. Before sectioning, blocks were frozen in O.C.T. Compound (Tissue-Tek) and were brought up to about −20 °C, then sectioned at 10 microns on a Cryotome. Slides were stored at −80 °C until stained. Liver cryosections were stained with hematoxylin and Oil red O [32]. Bright field oil red images were acquired using a Zeiss LSM 800 microscope connected to a color XXX Cannon camera. Fluorescence imaging was performed with a laser scanning Zeiss LSM 800 confocal microscope with a 63x (1.3 numerical aperture) oil immersion objective using Zeiss Zen Black software. The density of oil red per area was measured in at least 10 slices per animal (n=3 per group) using the ImageJ software.

2.5. Study of mitochondrial function.

For mitochondrial studies, animals were slaughtered under anesthesia with isoflurane, livers removed, and immediately weighed. Livers were washed extensively with cold PBS until

they were free of blood and a biopsy of about 10 mg was obtained for further analysis. For respiration analysis, liver biopsies were permeabilized as described previously [39] with minor modifications. Fresh liver biopsies (4–5 mg) were permeabilized in ice-cold MIR05 medium with saponin (25 mg mL-1) for 10 min; then, biopsies were transferred to a petri dish and washed thoroughly with at least 20 mL of ice-cold modified MIR05 medium per sample. Cell respiration and mitochondrial function were immediately analyzed using an Oxygraph 2 K (Oroboros Instruments Corp). Oxygen consumption was recorded at 37°C in permeabilized liver biopsies, with the rate of oxygen consumption calculated employing the equipment software (DataLab). Oxygen consumption is expressed as pmol of $O_2 \cdot s^{-1} \cdot mL^{-1}$ being the volume of the chamber 2.4 mL (V) in all cases, and sample biopsy weight expressed in mg [39,40].

Baseline measurements of biopsies' oxygen consumption were performed at the beginning of the assay followed by the sequential addition of 20 mM succinate for complex II evaluation and 4 mM ADP [39]. Inhibition of respiration by 2.5 μM antimycin A was subtracted from all other values before calculating the reported respiratory parameters as described [41–44]. State 4 was determined in the presence of complex II substrates, corresponding to the rate of oxygen consumption/ sample weight. State 3 was measured after the addition of ADP, which resembles the basal respiration of the tissue at saturating concentrations of substrates and ADP/ sample weight. The cell respiratory control ratio (RCR), corresponding to the state 3/state 4 ratio, represents the coupling between ATP synthesis and electron transport [39].

2.6. Enzymatic assays and western blot

For mitochondrial complexes activities and western blot analyses, the liver was homogenized in PBS using a Next Advance (Next Advance, Inc., NY) bullet blender with bullet size and time of homogenization following the protocols given by the company. 0.5 mm zirconium oxide beads were added using a volume of beads equivalent to 1x the volume of the sample and samples homogenized for 3 minutes at a speed of 8. The supernatant was separated from the beads and further used for the necessary experimental procedures (18).

ATP synthase (ATPase) activity was determined by monitoring NADH oxidation at 340 nm (ε= 6.22 $mM^{-1} \cdot cm^{-1}$)) in a coupled reaction of pyruvate kinase and lactate dehydrogenase [45]. The reaction mixture contained liver homogenate, 2.5 mM ATP, 50 mg/mL pyruvate kinase, 50 mg/mL lactate dehydrogenase in 50 Mm Hepes, pH 8 [46]. Succinate dehydrogenase (SDH) was measured spectrophotometrically in the presence of 20 mM 2,6-dichlorophenol indophenol (DCPIP), 15 mM succinate, and 2 mM potassium cyanide. Reduction of DCPIP was followed at 600 nm (ε = 20.5 $mM^{-1} \cdot cm^{-1}$) [47]. The protein content of the liver homogenates was determined by Bradford [48] and the mitochondrial complexes' activities were reported per mg of tissue protein.

Liver proteins were separated by 10% SDS–PAGE as reported [49]. Gels were transferred to nitrocellulose membrane and proteins electroblotted by a semi-dry Trans-Blot cell. Membranes were incubated with a polyclonal antibody against HO-1 in a 1:1000 dilution or anti-GADPH diluted 1:2000. The LiCOR Odyssey system was used for immunodetection.

2.7. Statistical analysis.

The results are expressed as mean ± standard error of the means with values of at least three independent experiments and analyzed with GraphPad Prism. Statistical analyzes were performed by Student t-test or ANOVA followed by Tukey post-test. Differences were significant when $p \leq 0.05$. In the case of histological studies, Kruskal-Wallis followed by Dunn's multiple comparison tests were used for statistical analysis with $p < 0.05$ considered significant.

3. RESULTS

3.1. EVOO consumption increases nitro-fatty levels in plasma

Considering our previous studies demonstrating the formation of NO_2-OA in EVOO under in vitro model of gastric acidic conditions [15], we assessed the plasmatic levels of NO_2-OA after EVOO supplementation. Plasma from the HF animals exhibited lower levels of NO_2-OA when compared to ND mice (2.27 ± 0.05 fmol NO_2-OA/mg protein vs 1.93 ± 0.08 fmol NO_2-OA/mg protein; Figure 1). Supplementation with 10% EVOO restored plasmatic NO_2-OA levels (2.23 ± 0.13 fmol NO_2-OA/mg protein). Importantly, NO_2-OA levels were even greater when EVOO supplementation was in conjunction with NO_2^-, a condition where lipid nitration was expected favored (Figure 1). These levels represent a 30% increase of NO_2-OA compared to the HF condition (2.53 ± 0.12 fmol NO_2-OA/mg protein vs 1.93 ± 0.08 fmol NO_2-OA/mg protein).

3.2. Effects of NO_2-OA formation on hemoxygenase-1 expression.

To elucidate whether the formed NO_2-OA reaches the liver, heme oxygenase-1 (HO-1) expression was tested (Figure 2). HO-1 is an inducible enzyme, which plays an important role in both acute and chronic disorders due to its anti-inflammatory, and antioxidant properties [50]. The activation of HO-1 expression is regulated by multiple transcription factors sensitive to functional changes induced by electrophilic species, such as NO_2-FA [27,51]. In both ND and HF mice, HO-1 expression was induced by EVOO supplementation being greater when administered in addition to NO_2^- (Figure 2). This evidences the relationship between the generation of these compounds and the expression of HO-1 in the liver.

3.3. EVOO supplementation reduces body and liver weight

As shown in Table 1, after 12 weeks, mice fed with HF gained more weight compared to those with ND (5.8 ± 0.20 g vs 1.33 ± 0.21 g). Supplementation of the diet with EVOO decreased by almost 20% the gain of weight in the HF groups either in the absence or presence of NO_2^- (Table 1). Fatty liver is considered when the organ weight increases at least 5% from normal conditions [6,52]. The livers from the mice of the different groups were obtained after 12 weeks of diet treatment. Liverś weight was significantly greater (43%) in the HF mice compared to ND (Table 1). Notably, supplementation with 10% EVOO led to a lower weight increase being about 23 % lighter compared to those from HF (Table 1). Nitrite addition did not add any effect on neither ND nor HF liver weight, when compared to their corresponding EVOO supplemented groups (Table 1). No differences in

food intake during the experimental time were observed between the different groups (data not shown).

3.4. EVOO decreases liver fat accumulation in HF mice

Accumulation of lipids led to an increase in liver weight. We analyzed macroscopic and microscopic aspects of livers from the different experimental groups (Figures 3 and 4). Livers from HF mice resemble typical steatosis (Figure 3B), with the loss of the robust red healthy color compared to ND (Figure 3A). Supplementation with EVOO improves the liver appearance (Figure 3C) while EVOO + NO_2^- treatment restores the red color indicative of less fat accumulation (Figure 3D). Following the liverś weight, livers from ND and HF groups supplemented with EVOO were softer than those from HF. We did not observe any differences in the macroscopic aspect of livers from ND groups supplemented with EVOO in the absence or presence of NO_2^- compared to the ND condition (data not shown).

Fat accumulation in HF mice was observed histologically using oil red. Positive lipid droplets accumulated in HF livers sections compared to the ND group (Figures 4A and B). The lipid accumulation in the liver is indicative of the development of NAFLD characterized by simple steatosis. Supplementation with EVOO led to a reduction of 40% in hepatic lipid accumulation in the HF groups (Figure 4B). Finally, EVOO did not show any improvement in fat accumulation in ND groups (Figure 4).

3.5. EVOO improves mitochondrial function

Lipid toxicity due to the consumption of a HF diet causes mitochondrial dysfunction which produces oxidative stress and is related to apoptosis, inflammation, and other cellular disorders [4,6]. Respiratory analyses were carried out in liver biopsies, to assess electron transport chain activity and oxidative phosphorylation. Hepatic mitochondrial oxygen consumption rates in liver biopsies from mice under ND or HF diet were analyzed by high-resolution respirometry. Complex II-dependent respiration was diminished in HF animals (Figure 5). Oxygen consumption rates as well as oxidative phosphorylation (RCR values) were lower in HF mice than ND (Figure 5). Notably, conditions that stimulate NO_2-FA formation (EVOO + NO_2^-) significantly improved mitochondrial function at both state 3 and RCR (Figures 5B and 5C). Moreover, supplementation with EVOO also potentiated oxidative phosphorylation in ND mice compared to the control group (Figure 5C).

Considering the observed respiration data, mitochondrial respiratory chain complexes were analyzed (Figure 6). Complex II (succinate dehydrogenase, SDH) and complex V (ATP synthase, ATPase) activities were lowered by the HF diet (Figure 6). Both were restored by 10% EVOO supplementation and complexes' activities increased at conditions of NO_2-FA formation (Figure 6). Besides, supplementation of ND with EVOO improved mitochondrial activity at both complex V and RCR compared to controls (Figures 5C and 6B).

4. DISCUSSION

Herein, we examined the influence of EVOO supplementation in a murine model of NAFLD induced by the consumption of a HF diet. Currently, it is widely accepted that HF-diet-induced obesity in experimental animals resembles conditions of overnutrition and physical

inactivity that are major risk factors for the development of metabolic syndrome in humans [53,54]. Non-alcoholic fatty liver disease is emerging as the most common chronic liver condition in the Western world, is a condition with a wide spectrum of symptoms from simple steatosis to a more severe clinical status by the appearance of inflammation with or without fibrosis [2,52,55,56]. Our results can be analyzed according to the hypothesis of the "two-hit" for the establishment and outcome of the disease. The "first hit" involves the accumulation of fat in the liver while the "second hit" includes oxidative stress and mitochondrial dysfunction [57]. An excessive fat deposition in hepatocytes in the absence of significant alcohol consumption characterizes hepatic steatosis which is defined as an excess of intrahepatic fat greater than 5% of the liverś volume [1,5,6]. Other considerations to analyze the effects of a HF diet on the appearance of NAFLD are connected to the *in vivo* model. Recent work in the area discussed the relevance of the mice strain, the gender and age of the animals used, and the duration of the study when using high-fat diets [54]. This study observed that the treatment for 12 weeks with a HF diet containing 40% fat (60% of the calories derived from fat) rich in saturated fatty acids (SFAs) lead to an increase in body weight and fat accumulation in the liver characteristic of NAFLD. Diets rich in SFAs in mice C57B1/6j cause obesity with a decreased in basal metabolic rates, higher body weights as well as fat gain when compared to diets rich in unsaturated fatty acids [54,58–60]. We observed that female C57B1/6j mice fed with HF, as well as their livers, gained weight when compared to the ND group. Moreover, liver macroscopic alterations (color and texture) were also observed, and histological data confirmed the characteristic fat accumulation of liver steatosis. Interestingly, NAFLD associated fat liver accumulation and body weight gain, were lower when HF mice were supplemented with 10% EVOO (w/w).

The health benefits of EVOO are attributed to its lipid profile, mainly the monounsaturated/polyunsaturated fatty acids ratio, and the presence of bioactive compounds such as polyphenols [61–64]. Among EVOOs' biological benefits have been described as a decrease in cholesterol levels and low-density lipoprotein oxidation, decrease in inflammation, improvement of endothelial dysfunction, blood pressure, thrombosis, hyperglycemia, and NAFLD among others [12,65–73]. In addition to the consumption of EVOO, the Mediterranean diet contains large quantities of vegetables rich in NO_2^- and nitrate (NO_3^-). The latter is widely accepted to exert nitric-oxide (·NO)-like bioactivities at the cardiovascular system as a reduction of blood pressure, inhibition of platelet aggregation, and protection against ischemia-reperfusion [18,74–77]. Previous reports from our lab showed that EVOO is a source of unsaturated fatty acids for NO_2-FA formation under acidic gastric conditions [15]. Moreover, NO_2-FA incorporated into complex lipids can be released by lipase hydrolysis and be distributed to different tissues thus modulating cell signaling [78,79]. Oral administration of NO_2-FA results in their absorption and distribution to the kidney, liver, lungs, and heart [17,26]. However, less is known about the effect that dietary EVOO supplementation in the presence of NO_2^- can exert to stimulate NO_2-FA formation and biological actions. Our experimental conditions present all the substrates to perform endogenous lipid nitration, thus plasma NO_2-FA levels were quantified. Although gastric conditions stimulate NO_2-cLA formation as reported previously by our lab [15], their levels after EVOO supplementation were below the limit of quantification probably due to the low concentration of conjugated linoleic acid in EVOO (less than 0.2%) [15]. NO_2-OA levels

were lower in the HF condition when compared to ND whereas EVOO supplementation reversed this decrease. Moreover, in the condition of HF supplemented with EVOO plus NO_2^-, NO_2-OA concentration was higher not only than the condition of HF plus EVOO but even to the ND group. Since NO_2-FA exert antioxidant and anti-inflammatory effects including inhibition of pro-inflammatory transcription factors or the release of pro-inflammatory cytokines, their capacity to induce the expression of antioxidant phase II enzymes was determined as a marker of their protective actions [27]. Liver HO-1 expression was increased in animals supplemented with EVOO even when the animals were under ND. By the increased plasmatic levels of NO_2-OA, supplementation with EVOO in the presence of NO_2^- presented the highest expression of HO-1. Considering the extensive reports in the literature about the effects induced by NO_2-FA on HO-1 expression and protein levels, and how these changes exert protection on inflammatory processes [27,51,80,81], our data support that stimulating NO_2-FA formation is a novel mechanism to explain the beneficial effects of EVOO consumption.

Liver lipid accumulation causes the organ weight increase while hepatocyte toxicity is related to mitochondrial dysfunction, oxidative stress, apoptosis, inflammation, and other cellular disorders [4,54,82,83]. Mitochondrial dysfunction plays an important role in the development of NAFLD since mitochondria are involved not only in fatty acid β-oxidation but also represent a key source of oxidant species [84]. Its relation with the “two-hit” hypothesis and involvement in the pathogenesis of the disease has been established [7,8]. Reduction of mitochondrial complex activity has been reported *in vivo* and *in vitro* models using SFAs diets, by decreasing the amount of the electron chain complexes subunits [85–87]. With this background, we analyzed hepatic mitochondrial respiration in HF mice at conditions where NO_2-FA formation is stimulated. Reduced succinate dependent-mitochondrial function measured by respiratory parameters (state 3 and RCR) and electron transport respiratory chain complexes activities were observed on HF mice livers compared to controls. When the HF diet was supplemented with EVOO plus NO_2^-, respiratory parameters, as well as complexes II and V enzymatic activities, were higher than HF and ND groups. Mitochondria is a hub for cell signaling processes in inflammation, and NO_2-FA modulate inflammatory outcomes at a mitochondrial function [28,40,88]. Liver mitochondrial function was improved in conditions where NO_2-OA levels increased. We and others have reported that NO_2-FA can protect mitochondria under inflammatory or ischemic pre-conditioning conditions [37,40,89]. The capacity of NO_2-FA to be incorporated into mitochondrial membranes to exert protective effects was demonstrated in kidney cells activated with Angiotensin-II [40] while a recent study shows the capacity of NO_2-OA to diminish hepatic triglycerides accumulation, liver damage and improve mitochondrial function [5]. One of the mechanisms by how NO_2-FA exert their effects on mitochondrial function is the formation of electrophilic adducts with critical cysteine, histidine, and lysine residues. Indeed, mitochondria have multiple targets for electrophilic modifications whose adduction can be cytoprotective at mitochondrial dysfunction conditions. Reports in the literature demonstrate the capacity of NO_2-OA to covalently modify, in a reversible manner, mitochondrial complex II affecting respiration and decreasing oxidant species formation [28]. Other changes include a metabolic shift to glycolysis, which improves cellular outcome involving covalent modifications by NO_2-FA of glycolytic enzymes, e.g. GAPDH [90]. The

effects exerted by NO_2-FA on mitochondria vary from inhibition of respiration [28] to uncoupling mitochondria by modifying UCP-2 [89] protecting hearts at ischemia/reperfusion conditions. The effects on liver cells were not previously analyzed, and the modifications needed to protect these cells at the mitochondrial level can be different. Previous reports in a Non-Alcoholic Hepatic Steatosis (NASH) model treated with NO_2-OA showed that lipid metabolism enzymes or transcription factors are modulated [32]. Some of these proteins are mitochondrial. Considering our results on mitochondrial function and mitochondrial complexes activities, future studies will be aimed to detect modifications of relevant mitochondrial proteins in our experimental model. Other authors propose NO_2-OA as a new drug candidate for NAFLD and other inflammatory diseases [26,33]. Thus, our results strongly suggest a positive correlation between NO_2-OA formation from EVOO and the observed improvement of mitochondrial function. The gastric acidic formation of NO_2-OA can lead to their absorption and tissue distribution being the liver one of its target organs where they can exert pleiotropic protective effects.

In summary, our results propose a novel nutraceutical value associated with the consumption of EVOO in conjunction with vegetables, as NO_2^- sources. Overall, our data support that stimulating NO_2-FA formation is a novel mechanism to explain the beneficial effects of consuming EVOO. These conditions favor NO_2-FA formation and protective effects, as those observed by the improvement of mitochondrial function in NAFLD, which are in line with the health beneficial effects ascribed to the Mediterranean diet.

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5. REFERENCES

[1]. Vernon G, Baranova A, Younossi ZM. Systematic review : the epidemiology and natural history of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis in adults. Aliment Pharmacol Ther 2011;34:274–85. [PubMed: 21623852]

[2]. Adams LA, Lymp JF, St Sauver J, Sanderson SO, Lindor KD, Feldstein A, et al. The natural history of nonalcoholic fatty liver disease: a population-based cohort study. Gastroenterology 2005;129:113–21. [PubMed: 16012941]

[3]. Priore P, Cavallo A, Gnoni A, Damiano F, Gnoni G V, Siculella L. Modulation of hepatic lipid metabolism by olive oil and its phenols in nonalcoholic fatty liver disease. IUBMB Life 2015;67:9–17. [PubMed: 25631376]

[4]. Begriche K, Massart J, Robin M-A, Bonnet F, Fromenty B. Mitochondrial adaptations and dysfunctions in nonalcoholic fatty liver disease. Hepatology 2013;58:1497–507. [PubMed: 23299992]

[5]. Khoo NKH, Fazzari M, Chartoumpekis D V, Li L, Aparecida D, Arteel GE, et al. Electrophilic nitro-oleic acid reverses obesity-induced hepatic steatosis. Redox Biol 2019;22:101132. [PubMed: 30769284]

[6]. Nassir F, Ibdah JA. Role of mitochondria in nonalcoholic fatty liver disease. Int J Mol Sci 2014;15:8713–42. [PubMed: 24837835]

[7]. Grattagliano I, de Bari O, Bernardo TC, Oliveira PJ, Wang DQ, Portincasa P. Role of mitochondria in nonalcoholic fatty liver disease--from origin to propagation. Clin Biochem 2012;45:610–8. [PubMed: 22484459]

[8]. Grattagliano I, Russmann S, Diogo C, Bonfrate L, Oliveira J, Wang Q-H, et al. Mitochondria in Chronic Liver Disease. Curr Drug Targets 2011;12:879–93. [PubMed: 21269263]

[9]. Dos Santos GA, Ferreira MS, De Oliveira DN, De Oliveira V, Siqueira-Santos ES, Cintra DEC, et al. Identification of compounds from high-fat and extra virgin olive oil-supplemented diets in whole mouse liver extracts and isolated mitochondria using mass spectrometry. J Mass Spectrom 2015;50:951–8. [PubMed: 26349651]

[10]. Valenzuela R, Espinosa A, Llanos P, Hernandez-Rodas MC, Barrera C, Vergara D, et al. Anti-steatotic effects of an n-3 LCPUFA and extra virgin olive oil mixture in the liver of mice subjected to high-fat diet. Food Funct 2016;7:140–50. [PubMed: 26471014]

[11]. Jurado-Ruiz E, Varela LM, Luque A, Berna G, Cahuana G, Martinez-Force E, et al. An extra virgin olive oil rich diet intervention ameliorates the nonalcoholic steatohepatitis. Mol Nutr Food Res 2017;61.

[12]. Gorzynik-Debicka M, Przychodzen P, Cappello F, Kuban-Jankowska A, Marino Gammazza A, Knap N, et al. Potential Health Benefits of Olive Oil and Plant Polyphenols. Int J Mol Sci 2018;19.

[13]. Rocha BS, Gago B, Barbosa RM, Lundberg JO, Radi R, Laranjinha J. Intragastric nitration by dietary nitrite: implications for modulation of protein and lipid signaling. Free Radic Biol Med 2012;52:693–8. [PubMed: 22154654]

[14]. Lundberg JO, Weitzberg E. Biology of nitrogen oxides in the gastrointestinal tract. Gut 2013;62:616–29. [PubMed: 22267589]

[15]. Fazzari M, Trostchansky A, Schopfer FJ, Salvatore SR, Sanchez-Calvo B, Vitturi D, et al. Olives and olive oil are sources of electrophilic fatty acid nitroalkenes. PLoS One 2014;9:e84884. [PubMed: 24454759]

[16]. Delmastro-Greenwood M, Freeman BA, Wendell SG. Redox-dependent anti-inflammatory signaling actions of unsaturated fatty acids. Annu Rev Physiol 2014;76:79–105. [PubMed: 24161076]

[17]. Khoo NKH, Schopfer FJ. Nitrated fatty acids : from diet to disease. Curr Opin Psychol 2019;9:67–72.

[18]. Lundberg JO, Carlström M, Weitzberg E. Metabolic Effects of Dietary Nitrate in Health and Disease. Cell Metab 2018.

[19]. Cui T, Schopfer FJ, Zhang J, Chen K, Ichikawa T, Baker PR, et al. Nitrated fatty acids: Endogenous anti-inflammatory signaling mediators. J Biol Chem 2006;281:35686–98. [PubMed: 16887803]

[20]. Schopfer FJ, Cipollina C, Freeman BA. Formation and signaling actions of electrophilic lipids. Chem Rev 2011;111:5997–6021. [PubMed: 21928855]

[21]. Trostchansky A, Rubbo H. Nitrated fatty acids: mechanisms of formation, chemical characterization, and biological properties. Free Radic Biol Med 2008;44:1887–96. [PubMed: 18395528]

[22]. Freeman BA, Baker PR, Schopfer FJ, Woodcock SR, Napolitano A, d'Ischia M. Nitro-fatty acid formation and signaling. J Biol Chem 2008;283:15515–9. [PubMed: 18285326]

[23]. Schopfer FJ, Khoo NKH. Nitro-Fatty Acid Logistics: Formation, Biodistribution, Signaling, and Pharmacology. Trends Endocrinol Metab 2019;30:505–19. [PubMed: 31196614]

[24]. Ferreira AM, Trostchansky A, Ferrari M, Souza JM, Rubbo H. Nitroalkenes: synthesis, characterization, and effects on macrophage activation. Methods Enzym 2008;441:33–51.

[25]. Deen AJ, Sihvola V, Harkonen J, Patinen T, Adinolfi S, Levonen AL. Regulation of stress signaling pathways by nitro-fatty acids. Nitric Oxide 2018.

[26]. Schopfer FJ, Vitturi DA, Jorkasky DK, Freeman BA. Nitro-fatty acids: New drug candidates for chronic inflammatory and fibrotic diseases. Nitric Oxide 2018.

[27]. Geisler AC, Rudolph TK. Nitroalkylation: a redox sensitive signaling pathway. Biochim Biophys Acta 2012;1820:777–84. [PubMed: 21723375]

[28]. Koenitzer JR, Bonacci G, Woodcock SR, Chen C-S, Cantu-Medellin N, Kelley EE, et al. Fatty acid nitroalkenes induce resistance to ischemic cardiac injury by modulating mitochondrial respiration at complex II. Redox Biol 2016;8:1–10. [PubMed: 26722838]

[29]. Klinke A, Moller A, Pekarova M, Ravekes T, Friedrichs K, Berlin M, et al. Protective effects of 10-nitro-oleic acid in a hypoxia-induced murine model of pulmonary hypertension. Am J Respir Cell Mol Biol 2014;51:155–62. [PubMed: 24521348]

[30]. Liu Y, Jia Z, Liu S, Downton M, Liu G, Du Y, et al. Combined losartan and nitro-oleic acid remarkably improves diabetic nephropathy in mice. Am J Physiol Ren Physiol 2013;305:F1555–62.

[31]. Liu S, Jia Z, Zhou L, Liu Y, Ling H, Zhou SF, et al. Nitro-oleic acid protects against adriamycin-induced nephropathy in mice. Am J Physiol Ren Physiol 2013;305:F1533–41.

[32]. Rom O, Xu G, Guo Y, Zhu Y, Wang H, Zhang J, et al. Nitro-fatty acids protect against steatosis and fibrosis during development of nonalcoholic fatty liver disease in mice. EBioMedicine 2019;41:62–72. [PubMed: 30772307]

[33]. Yang RX, Fan JG. Nitro-oleic acid as a new drug candidate for non-alcoholic steatohepatitis. EBioMedicine 2019;42:32–3. [PubMed: 30948353]

[34]. Zhang J, Villacorta L, Chang L, Fan Z, Hamblin M, Zhu T, et al. Nitro-oleic acid inhibits angiotensin II-induced hypertension. Circ Res 2010;107:540–8. [PubMed: 20558825]

[35]. Kirk EA, Sutherland P, Wang SA, Chait A, LeBoeuf RC. Dietary isoflavones reduce plasma cholesterol and atherosclerosis in C57BL/6 mice but not LDL receptor-deficient mice. J Nutr 1998;128:954–9. [PubMed: 9614153]

[36]. Trostchansky A, Mastrogiovanni M, Miquel E, Rodriguez-Bottero S, Martinez-Palma L, Cassina P, et al. Profile of Arachidonic Acid-Derived Inflammatory Markers and Its Modulation by Nitro-Oleic Acid in an Inherited Model of Amyotrophic Lateral Sclerosis. Front Mol Neurosci 2018;11:131. [PubMed: 29760648]

[37]. Schopfer FJ, Batthyany C, Baker PR, Bonacci G, Cole MP, Rudolph V, et al. Detection and quantification of protein adduction by electrophilic fatty acids: mitochondrial generation of fatty acid nitroalkene derivatives. Free Radic Biol Med 2009;46:1250–9. [PubMed: 19353781]

[38]. Tsikas D, Zoerner AA, Mitschke A, Gutzki FM. Nitro-fatty acids occur in human plasma in the picomolar range: a targeted nitro-lipidomics GC-MS/MS study. Lipids 2009;44:855–65. [PubMed: 19701657]

[39]. Garcia-Roche M, Casal A, Carriquiry M, Radi R, Quijano C, Cassina A. Respiratory analysis of coupled mitochondria in cryopreserved liver biopsies. Redox Biol 2018;17:207–12. [PubMed: 29704825]

[40]. Sanchez-Calvo B, Cassina A, Rios N, Peluffo G, Boggia J, Radi R, et al. Nitro-Arachidonic Acid Prevents Angiotensin II-Induced Mitochondrial Dysfunction in a Cell Line of Kidney Proximal Tubular Cells. PLoS One 2016;11:e0150459. [PubMed: 26943326]

[41]. Brand MD, Nicholls DG. Assessing mitochondrial dysfunction in cells. Biochem J 2011;435:297–312. [PubMed: 21726199]

[42]. Hill BG, Benavides GA, Lancaster JR, Ballinger S, Dell'Italia L, Zhang J, et al. Integration of cellular bioenergetics with mitochondrial quality control and autophagy. Biol Chem 2012;393:1485–512. [PubMed: 23092819]

[43]. Pesta D, Gnaiger E. High-Resolution Respirometry: OXPHOS Protocols for Human Cells and Permeabilized Fibers from Small Biopsies of Human Muscle. In: Palmeira CM, Moreno AJ, editors. Mitochondrial Bioenerg. Methods Protoc, Totowa, NJ: Humana Press; 2012, p. 25–58.

[44]. Salabei JK, Gibb AA, Hill BG. Comprehensive measurement of respiratory activity in permeabilized cells using extracellular flux analysis. Nat Protoc 2014;9:421–38. [PubMed: 24457333]

[45]. Pullman ME, Penefsky HS, Datta A, Racker E. Partial resolution of the enzymes catalyzing oxidative phosphorylation. I. Purification and properties of soluble dinitrophenol-stimulated adenosine triphosphatase. J Biol Chem 1960;235:3322–9. [PubMed: 13738472]

[46]. Cassina A, Radi R. Differential inhibitory action of nitric oxide and peroxynitrite on mitochondrial electron transport. Arch Biochem Biophys 1996;328:309–16. [PubMed: 8645009]

[47]. Singer TP. Determination of the activity of succinate, NADH, choline, and alpha-glycerophosphate dehydrogenases. Methods Biochem Anal 1974;22:123–75. [PubMed: 4155042]

[48]. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54. [PubMed: 942051]

[49]. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5. [PubMed: 5432063]

[50]. Yet SF, Layne MD, Liu X, Chen YH, Ith B, Sibinga NES, et al. Absence of heme oxygenase-1 exacerbates atherosclerotic lesion formation and vascular remodeling. FASEB J 2003;17:1759–61. [PubMed: 12958201]

[51]. Wright MM, Schopfer FJ, Baker PR, Vidyasagar V, Powell P, Chumley P, et al. Fatty acid transduction of nitric oxide signaling: nitrolinoleic acid potently activates endothelial heme oxygenase 1 expression. Proc Natl Acad Sci U S A 2006;103:4299–304. [PubMed: 16537525]

[52]. Adams LA, Angulo P, Lindor KD. Nonalcoholic fatty liver disease. CMAJ 2005;172:899–905. [PubMed: 15795412]

[53]. Sharma RS, Harrison DJ, Kisielewski D, Cassidy DM, McNeilly AD, Gallagher JR, et al. Experimental Nonalcoholic Steatohepatitis and Liver Fibrosis Are Ameliorated by Pharmacologic Activation of Nrf2 (NF-E2 p45-Related Factor 2). Cmgh 2018;5:367–98. [PubMed: 29552625]

[54]. Kakimoto PA, Kowaltowski AJ. Effects of high fat diets on rodent liver bioenergetics and oxidative imbalance. Redox Biol 2016;8:216–25. [PubMed: 26826574]

[55]. Angulo P Nonalcoholic fatty liver disease. N Engl J Med 2002;346:1221–31. [PubMed: 11961152]

[56]. Lindenmeyer CC, McCullough AJ. The Natural History of Nonalcoholic Fatty Liver Disease-An Evolving View. Clin Liver Dis 2018;22:11–21. [PubMed: 29128051]

[57]. Day CP, James OF. Steatohepatitis: a tale of two “hits”? Gastroenterology 1998;114:842–5. [PubMed: 9547102]

[58]. Pan DA, Storlien LH. Dietary lipid profile is a determinant of tissue phospholipid fatty acid composition and rate of weight gain in rats. J Nutr 1993;123:512–9. [PubMed: 8463854]

[59]. Takeuchi H, Matsuo T, Tokuyama K, Shimomura Y, Suzuki M. Diet-induced thermogenesis is lower in rats fed a lard diet than in those fed a high oleic acid safflower oil diet, a safflower oil diet or a linseed oil diet. J Nutr 1995;125:920–5. [PubMed: 7722695]

[60]. Kien CL, Bunn JY, Ugrasbul F. Increasing dietary palmitic acid decreases fat oxidation and daily energy expenditure. Am J Clin Nutr 2005;82:320–6. [PubMed: 16087974]

[61]. Terés S, Barceló-Coblijn G, Benet M, Álvarez R, Bressani R, Halver JE, et al. Oleic acid content is responsible for the reduction in blood pressure induced by olive oil. Proc Natl Acad Sci U S A 2008;105:13811–6. [PubMed: 18772370]

[62]. Chen X, Li L, Liu X, Luo R, Liao G, Liu J, et al. Oleic acid protects saturated fatty acid mediated lipotoxicity in hepatocytes and rat of non-alcoholic steatohepatitis. Life Sci 2018;203:291–304. [PubMed: 29709653]

[63]. Marcelino G, Hiane PA, Freitas K de C, Santana LF, Pott A, Donadon JR, et al. Effects of olive oil and its minor components on cardiovascular diseases, inflammation, and gut microbiota. Nutrients 2019;11:4–6.

[64]. Gaforio JJ, Visioli F, Alarcón-De-la-lastra C, Castañer O, Delgado-Rodríguez M, Fitó M, et al. Virgin olive oil and health: Summary of the iii international conference on virgin olive oil and health consensus report, JAEN (Spain) 2018. Nutrients 2019;11.

[65]. Esposito K, Marfella R, Ciotola M, et al. Effect of a mediterranean-style diet on endothelial dysfunction and markers of vascular inflammation in the metabolic syndrome: A randomized trial. JAMA 2004;292:1440–6. [PubMed: 15383514]

[66]. Bondia-Pons I, Schroder H, Covas MI, Castellote AI, Kaikkonen J, Poulsen HE, et al. Moderate consumption of olive oil by healthy European men reduces systolic blood pressure in non-Mediterranean participants. J Nutr 2007;137:84–7. [PubMed: 17182805]

[67]. Frankel EN. Nutritional and biological properties of extra virgin olive oil. J Agric Food Chem 2011;59:785–92. [PubMed: 21210703]

[68]. Rozati M, Barnett J, Wu D, Handelman G, Saltzman E, Wilson T, et al. Cardio-metabolic and immunological impacts of extra virgin olive oil consumption in overweight and obese older adults: a randomized controlled trial. Nutr Metab 2015;12:28.

[69]. Perez-Martinez P, Garcia-Rios A, Delgado-Lista J, Perez-Jimenez F, Lopez-Miranda J. Mediterranean diet rich in olive oil and obesity, metabolic syndrome and diabetes mellitus. Curr Pharm Des 2011;17:769–77. [PubMed: 21443484]

[70]. Abenavoli L, Milanović M, Milić N, Luzza F, Abenavoli L, Milanović M, et al. Olive oil antioxidants and non-alcoholic fatty liver disease. Expert Rev Gastroenterol Hepatol 2019;13:739–49. [PubMed: 31215262]

[71]. Covas MI, de la Torre R, Fito M. Virgin olive oil: a key food for cardiovascular risk protection. Br J Nutr 2015;113 Suppl:S19–28. [PubMed: 26148918]

[72]. Reboredo-Rodríguez P, Figueiredo-González M, González-Barreiro C, Simal-Gándara J, Salvador MD, Cancho-Grande B, et al. State of the Art on Functional Virgin Olive Oils Enriched with Bioactive Compounds and Their Properties. Int J Mol Sci 2017;18:668.

[73]. Bullón-Vela V, Abete I, Tur JA, Pintó X, Corbella E, Martínez-González MA, et al. Influence of lifestyle factors and staple foods from the Mediterranean diet on non-alcoholic fatty liver disease among older individuals with metabolic syndrome features. Nutrition 2020;71:110620. [PubMed: 31838461]

[74]. Lundberg JO, Weitzberg E, Gladwin MT. The nitrate–nitrite–nitric oxide pathway in physiology and therapeutics. Nat Rev Drug Discov 2008;7:156. [PubMed: 18167491]

[75]. Lundberg JO, Weitzberg E. NO generation from inorganic nitrate and nitrite: Role in physiology, nutrition and therapeutics. Arch Pharm Res 2009;32:1119–26. [PubMed: 19727604]

[76]. Nadtochiy SM, Redman EK. Mediterranean diet and cardioprotection: the role of nitrite, polyunsaturated fatty acids, and polyphenols. Nutrition 2011;27:733–44. [PubMed: 21454053]

[77]. Karwowska M, Kononiuk A. Nitrates / Nitrites in Food — Risk for Nitrosative Stress and Benefits. Antioxidant 2020:1–17.

[78]. Fazzari M, Khoo NK, Woodcock SR, Jorkasky DK, Li L, Schopfer FJ, et al. Nitro-fatty acid pharmacokinetics in the adipose tissue compartment. J Lipid Res 2017;58:375–85. [PubMed: 27913584]

[79]. Buchan GJ, Bonacci G, Fazzari M, Salvatore SR, Gelhaus Wendell S. Nitro-fatty acid formation and metabolism. Nitric Oxide 2018;79:38–44. [PubMed: 30006146]

[80]. Bonacci G, Baker PR, Salvatore SR, Shores D, Khoo NK, Koenitzer JR, et al. Conjugated linoleic acid is a preferential substrate for fatty acid nitration. J Biol Chem 2012;287:44071–82. [PubMed: 23144452]

[81]. Mollenhauer M, Mehrkens D, Rudolph V. Nitrated fatty acids in cardiovascular diseases. Nitric Oxide 2018;78:146–53.

[82]. James AM, Collins Y, Logan A, Murphy MP. Mitochondrial oxidative stress and the metabolic syndrome. Trends Endocrinol Metab 2012;23:429–34. [PubMed: 22831852]

[83]. Tahara EB, Navarete FDT, Kowaltowski AJ. Tissue-, substrate-, and site-specific characteristics of mitochondrial reactive oxygen species generation. Free Radic Biol Med 2009;46:1283–97. [PubMed: 19245829]

[84]. Fromenty B, Robin MA, Igoudjil A, Mansouri A, Pessayre D. The ins and outs of mitochondrial dysfunction in NASH. Diabetes Metab 2004;30:121–38. [PubMed: 15223984]

[85]. Garcia-Ruiz I, Fernandez-Moreira D, Solis-Munoz P, Rodriguez-Juan C, Diaz-Sanjuan T, Munoz-Yague T, et al. Mitochondrial complex I subunits are decreased in murine nonalcoholic fatty liver disease: implication of peroxynitrite. J Proteome Res 2010;9:2450–9. [PubMed: 20222668]

[86]. Garcia-Ruiz I, Solis-Munoz P, Fernandez-Moreira D, Grau M, Colina F, Munoz-Yague T, et al. High-fat diet decreases activity of the oxidative phosphorylation complexes and causes nonalcoholic steatohepatitis in mice. Dis Model Mech 2014;7:1287–96. [PubMed: 25261569]

[87]. Garcia-Ruiz I, Solis-Munoz P, Fernandez-Moreira D, Munoz-Yague T, Solis-Herruzo JA. In vitro treatment of HepG2 cells with saturated fatty acids reproduces mitochondrial dysfunction found in nonalcoholic steatohepatitis. Dis Model Mech 2015;8:183–91. [PubMed: 25540128]

[88]. Koenitzer JR, Freeman BA. Redox signaling in inflamation: interactions of endogenous electrophiles and mitochondria in cardiovascular disease. Ann N Y Acad Sci 2010;1203:45–52. [PubMed: 20716282]

[89]. Nadtochiy SM, Baker PR, Freeman BA, Brookes PS. Mitochondrial nitroalkene formation and mild uncoupling in ischaemic preconditioning: implications for cardioprotection. Cardiovasc Res 2009;82:333–40. [PubMed: 19050010]

[90]. Batthyany C, Schopfer FJ, Baker PRS, Durán R, Baker LMS, Huang Y, et al. Reversible post-translational modification of proteins by nitrated fatty acids in vivo. J Biol Chem 2006;281:20450–63. [PubMed: 16682416]

Highlights

- EVOO supplementation in HF fed mice increase NO_2-FA levels
- Endogenous NO_2-FA formation decrease fat liver accumulation
- Liver mitochondrial function is improved by EVOO
- NO_2-FA can account for the health benefits associated with EVOO consumption

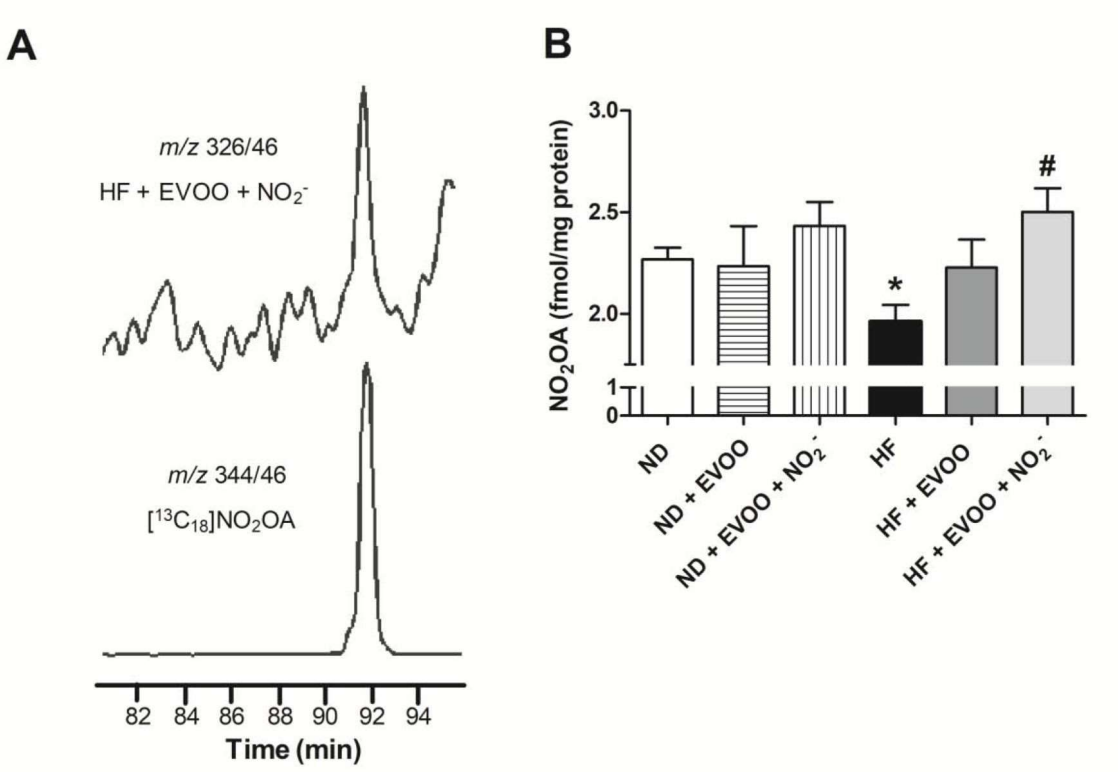


Figure 1. EVOO consumption favors the formation of nitro fatty acids.

Plasma from ND or HF fed mice, with or without EVOO supplementation in the absence or presence of 150 μM NO_2^-, was extracted with cold acetonitrile (1:4, v/v). Then, the presence and levels of NO_2-OA were analyzed by LC-ESI-MS/MS. (**A**) Nitro oleic acid (*m/z* 326/46) elution profile was determined using its ^{13}C labeled internal standard (*m/z* 344/46). Retention times and MS/MS spectra of the peaks (data not shown) confirmed the presence of NO_2-OA in plasma. (**B**) NO_2-OA levels were obtained using calibration curves with the internal standard. Plasmatic NO_2-OA concentrations normalized to the mg of plasma proteins are plotted. The control groups of ND and HF did not show differences with the corresponding groups in the presence of NO_2^- (data not shown). Results shown correspond to the mean ± SEM, n=10. * $p< 0.05$ respect to ND conditions; # $p< 0.05$ respect to HF + NO_2^- condition.

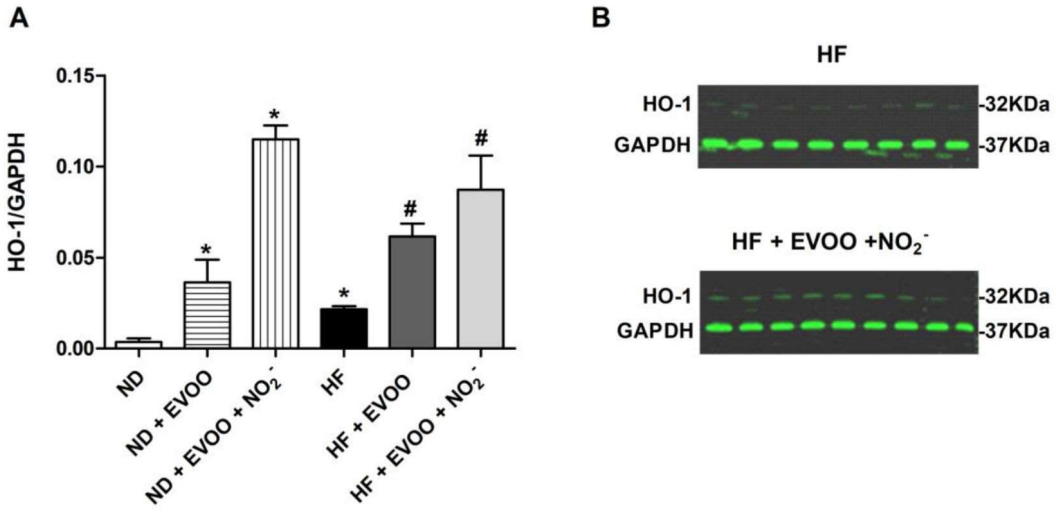


Figure 2. EVOO supplementation increases liver HO-1 expression.

(**A**) Western blot analysis of liver homogenates for HO-1 expression was done. The relative density of HO-1 to GAPDH of the observed bands was plotted as the mean ± SEM, n=8. (**B**) Representative results from HF and HF+EVOO+NO_2^- groups. * express significant differences relative to ND conditions. # express significant differences relative to the HF diet ($p<0.05$).

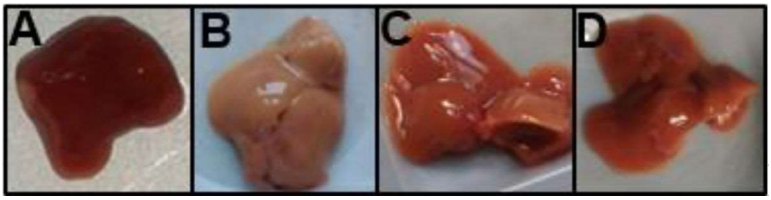


Figure 3. Olive oil supplementation decreases fat accumulation in mice liver.

After 12 weeks of diet treatment, mice were euthanized and livers obtained as explained in the Methods section. Changes in the livers' color from red to a light red-white color were used as an index of fat accumulation. Pictures correspond to ND (**A**); HF (**B**), HF with 10% EVOO supplementation (**C**); HF with NO_2^- and EVOO (**D**). Pictures shown are one representative for each group of n=10 as explained in the Methods section.

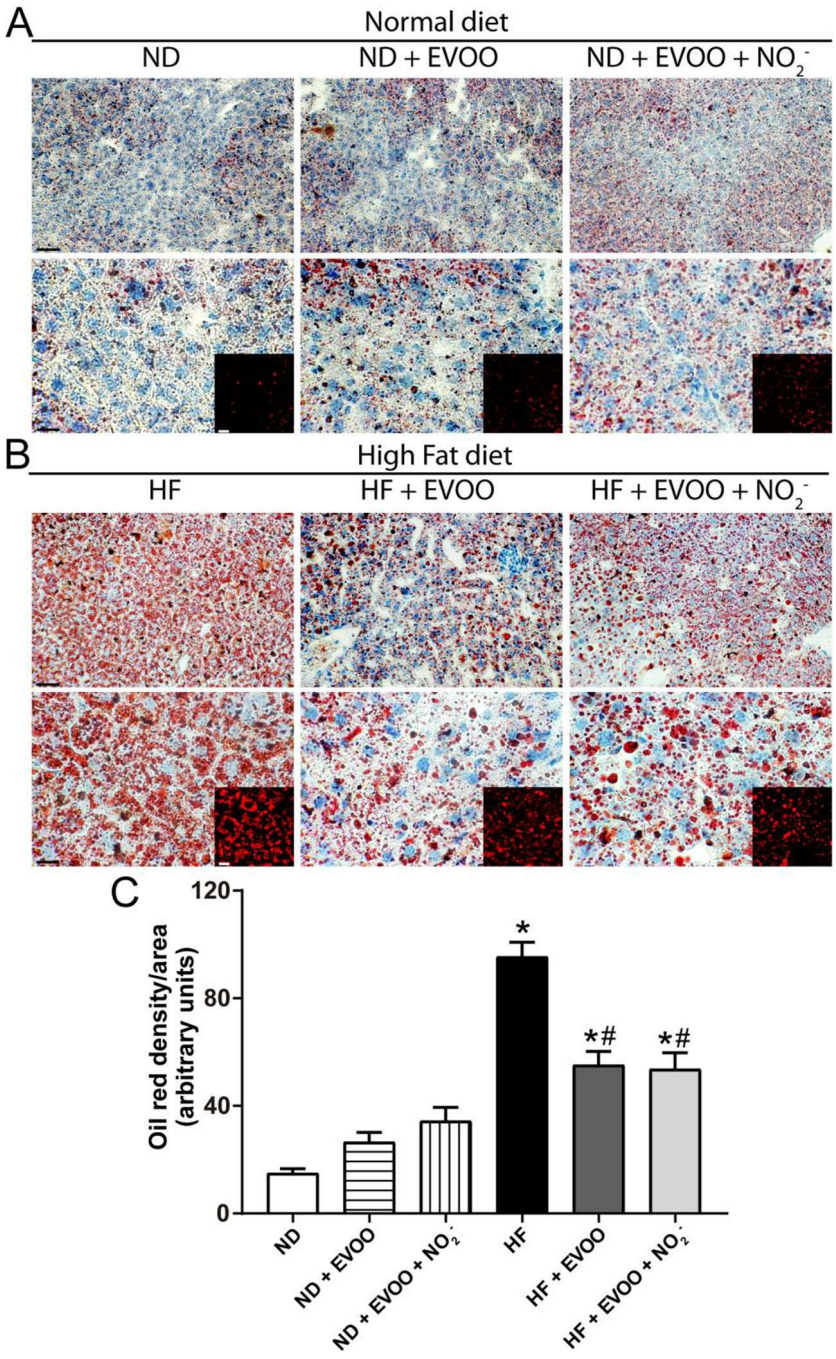


Figure 4. Liver histology in the normal and high-fat diet-fed animals subjected to EVOO plus nitrite supplementation.

Liver 30 μm cryosections were stained with hematoxylin and the lysochrome diazo dye Oil red O. (A) Representative bright-field microscopic images showing the Oil red O (red) and hematoxylin (blue) in liver sections of mice fed with normal diet (ND, left panels), ND plus olive oil (EVOO, middle panels) or ND plus EVOO plus nitrite (NO_2^-) (right panels). Lower panels show a higher magnification of the liver histology. Insets in lower panels show a representative confocal microphotograph of Oil red O staining. Scale bars: 40 μm in upper panels, 20 μm in lower panels, 10 μm in insets. (B) Representative bright-field microscopic

images showing the Oil red O (red) and hematoxylin (blue) staining in liver sections of mice fed with high-fat diet (HF, left panels), HF plus EVOO (middle panels) or HF plus EVOO plus NO_2^- (right panels). Insets in lower panels show a representative confocal microphotograph of Oil red O staining. Scale bars: 40 μm in upper panels, 20 μm in lower panels, 10 μm in insets. **(C)** The graph shows the quantitative analysis of Oil red O intensity among groups. Data are expressed as mean ± SEM.*express significant differences relative to ND conditions with $p<0.0001$ and $^{\#}$express significant differences relative to ND conditions with $p=0.027$ and 0.0056 for HF+EVOO and HF+EVOO+NO_2^-, respectively.

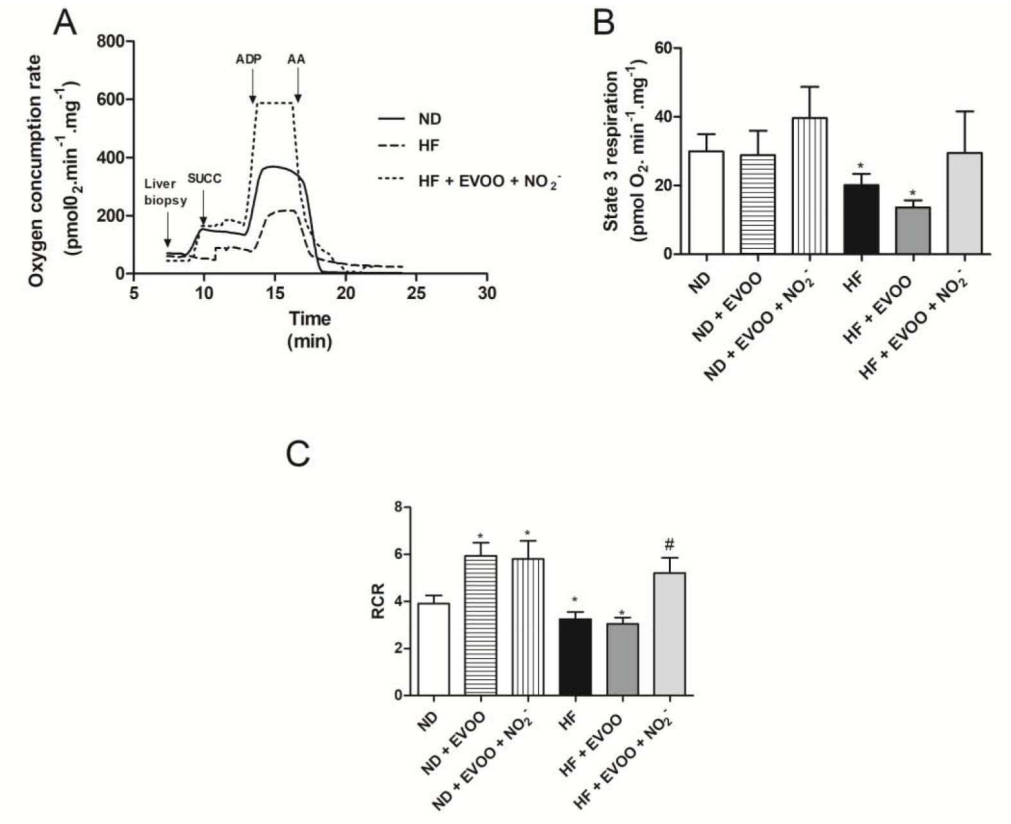


Figure 5. Stimulation of NO_2-FA formation spare liver mitochondria.

Liver biopsies were permeabilized with digitonin and mitochondrial function analyzed by high-resolution respirometry at 37°C. (**A**) Representative registers of oxygen consumption. Arrows indicate the addition of the complex II substrate succinate and ADP to perform oxidative phosphorylation. To confirm that oxygen consumption was due to mitochondria, the addition of antimycin A was done. State 3 (**B**) and tissue RCR (**C**) were determined. At least 5 animals per group were tested and the results shown correspond to the mean ± SEM. * express significant differences relative to ND conditions. # express significant differences relative to the HF diet ($p<0.05$).

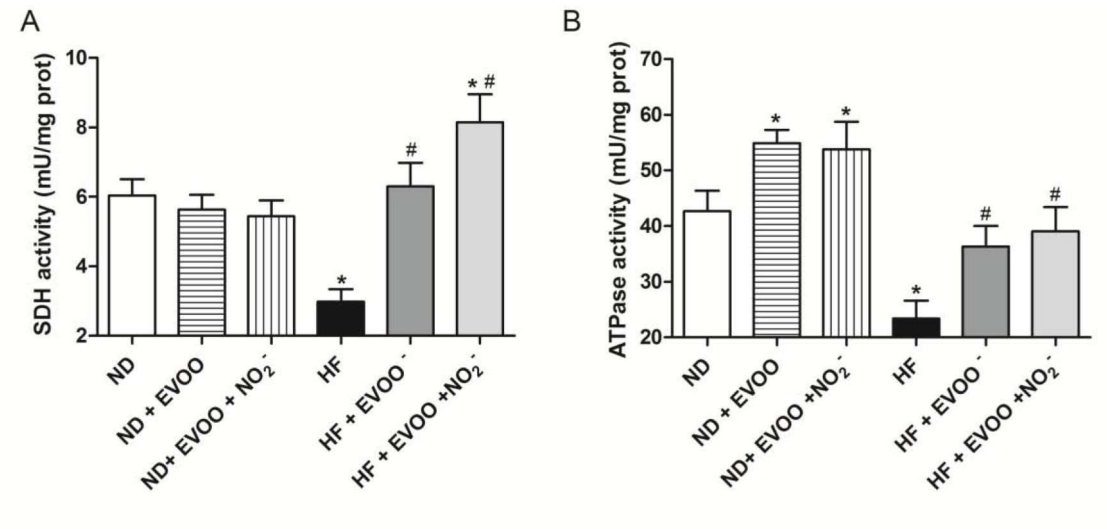
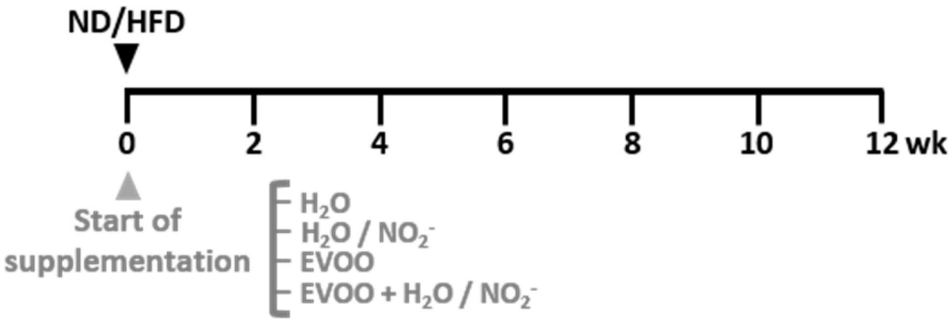


Figure 6. Increasing plasma NO_2-OA recovers mitochondrial complex II and V activities in HF mice.

Liver homogenates were used to analyze (**A**) succinate dehydrogenase (complex II) and (**B**) ATP synthase (complex V) activities. In all groups at least 8 animals were tested and the results shown correspond to the mean ± SEM. * express significant differences relative to ND conditions. # express significant differences relative to the HF diet ($p<0.05$).



Scheme 1.
Experimental design.

Table 1.

Body and liver weights gain.

Group	Weight gain (g)	Liver weight (g)
ND	$1.33 \pm 0.21_{a}$	$1.16 \pm 0.05_{a}$
ND + EVOO	$2.00 \pm 0.31_{a}$	$1.13 \pm 0.04_{a}$
ND + NO_{2-} + EVOO	$1.78 \pm 0.28_{a}$	$1.16 \pm 0.05_{a}$
HF	5.80 ± 0.20	1.66 ± 0.10
HF + EVOO	$4.75 \pm 0.25_{ab}$	$1.27 \pm 0.01_{a}$
HF + NO_{2-}	+ EVOO $4.57 \pm 0.20_{ab}$	$1,39 \pm 0.04_{a}$