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Protective Effects of Nitrated Fatty Acids from Olive Oil on Cardiovascular Risk in Non-Alcoholic Fatty Liver Disease

Sánchez-Calvo, B. [1]; Cassina, A. [2]; Rubbo, H. [2]; Trostchansky, A. [2]

[1]*Universidad de la República, Escuela de Nutrición, Unidad Académica Departamento de Nutrición Básica, Montevideo. Uruguay*

[2]*Universidad de la República, Facultad de Medicina, Centro de Investigaciones Biomédicas, Montevideo. Uruguay*

Abstract

Non-alcoholic fatty liver disease (NAFLD) is defined by the deposition of fat in the liver without alcohol consumption. Moreover, it is the most prevalent chronic liver disease worldwide and is associated with an increased risk of cardiovascular disease. Olive oil is one of the main sources of lipids in the Mediterranean diet and may reduce cardiovascular risk, although the mechanism has not yet been elucidated. Olive oil is a source of nitrated fatty acids (NO_2-FA) that are formed during digestion and are bioactive molecules with potent anti-inflammatory actions and cytoprotective effects. The aim of this work was to study the capacity of NO_2-FA derived from olive oil in protecting mitochondrial dysfunction in cardiomyocytes in NAFLD. We used a murine model of NAFLD induced by a high-fat diet, supplemented with and without nitrite, as well as with and without olive oil. Heart biopsies were used to analyze mitochondrial function using high-resolution respirometry and the enzymatic activity of the mitochondrial transport chain complexes. As a result, we observed that the supplementation of olive oil in the presence of nitrite, a condition that favors the formation of NO_2-FA, improves mitochondrial function in heart biopsies, which correlates with the improvement in ATPase activity in that tissue. These compounds may be partly responsible for the beneficial effects of this functional food.

Keywords: cellular respiration, heart, liver, nitroalkenes, olive oil

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Juliano Garavaglia
Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), Porto Alegre, Brasil

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Correspondence

Beatriz Sánchez
beatriz.sanchez@nutricion.edu.uy

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Rol de los ácidos grasos nitrados derivados del aceite de oliva en la protección del riesgo cardiovascular en la enfermedad de hígado graso no alcohólico

Resumen

La enfermedad de hígado graso no alcohólico (EHGNA) se caracteriza por la acumulación de grasa en el hígado en ausencia de consumo de alcohol y es la enfermedad crónica hepática más común en el mundo, que da lugar a un incremento del riesgo cardiovascular. El aceite de oliva es una de las principales fuentes de grasas de la dieta mediterránea y puede disminuir el riesgo cardiovascular, pero aún el mecanismo no ha sido dilucidado. El aceite de oliva es fuente de ácidos grasos nitrados (NO_2-FA) que se forman tras la ingesta y son moléculas de potente acción antiinflamatoria y efecto citoprotector. El objetivo de este trabajo fue estudiar el rol de estos NO_2-FA derivados del aceite de oliva en la protección de la disfunción mitocondrial del cardiomiocito en la EHGNA. Para ello utilizamos un modelo murino de EHGNA inducido con dieta rica en grasa, suplementados con y sin nitrito, como también con y sin aceite de oliva. Se utilizaron biopsias de corazón para analizar la función mitocondrial usando estudios de consumo de oxígeno de alta resolución y la actividad enzimática de los complejos de la cadena de transporte mitocondrial. Como resultado, observamos que la suplementación de aceite de oliva en presencia de nitrito, condición en la que se favorece la formación de NO_2-FA, mejora la función mitocondrial en biopsias de corazón, que se correlaciona con la mejora en la actividad de la ATPasa en dicho tejido. Estos compuestos serían en parte responsables de los efectos beneficiosos de este alimento funcional.

Palabras clave: respiración celular, corazón, hígado, nitroalquenos, aceite de oliva

Papel dos ácidos graxos nitrados derivados do azeite na proteção do risco cardiovascular na doença hepática gordurosa não alcoólica

Resumo

A doença hepática gordurosa não alcoólica (DHGNA) é caracterizada pelo acúmulo de gordura no fígado na ausência do consumo de álcool e é a doença hepática crônica mais comum no mundo, levando a um risco cardiovascular aumentado. O azeite é uma das principais gorduras da dieta mediterrânica e pode reduzir o risco cardiovascular, mas o mecanismo ainda não foi elucidado. O azeite é fonte de ácidos graxos nitrados que se formam após a ingestão e são moléculas com poderosa ação anti inflamatória e efeito citoprotetor. O objetivo deste trabalho foi estudar o papel desses nitroalcenos derivados do azeite na proteção da disfunção mitocondrial de cardiomiócitos na DHGNA. Para isso, utilizamos um modelo murino de DHGNA induzido com dieta rica em gordura, suplementada com e sem nitrito, bem como com e sem azeite, num total de 8 grupos. Biópsias cardíacas foram utilizadas para analisar a função mitocondrial usando Oxygraph 2 K e seu extrato para determinar a atividade enzimática dos complexos da cadeia de transporte. Como resultados, observamos que a suplementação de azeite na presença de nitrito, condição em que é favorecida a formação de ácidos graxos nitrados, melhora a função mitocondrial nas biópsias cardíacas que se correlaciona com a melhora da atividade ATPase no referido tecido. Estes compostos seriam parcialmente responsáveis pelos efeitos benéficos deste alimento funcional.

Palavras-chave: respiração celular, coração, fígado, nitroalcenos, azeite

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is a highly prevalent metabolic disorder, closely associated with obesity, insulin resistance, and type 2 diabetes (Byrne & Targher, 2015; Caussy et al., 2021; Kasper et al., 2021; Targher et al., 2024). Beyond its hepatic complications, NAFLD has emerged as a major risk factor for cardiovascular disease (CVD), which is the leading cause of mortality among these patients (Duell et al., 2022). The mechanisms linking NAFLD and CVD are multifaceted and include systemic inflammation, oxidative stress, dyslipidemia, endothelial dysfunction, and mitochondrial impairment (Deprince et al., 2020; Kasper et al., 2021).

Atherogenic dyslipidemia plays a key role in the cardiovascular risk associated with NAFLD. Patients commonly present with elevated triglycerides, reduced HDL-C, and an increased proportion of small, dense LDL particles, which contribute to atherosclerosis and coronary artery disease. Hepatic fat accumulation further exacerbates this risk by disrupting lipoprotein secretion, increasing the circulating burden of atherogenic particles, and promoting foam cell formation. Additionally, chronic low-grade inflammation and oxidative stress, driven by cytokines such as IL-6 and TNF-α, contribute to vascular dysfunction. This pro-inflammatory state, coupled with insulin resistance, impairs nitric oxide bioavailability, leading to endothelial dysfunction, hypertension, and arterial stiffness (Deprince et al., 2020; Kasper et al., 2021; Santos et al., 2019).

Mitochondrial dysfunction is another key factor in NAFLD pathophysiology. Studies suggest that patients with NAFLD exhibit impaired oxidative phosphorylation, leading to excessive reactive oxygen species (ROS) production and mitochondrial DNA damage (Deprince et al., 2020). This metabolic stress not only contributes to hepatocellular injury but also compromises cardiac function, increasing susceptibility to ischemic damage and heart failure (Byrne & Targher, 2015; Targher et al., 2024).

Extra virgin olive oil (EVOO), a fundamental component of the Mediterranean diet, has been extensively studied for its metabolic and cardiovascular benefits (Bulotta et al., 2014; Ditano-Vázquez et al., 2019; Gaforio et al., 2019; Guasch-Ferré et al., 2014; Reboredo-Rodríguez et al., 2017). Its protective effects are attributed to its high content of monounsaturated fatty acids (MUFAs) and bioactive polyphenols, which modulate lipid metabolism, improve mitochondrial function, and reduce oxidative stress. Among its bioactive components, nitro-fatty acids (NO_2-FAs) have gained significant attention due to their potent anti-inflammatory, antioxidant, and vasodilatory properties (Khoo & Schopfer, 2019; Schopfer et al., 2018).

NO_2-FAs are formed in the gastrointestinal tract through non-enzymatic reactions between unsaturated fatty acids and nitric oxide derivatives, a process that can be enhanced by dietary intake (Fazzari et al., 2014; Khoo & Schopfer, 2019). Among these products, nitroalkenes, which present the nitro group bonded to a double bond of the carbon chain, exert pleiotropic effects by modulating key signaling pathways, such as activating nuclear factor erythroid 2-related factor 2 (Nrf2) and inhibiting nuclear factor kappa B (NF-κB), thereby reducing inflammation and oxidative damage (Kansanen et al., 2009, 2011; Sharma et al., 2017). In NAFLD, EVOO-derived NO_2-FAs have been shown to enhance mitochondrial respiration, reduce hepatic lipid accumulation, and attenuate inflammation, mitigating disease progression (Khoo et al., 2019; Sánchez-Calvo et al., 2021; Valenzuela et al., 2016).

Beyond their hepatic effects, NO_2-FAs also hold significant promise for cardiovascular health. These electrophilic lipids reduce ischemic injury by modulating mitochondrial respiration, particularly at complex II, limiting oxidative stress and promoting metabolic adaptations that improve tissue survival (Koenitzer et al., 2016). Additionally, NO_2-FAs contribute to vascular homeostasis by enhancing endothelial function and reducing arterial stiffness (Khoo & Schopfer, 2019; Schopfer et al., 2018). Their ability to target multiple mechanisms underlying CVD suggests that NO_2-FAs derived from olive oil may represent a novel therapeutic approach for patients with NAFLD at high cardiovascular risk.

Given the intricate relationship between hepatic metabolism and cardiovascular health, understanding the role of NO_2-FAs in cardio protection is of paramount importance. This study aims to explore the cardiovascular implications of NO_2-FAs derived from olive oil in the context of NAFLD, focusing on their potential to modulate mitochondrial function in the heart, ultimately lowering CVD risk in this animal model.

2. Materials and Methods

2.1 Animal Model

C57Bl/6J female mice, aged 6 to 8 weeks, were obtained from the Dirección de Laboratorios Veterinarios (DILAVE, Uruguay) and housed at the Bioterio of Facultad de Medicina, Universidad de la República (Udelar), Uruguay. All procedures involving animals adhered to the guidelines outlined in the Guide for the Care and Use of Laboratory Animals and were approved by the Comisión Honoraria de Experimentación Animal (CHEA, Protocol No. 0011/11). The mice were randomly assigned to one of six groups (ten animals per group) and treated for 12 weeks according to their respective dietary conditions, as previously described (Sánchez-Calvo et al., 2021). Three groups received a normal chow diet (ND), with the following variations: ND as control, ND enriched with 10% (w/w) EVOO, or ND with both 150 µM NO_2^- and 10% (w/w) EVOO. NAFLD was induced in the remaining three groups through a high-fat diet (HF), in which 60% of the adjusted caloric intake was derived from fat (beef tallow as the fat source). These HF groups followed the same dietary patterns as their ND counterparts. EVOO used in the study was from the Frantoio variety from Jaén (Spain), and all animals had unrestricted access to food and water. At the conclusion of the study, mice were euthanized under isoflurane anesthesia. The HFD-induced NAFLD model was previously validated by our group in the same experimental setting, including EVOO and nitrite supplementation (Sánchez-Calvo et al., 2021). Cardiac samples analyzed in the present work were obtained from the same animals and from the same experiment reported in that study.

2.2 Study of Mitochondrial Function

For mitochondrial analysis, animals were humanely euthanized under isoflurane anesthesia, and hearts were excised. The excised hearts were thoroughly rinsed with cold PBS to remove residual blood, and a biopsy sample of approximately 10 mg was collected for further experiments. For respiration assessments, heart biopsies were permeabilized following previously established protocols with slight modifications (García-Roche et al., 2018; Sánchez-Calvo et al., 2016). Specifically, fresh heart biopsies were incubated in an ice-cold MIR05 medium containing saponin (25 mg mL^{-1}) for 10 minutes. Subsequently, the samples were transferred to a petri dish and washed with MIR05 medium. Cellular respiration was analyzed using an Oxygraph 2K system (Oroboros Instruments Corp). Oxygen consumption measurements were recorded at 37 °C in permeabilized heart biopsies, with the respiration rate calculated using the instrument's software (DataLab). Oxygen consumption rates were expressed in pmol $O_2 \cdot s^{-1} \cdot mL^{-1}$, and biopsy weight measured in mg (Brand & Nicholls, 2011; Hill et al., 2012; Pesta & Gnaiger, 2012; Salabei et al., 2014). The respiratory control ratio (RCR), defined as the state 3/state 4 ratio, was used to assess the coupling between ATP synthesis and electron transport (Sánchez-Calvo et al., 2016), and was calculated as described in previous studies (García-Roche et al., 2018).

2.3 Enzymatic Assays and Western Blot

For mitochondrial complex activity assays and western blot analysis, heart tissue was homogenized in PBS using a Bullet Blender (Next Advance, Inc., NY) with zirconium oxide beads (0.5 mm). The volume of beads was equal to the sample volume, and homogenization was carried out at a speed of 8 for 3 minutes. The supernatant was separated from the beads and used for subsequent experimental analyses.

ATP synthase (ATPase) activity was measured by monitoring NADH oxidation at 340 nm (ε = 6.22 $mM^{-1} \cdot cm^{-1}$) in a coupled reaction with pyruvate kinase and lactate dehydrogenase (Pullman et al., 1960). The reaction mixture contained heart homogenate, ATP, pyruvate kinase, and lactate dehydrogenase in 50 mM Hepes buffer (pH 8), as described in Cassina and Radi (1996). Succinate dehydrogenase (SDH) activity was assessed spectrophotometrically using a reaction mixture containing 2,6-dichlorophenol indophenol (DCPIP), succinate, and potassium cyanide. DCPIP reduction was monitored at 600 nm (ε = 20.5 $mM^{-1} \cdot cm^{-1}$) (Singer, 1974). The

protein content in heart homogenates was determined using the Bradford assay (Bradford, 1976), and mitochondrial complex activities were normalized per mg of protein.

Heart proteins were resolved using 10% SDS-PAGE as described previously (Laemmli, 1970). Following electrophoresis, proteins were transferred onto a nitrocellulose membrane using a semi-dry electroblotting system. The membranes were incubated with a polyclonal antibody against N-Tyr (1:1000 dilution) or anti-GAPDH (1:2000 dilution). Immunodetection was performed using the LiCOR Odyssey system.

2.4 Statistical Analysis

Results were expressed as mean ± standard error of the mean (SEM), based on at least three independent experiments, and analyzed using GraphPad Prism 8.0. Statistical comparisons were conducted using either Student's t-test or ANOVA followed by Tukey's post hoc test. Statistical significance was set at $p \leq 0.05$. For histological analyses, the Kruskal-Wallis test followed by Dunn's multiple comparison test was employed, with $p < 0.05$ considered significant.

3. Results

3.1 EVOO Improves Heart Mitochondrial Function

Cellular respiration in cardiac biopsies, assessed through the respiratory control ratio (RCR), was significantly reduced in the HF group compared to the ND group, indicating mitochondrial dysfunction (Figure 1). Supplementation with 10% EVOO improved RCR. In conditions where NO_2-FA formation is enhanced as previously reported by us (Sánchez-Calvo et al., 2021), EVOO further enhanced this protective effect, restoring mitochondrial function to levels similar to those of the ND group (Figure 1).

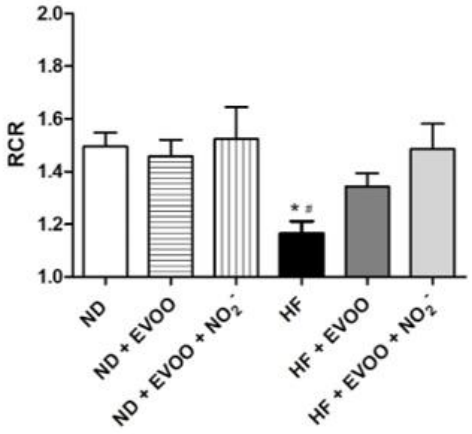


Figure 1. EVOO ameliorate heart mitochondrial function. Heart biopsies were analyzed by high-resolution respirometry (OROBOROS)

The respiratory parameter was determined as cell respiratory control ratio (RCR). At least 8 animals were tested per group and the results shown correspond to mean ± SEM. * significant differences relative to ND conditions. # significant differences relative to HF diet ($p < 0.05$).

3.2 Effects of EVOO and NO_2-FA on Mitochondrial Complex II and V Activities

Complex II (SDH) activity remained unchanged between groups, indicating that this enzyme may be less sensitive to the metabolic alterations induced by the HF diet (Figure 2A). On the other hand, EVOO supplementation partially restored ATPase activity, while the formation of NO_2-FA in the HF + EVOO + NO_2- group further

enhanced this recovery, surpassing the effect observed with EVOO alone (Figure 2B). Additionally, EVOO not only improved complex V activity but also contributed to the overall enhancement of mitochondrial function, as reflected by the increased RCR shown above (Figure 1). The presence of NO_2-FA further potentiated these benefits, suggesting an additional protective mechanism that enhances the efficiency of the myocardial electron transport chain.

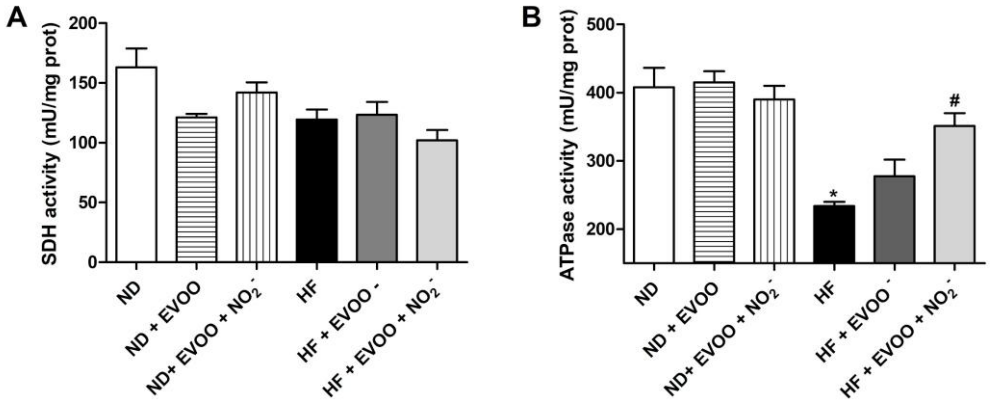


Figure 2. Effects of a high-fat (HF) diet, extra virgin olive oil (EVOO), and nitrite supplementation on mitochondrial complex II and complex V activities in cardiac tissue

We analyzed Complex II (SDH activity, A), and ATPase activity (B). At least 8 animals were tested per group and the results shown correspond to mean ± SEM. * significant differences relative to ND conditions. # significant differences relative to HF diet ($p < 0.05$).

3.3 EVOO Reduces Cardiac Oxidative Stress in a NAFLD Model

Tyrosine nitration serves as a key indicator of oxidative stress, and its presence in proteins is widely recognized as evidence of oxidative species formation. Furthermore, in pathological conditions characterized by increased production of reactive nitrogen species, elevated levels of 3-nitrotyrosine have been observed across various diseases. In this NAFLD model at the cardiac tissue level, as shown in Figure 3, there is an increase in nitro-tyrosine, which is reversed to basal levels with EVOO supplementation, both in the presence and absence of NO_2-. This suggests that the observed effect is independent of the formation of nitrated fatty acids and may be attributed to another bioactive component of olive oil.

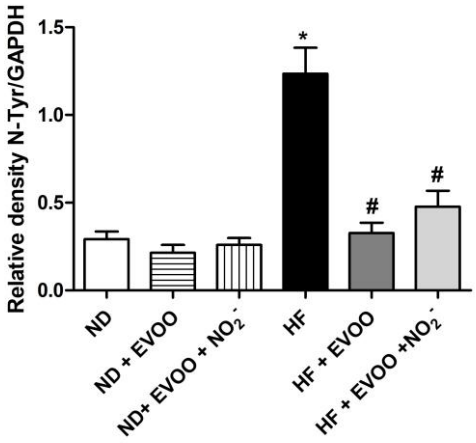


Figure 3. EVOO decreases nitro-tyrosine levels in cardiac tissue

At least 8 animals were tested per group and the results shown correspond to mean ± SEM. *significant differences relative to ND conditions. # significant differences relative to HF diet ($p < 0.05$).

4. Discussion

NAFLD has been strongly associated with an increased risk of CVD, with mitochondrial dysfunction playing a key role in this relationship (Byrne & Targher, 2015; Caussy et al., 2021; Deprince et al., 2020; Duell et al., 2022; Kasper et al., 2021; Targher et al., 2024). Mitochondrial alterations in NAFLD contribute to impaired energy metabolism, oxidative stress, and endothelial dysfunction, all of which are linked to a higher risk of cardiovascular complications. In this context, it should be noted that the HFD-induced NAFLD model used in the present study has been previously validated by our group under the same experimental conditions. In that earlier work, comprehensive hepatic metabolic and mitochondrial analyses demonstrated liver steatosis, mitochondrial dysfunction, and the involvement of nitro-fatty acid (NO_2-FA) formation derived from extra virgin olive oil (EVOO) and nitrite supplementation (Sánchez-Calvo et al., 2021). The cardiac samples analyzed here were obtained from the same animals and the same experiment, allowing the present results to be interpreted as an extension of a well-characterized NAFLD model to cardiac tissue. Against this background, we observed that HF feeding induced marked mitochondrial dysfunction in cardiac tissue, as evidenced by a significant reduction in the respiratory control ratio (RCR) and ATPase (complex V) activity. These findings are consistent with the "two-hit" hypothesis, where lipid accumulation in the liver (first hit) leads to oxidative stress and mitochondrial dysfunction (second hit), exacerbating systemic metabolic dysfunction and increasing cardiovascular risk (Day & James, 1998).

The decrease in RCR observed in the HF group suggests a compromised mitochondrial efficiency, likely due to impaired oxidative phosphorylation and increased electron leakage. This mitochondrial dysfunction could lead to reduced ATP production and increased oxidative stress, both of which are detrimental to cardiac function. Previous studies have reported that diets rich in saturated fatty acids (SFAs), such as those used to induce NAFLD in murine models, reduce basal metabolic rates and impair mitochondrial function, contributing to weight gain and fat accumulation in both hepatic and extrahepatic tissues (Kakimoto & Kowaltowski, 2016; Kien et al., 2005; Sánchez-Calvo et al., 2021; Takeuchi et al., 1995). Our results align with these findings, reinforcing the notion that HF diets not only impair liver function but also negatively affect mitochondrial efficiency in the heart.

Furthermore, complex V (ATPase) activity was significantly reduced in the HF group, indicating impaired ATP synthesis capacity, which is particularly critical in high-energy-demand tissues such as the myocardium. EVOO supplementation partially restored ATPase activity, while the formation of NO_2-FA in the HF + EVOO + NO_2- group further enhanced this recovery, suggesting that the cardioprotective effects of EVOO might be potentiated by the presence of NO_2-FA. These results are in line with previous findings showing that NO_2-FA can modulate mitochondrial function by covalently modifying key proteins, including components of the electron transport chain, leading to improved respiration and reduced oxidative stress (Koenitzer et al., 2016; Sánchez-Calvo et al., 2016).

Interestingly, complex II (SDH) activity remained unchanged across groups, suggesting that this enzyme may be less sensitive to the metabolic alterations induced by the HF diet compared to ATPase. Given that complex II plays a dual role in both the electron transport chain and the tricarboxylic acid cycle, its stability suggests that the HF diet primarily affects ATP production rather than upstream metabolic pathways. Previous studies have demonstrated that mitochondrial dysfunction in NAFLD is often associated with decreased complex activity, particularly in complex V, which aligns with our findings (García-Roche et al., 2018; García-Ruiz et al., 2010, 2015).

A key aspect of our study is the role of EVOO in restoring mitochondrial function. The beneficial effects of EVOO have been attributed to its high monounsaturated/polyunsaturated fatty acid ratio and the presence of bioactive compounds such as polyphenols (Bulotta et al., 2014; Gaforio et al., 2019; Gorzynik-Debicka et al., 2018; Mancebo-Campos et al., 2023; Reboredo-Rodríguez et al., 2017; Terés et al., 2008; Visioli et al., 2018). Previous research has demonstrated that EVOO consumption is associated with reductions in cholesterol levels, inflammation, and oxidative stress, as well as improvements in endothelial function and blood pressure (Abenavoli et

al., 2019; Bullón-Vela et al., 2020; Guasch-Ferré et al., 2014; Reboredo-Rodríguez et al., 2017). Specifically in this NAFLD model, EVOO has been shown to have a positive effect on liver tissue, improving mitochondrial function, and increasing the activity of the SDH complex and that of ATPase (Sánchez-Calvo et al., 2021). Our findings further support the idea that EVOO can mitigate the mitochondrial dysfunction induced by NAFLD in heart tissue, restoring both RCR and ATPase activity. Furthermore, in pathological situations where there is an increase in reactive nitrogen and oxygen species, as occurs in NAFLD, nitro-tyrosine levels increase (Castro et al., 2011; Mazzoli et al., 2019), which are markers of oxidative stress. In this model, EVOO decreases this biomarker, which is related to improved mitochondrial function.

Moreover, the formation of NO_2-FA appears to enhance the protective effects of EVOO. Previous studies have shown that NO_2-FA can be formed endogenously in the gastrointestinal tract when EVOO is consumed alongside nitrate-rich vegetables, as in the Mediterranean diet (Buchan et al., 2018; Fazzari et al., 2014; Khoo & Schopfer, 2019; Schopfer & Khoo, 2019). These electrophilic lipids have been reported to exert anti-inflammatory and antioxidant effects by modulating key signaling pathways, including the inhibition of pro-inflammatory transcription factors and the induction of phase II antioxidant enzymes such as HO^{-1} (Geisler & Rudolph, 2012; Rudolph et al., 2010; Sánchez-Calvo et al., 2021; Wright et al., 2009). Additionally, NO_2-FA incorporated into complex lipids can be released through lipase hydrolysis and distributed to various tissues, where they influence cell signaling and are incorporated into mitochondrial membranes to exert protective effects (Buchan et al., 2018; Fazzari et al., 2017; Sánchez-Calvo et al., 2016). Oral administration of NO_2-FA or EVOO combined with nitrite facilitates their absorption and subsequent distribution to the plasma, kidneys, liver, lungs, and heart (Khoo & Schopfer, 2019; Sánchez-Calvo et al., 2021; Schopfer et al., 2018).

NO_2-FAs also hold significant promise for cardiovascular health. These electrophilic lipids reduce ischemic injury by modulating mitochondrial respiration, particularly at complex II, limiting oxidative stress and promoting metabolic adaptations that improve tissue survival (Koenitzer et al., 2016). Additionally, NO_2-FAs contribute to vascular homeostasis by enhancing endothelial function and reducing arterial stiffness (Khoo & Schopfer, 2019; Schopfer et al., 2018). Their ability to target multiple mechanisms underlying CVD suggests that NO_2-FAs derived from olive oil may represent a novel therapeutic approach for patients with NAFLD at high cardiovascular risk.

5. Conclusions

Our study highlights the crucial role of mitochondrial dysfunction in the NAFLD-CVD axis, emphasizing how a high-fat diet impairs cardiac mitochondrial efficiency by reducing RCR and ATPase activity. These findings reinforce the idea that NAFLD extends beyond the liver, affecting systemic metabolism and increasing cardiovascular risk. Importantly, our results demonstrate that EVOO supplementation partially restores mitochondrial function, while the formation of NO_2-FA further enhances these protective effects. This suggests that NO_2-FA formed endogenously or through dietary strategies may serve as a novel mechanism through which EVOO exerts its cardioprotective and metabolic benefits. Given their ability to modulate mitochondrial function, oxidative stress, and inflammation, NO_2-FAs represent a promising therapeutic approach for patients with NAFLD at high cardiovascular risk.

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Transparency of Data

Data not available: The data set that supports the results of this study is not publicly available.

Author Contribution Statement

	B Sánchez-Calvo	A Cassina	H Rubbo	A Trostchansky
Conceptualization	■	■		■
Investigation	■			
Methodology		■		■
Project administration				■
Writing – original draft	■			
Writing – review and editing	■		■	■

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