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Oleuropein overrides liver damage in steatotic mice

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ABSTRACT

Nonalcoholic fatty liver disease (NAFLD) is a common disease in which oxidative stress plays a main role in causing organ damage. Oleuropein (Ole) is a phenolic compound with both significant anti-inflammatory and antioxidant properties.

The aim of the present work has been focused on investigating the mechanisms by which Ole is able to improve liver damage, in the presence of hepatic steatosis.

We evaluated the effects of Ole in female and male mice fed normal diet (ND) or high fat diet (HFD) for 8 weeks, adding or not Ole for the following 8 weeks.

Ole induced a decrease in body, liver and heart weights and had an anti-inflammatory and antioxidant effects in HFD mice. Interestingly, in presence of the unhealthy diet, antioxidant effects of Ole overcome sex-related differences, improving liver damage.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is currently the most common hepatic disease worldwide. In Western countries, the prevalence of NAFLD in the general population is 20–30%.

Nonalcoholic steatohepatitis (NASH) is characterized by hepatocellular ballooning, inflammation and oxidative stress, which are the main causes of NAFLD progression, resulting in higher liver fibrosis (Araújo, Rosso, Bedogni, Tiribelli, & Bellentani, 2018; Czaja, 2007).

Even if it has been well documented that the progression of NAFLD versus NASH is improved by lifestyles (e.g., high calorie intake, sedentary habit), patients often do not change their habits or, at the best, take time (Araújo et al., 2018). Unfortunately, if no proper approach is engaged, NASH related liver damage may progress, leading to liver cirrhosis as well as liver cancer (Czaja, 2007). In this context, it could be important to identify anti-inflammatory and antioxidant molecules able to counteract the progression of this disease.

Reactive oxygen species (ROS), such as superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), are generated within cells by cellular metabolic activities such as cell survival, stressor responses and inflammation (Masarone et al., 2018). Impaired antioxidant defence system promotes the accumulation of oxidative stress inducing tissue damage and promoting the activation of a pro-inflammatory program leading to secretion of inflammatory cytokines, such as: IL1-α, IL-6, IL-2, TNF-α and G-CSF (Stojsavljević, Gomerčić Palčić, Virović Jukić, Smirčić Duvnjak, & Duvnjak, 2014).

Several biomarkers are used to evaluate oxidative stress. Superoxide dismutase (SOD) family activity, reported to be altered in many syndromes associated with oxidative stress impairment, is an excellent and sensitive biomarker (Ighodaro & Akinloye, 2018; Wang et al., 2018). SOD1 and SOD2 belong to the superoxide dismutase family. The free radical scavenger Cu, Zn superoxide dismutase (SOD1) represents 90% of the total SOD activity and is primarily located in the cytosol, but it exerts its antioxidant activity translocating in the nucleus and activating the expression of antioxidant enzyme genes. SOD2, is a manganese-containing enzyme located in the mitochondrial matrix. SOD2 is believed to represent the best protection against mitochondrial superoxide. This protein, as well as SOD1, binds to the superoxide and transforms it to hydrogen peroxide (Ighodaro & Akinloye, 2018; Tsang, Liu, Thomas, Zhang, & Zheng, 2014; Wang et al., 2018).

One of the specific features of NAFLD is the existence of mitochondrial dysfunction (Begriche, Massart, Robin, Bonnet, &

Abbreviations: NAFLD, nonalcoholic fatty liver disease; Ole, Oleuropein; ND, normal diet; HFD, high fat diet; NASH, nonalcoholic steatohepatitis;; ROS, reactive oxygen species

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Fromenty, 2013; Caldwell et al., 1999; Grattagliano et al., 2012). Mitochondrial alterations negatively influence the equilibrium between antioxidant and pro-oxidant systems, inducing the accumulation of ROS and consequently the progression of liver damage (He & Zuo, 2015).

Sirtuins are a conserved family of nicotinamide adenine dinucleotide (NAD+)-dependent protein deacetylases and control ROS levels especially during caloric restriction (Ding, Bao, & Deng, 2017; Nassir & Ibdah, 2016). Among other members of this family, SIRT1 and SIRT3 are mainly involved in NAFLD pathogenesis: SIRT1 activating, in the nucleus, the expression of antioxidant enzyme genes dependent on several transcription factors, and SIRT3 by its participation in mitochondria β-oxidation of free fatty acids (FAs). During NAFLD progression, SIRT1 and SIRT3 activities have been related to metabolic challenges, inflammatory signals and defense against oxidative stress (Kendrick et al., 2011; Yoshino & Imai, 2011). However, although both Sirtuins have been associated to NAFLD/NASH pathogenesis, their specific roles still remain not completely defined.

Natural phytochemicals such as polyphenols are associated with the mitigation of oxidative stress and the reduction of inflammation in chronic inflammatory diseases. Oleuropein (Ole), a polyphenol compound, is mainly present in the leaves and fruits of the olive tree and holds both antioxidant and anti-inflammatory effects (Barbaro et al., 2014; Visioli & Bernardini, 2011). Olive leaf extracts have therapeutic effects (Visioli & Bernardini, 2011). Ole has hepato/cardio-protective, antioxidant, anti-inflammatory, cardio-protective, anti-hypertensive and anticancer properties (Barbaro et al., 2014; Buckland & Gonzalez, 2015; Porcu et al., 2018; Santangelo et al., 2018). However, the antioxidant properties of Ole are still under investigation, showing contrasting mechanisms. In fact, in thyroid cancer cells Ole is able to inhibit cell proliferation reducing ROS levels, whereas in prostate cancer cells it displays pro-oxidant effects. What is more, Ole displays opposite behaviours in normal respect to cancer cells (Acquaviva et al., 2012; Hashmi, Khan, Hanif, Farooq, & Perveen, 2015; Imran et al., 2018; Omar, 2010). Interestingly enough, hydroxytyrosol, a metabolite of Ole, has in its structure an aromatic ring, that is a common characteristic of estrogens. In keeping with this aspect, the afore mentioned phenolic compound has gender specific effects, likely at the level of estrogen receptor (ER) binding (Chimento et al., 2014).

According to previous literature data, it is evident that more studies are needed to determine the exact mechanism by which Ole exerts its antioxidant effects.

We asked if, in presence of hepatic steatosis, Ole-related improvement of liver damage is associated with an increase of antioxidant defenses, evaluating the activity of SOD family and catalase (CAT) enzymes.

2. Materials and methods

2.1. Cell Culture

The human hepatoma cell line, HepG2, purchased from American Type Culture Collection (ATCC, Manassas, VA), was maintained in Dulbecco's modified Eagle's medium (DMEM; Lonza East Rutherford, NJ, USA), supplemented with 1% L-glutamine, 10,000 U/mL of penicillin and streptomycin (Lonza, East Rutherford, NJ, USA) and 10% fetal bovine serum (Gibco, Milan, Italy) at 37 °C in a humidified atmosphere of 5% CO_2. Long-chain FAs, palmitic acid (PA; 16:0) and oleic acid (OA; 18:1) (Sigma-Aldrich, Milan, Italy) were dissolved in methanol (MetOH) 99%. Steatosis was induced as previously described by Ricchi et al. (2009). Briefly, cell cultures were incubated with DMEM containing 10% of Charcoal stripped fetal bovine serum (Lonza, East Rutherford, NJ, USA), 1% bovine serum albumin (BSA), and 1% L-glutammine, supplemented with a solution of FAs containing 0.16 mM PA and 0.33 mM OA in a 1:2 M ratio at a final concentration of 0.5 mM for 24 h (FA-HepG2). Cells incubated with MetOH were considered as control.

After 24 h, Oleuropein was added to HepG2 cells at the following concentrations: 10, 25, 50 and 100 μM. Oleuropein (Sigma-Aldrich cat. 12247, Milan, Italy) stock solution was prepared in water and added at the final concentration of 50 μM to HepG2 cells treated with or without FAs for 24 hrs.

2.2. Evaluation of cell toxicity

Control and treated cells were plated at the final concentration of 10^4 cells per well in a 96-well microplate, and allowed to grow. At the end of FAs and Ole treatments, the supernatant was aspirated and 100 μL medium mixed with MTS reagent (Promega cat. G3580, WI, USA) were added to each well, according to the manufacturer's protocol. Wells only containing 200 μL medium were used as blanks and were subtracted as background from each sample. Results obtained in treated cells were expressed as relative respect to untreated cells.

2.3. AdipoRed assay

The intracellular increase of lipid content was evaluated by AdipoRed assay (Lonza cat. PT-7009, Walkersville, MD, USA); according to the manufacturer's protocol. This assay exploits the properties of the dye Nile Red that binds lipids and becomes fluorescent when it is in a hydrophobic environment. Cells were seeded at the final concentration of 10^4 cells in 96 black plates and treated for 24 h with FAs, after that different concentrations (10, 25, 50, 100 μM) of Ole were added for 48 h. At the end of incubation, cells were washed with phosphate buffered saline (PBS) and incubated with AdipoRed for 10 min. After incubation, fluorescence was evaluated and expressed as relative fluorescence units (RFLU) per mg of protein and depicted as fold of increase vs control (vehicle treated cells).

2.4. Mice experimental protocol

We used the HFD fed C57BL/6J murine model, treated with 0.03% of Ole, the most commonly used concentration (Kim, Choi, & Park, 2010; Kuem, Song, Yu, Yun, & Park, 2014). Oleuropein was dissolved in water according to the manufacturer's instructions and administered by oral gavage. C57BL/6J mice, purchased from Charles River Laboratories International, Inc. (Wilmington, USA) were housed in wire mesh cages maintained at controlled (21 ± 1 °C) temperature room with a 12 h light–dark cycle. Mice had ad libitum access to food and water.

After one week of acclimation, 32 mice were randomly divided into 4 groups (8 mice for each group, 4 male and 4 female) and fed with one of the following diets: 16 weeks ND (Teklad Global 2018, Harlan Laboratories Indianapolis, IN, USA; ND group), 8 weeks of ND + 8 weeks of ND and 0.03% of Ole dissolved in water and daily administered by oral gavage (ND + Ole group); 16 weeks of HFD (TD.88137, Harlan Laboratories Indianapolis, IN, USA; HFD group), 8 weeks of HFD + 8 weeks of HFD and 0.03% of Ole dissolved in water (HFD + Ole group). High Fat Diet characterized by Protein kcal%15.2, Carbohydrate kcal% 42.7, Fat kcal% 42.0; Control group or ND fed with Normal Diet characterized by Protein kcal% 24.0, Carbohydrate kcal% 58.0 Fat kcal% 18.0.

After 16 weeks of feeding experimental diets, in presence or absence of Ole treatment, blood was collected from the abdominal veins of all C57BL/6 mice after fasting for 12 hrs.

The blood samples were incubated for 30 min at room temperature in serum separating tubes (BD Biosciences, NJ, USA). Serum was obtained by centrifugation at 1,500 × g for 15 min. Serum Total cholesterol, triglyceride (TRIG), high-density lipoprotein cholesterol (HDL-c), and low-density lipoprotein cholesterol (LDL-c) levels were analyzed by the automatic chemical analyzer (BS-120 Chemistry Analyzer; Mindray). Additionally, serum was analyzed for alanine aminotransferase (ALT) and aspartate aminotransferase (AST) by using an automatic biochemical analyzer (BS-120; Mindray). All assays were

conducted in duplicate using fresh serum.

After serum collection, mice were anesthetized and sacrificed according to institutional guidelines. Each organ was weighed, dissected and formalin-fixed for immune histological analysis or immediately frozen and stored at −80 °C until use for subsequent analysis.

All animal protocols were in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at the University of Florence, Italy (178/2013B, on 16 July 2013).

2.5. Histological analysis and immunohistochemistry for SOD1 and SOD2

Specimens were formalin-fixed, paraffin-embedded and sectioned in order to assess the histological features by Hematoxylin and Eosin (H& E) staining analysis, using a standard protocol.

Formalin-fixed, paraffin-embedded sections (4 μm thick) were mounted on positive charged glass slides. The slides were cooled and endogenous peroxidase were blocked with peroxidase block buffer (citric acid 0.04 M, $Na_2HPO_4x2H_2O$ 0.12 M, NaN_3 0.03 M and H_2O_2 at 1.5% v/v) for 10′ at room temperature. Then, the sections were incubated for 1 h at room temperature with rabbit polyclonal antibodies anti-SOD1 (Abcam, cat. ab16831; 1:100 dilution), anti-SOD2 (Abcam, cat. ab86087; 1:100 dilution).

The primary antibodies were visualized using the avidin-biotin-peroxidase complex method (UltraTek HRP Anti-polyvalent, ScyTek, Logan, UT) according to the instruction manual. 3,3′ diaminobenzidine was used as the enzyme substrate to observe the specific antibody localization, and Mayer hematoxylin was used as a nuclear counterstain. All samples were stained more than once and the results were highly reproducible. To assess differences in staining intensity, an immunoreactivity scoring system was applied. Intensity of staining was classified by both the percentages of the cells stained and the intensity of the staining. In this way, the final scores of 0 to 3 were obtained (0, negative; 1, weak; 2, moderate; 3, strong).

2.6. Cytokines analysis

Evaluation of cytokines levels in serum was performed by using the Bio-Plex multiplex biometric ELISA-based immunoassay (Bio-Rad Laboratories, Inc).

2.7. RNA extraction and cDNA synthesis

Total RNA was extracted from liver tissues using Trizol reagent (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. One μg of total RNA was reverse transcribed using the High-Capacity cDNA Reverse transcription Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer procedures.

2.8. Real-Time quantitative polymerase chain reaction PCR (Q-PCR) analysis

Q-PCR analysis was performed by 7500 Fast Real-Time PCR System (7500 Software v2.0.5, Applied Biosystems) using Power SYBR™ Green PCR Master Mix (Thermo Fisher Scientific, USA). For each sample, β-actin Ct values will be used for normalization purposes. Relative expression was calculated using the comparative cycle threshold (Ct) method ($2^{-\Delta Ct}$, ΔCt = Ct (target gene) - Ct (β-actin)). Data are expressed as fold induction (ND group vs HFD group) of $2^{-\Delta\Delta Ct}$ mean ± standard deviation. Primers were designed using the PrimerQuest software (IDT Integrated DNA Technologies, Coralville, IA, USA) and purchased from BIO-FAB research (Rome, Italy). The following primers were used: 5′ *β-Actin*, 5′-GGGTCAGAAGGACTCCT ATG-3′, 3′ *β-Actin*, 5′-GTAACAATGCCATGTTCA-3′; 5′ *SOD1*, 5′-CAAT ACACAAGGCTGTACCAGTGC-3′, 3′ *SOD1*, 5′- TCTCCAACATCGCTCTC TTCATCC-3′; 5′ *SOD2,* 5′-AAAGGAGAGTTGCTGGAGGCTATC-3′, 3′ *SOD2*, 5′-CGGAATAAGGCCTGTTGTTCCTTG-3′; 5′ *CAT*, 5′-GTGCGGA CATTCTACACAAAGGTG-3′, 3′ *CAT*, 5′-GTACTTGTCCAGAAGAGCCTG GAT-3′; 5′ *CCS*, 5′-CTTTTCCAGAACCCCAAG-3′, 3′ *CCS*, 5′-GGACCAA ATAACCTGACATG-3′.

2.9. Western blot analysis

Total protein extraction was performed by homogenizing cells and hepatic tissues in Ripa lysis buffer containing 1X protease and phosphatase inhibitors cocktail (Thermo Fisher Scientific Inc.). The homogenates, after 30 min of incubation on ice, were then centrifuged at 13,000 rpm for 30 min at 4 °C. Protein concentrations were determined using the Bradford Protein Assay (Bio-Rad, Hercules, CA, USA). Lysates obtained were analyzed in denaturing condition through SDS-PAGE and transferred onto nitrocellulose membranes (Amersham Bioscience, Little Chalfont, UK). Membranes were incubated with primary antibodies followed by horseradish peroxidase-conjugate secondary antibody (Jackson Laboratories, Ann Arbor, MI, USA) and visualized with ECL (Western nova 2.0, Cyanagen, Italy).

Primary antibodies, diluted according to the manufacturer instruction, were as follows: β-Actin (sc-47778, C4) purchased from Santa Cruz Biotechnology Inc. (Dallas, USA). Abcam (Cambridge, UK) provided the following primary antibodies: anti-SIRT1 (cat. ab12193; dil. 1:1000), anti-SIRT3 (cat. ab86671; dil. 1:1500), anti-SOD1 (cat. ab16831; dil. 1:1000), anti-SOD2 (cat. Ab86087 dil. 1:2000), anti-CAT (cat. ab16731; dil. 1:2000). The HRP Goat Anti-Rabbit IgG secondary antibody (cat. PI1000; dil. 1:1000) was purchased from Vector Laboratories, Inc. (Burlingame, CA, USA). Densitometric analysis of immunoblots was performed by ImageJ64 image processing software for electrophoresis gel analysis.

2.10. Superoxide dismutase, catalase enzymatic activity and lipid peroxidation assessments

Livers were lysed (100 mg/0.4 mL) as described by Bradford (1976). All spectrophotometric readings were carried out in quadruplicate by using a Lambda25 spectrophotometer (PerkinElmer, Inc., Waltham, MA, USA).

The tSOD (EC 1.15.1.1) activity was assayed by measuring its ability to inhibit the autoxidation of epinephrine (cat. E4375, Sigma-Aldrich, Milan, Italy), which was spectrophotometrically monitored at 480 nm at 30 °C, according to Sun and Zigman (1978). One unit of tSOD activity was assumed to halve the rate of epinephrine autoxidation. Measurements were carried out in quadruplicate.

The SOD1 inhibitor KCN was added at the concentration of 2.5 mM in order to evaluate the SOD2 isoenzyme activity.

The CAT (EC 1.11.1.6) activity was assayed by recording at 240 nm and 25 °C the disappearance of 10 mM hydrogen peroxide (cat. 21,676–3; Sigma-Aldrich), as described (Aebi, 1984). One unit of enzyme activity was defined as 1 μmol of H_2O_2 reduced/min at 25 °C. Measurements were carried out in five replicates.

The measurement of peroxidative damage was carried out by photometric detection (532 nm) of the thiobarbituric acid-conjugated adduct, by using the thiobarbituric acid reactive substances assay kit (cat. 10009055, Cayman Chemical Company, Ann Arbor, MI, USA), as described (Yagi, 1998). Briefly, livers (100 mg/0.4 mL) were lysed as described (Bradford, 1976); samples were added in triplicate to 1 vol of SDS and 40 vol of Colour Reagent, as suggested by the manufacturer. The reaction mixtures were incubated in quadruplicate for 1 h in boiling water and centrifuged at 1,600 × g for 10 min, at 4 °C. A linear calibration curve was computed from pure malondialdehyde (MDA)-containing reactions (range: 0–50 μM). Results were given as ng MDA/mg total protein.

Densitometric analysis of immunoblots were performed by ImageJ64 image processing software for electrophoresis gel analysis.

2.11. Statistical analysis

Results were expressed as mean ± standard deviation (SD). All in vitro experiments were performed in triplicate. A 2-tailed paired/unpaired Student *t* test was used to analyze in vitro data. The 2-tailed Mann-Whitney test and Factorial ANOVA with post-hoc Newman–Keuls/Tukey tests for multiple comparison were applied to compare animal groups (*vs ND mice; # vs HFD mice; § vs male mice). Statistical significance was assessed by p-value (P) thresholds: *P < 0.05; **P < 0.01; ***P < 0.001. All statistical analyses were performed with Prism software version 6 (GraphPad Software, San Diego, California).

3. Results

3.1. Viability of FA-HepG2 cells after Ole treatment

Before using Ole in *in vivo* model, we just wondered whether it might affect cell viability in presence of fat accumulation. Besides that, we look at the best effective concentration of Ole able to down-regulate the fat accumulation in FA-HepG2.

To this aim human hepatoma HepG2 cells were incubated with 0.5 mM of long chain FAs, as described in material and method, and increasing doses of Ole (ranging from 10 to 200 μM) were added. Cell viability and evaluation of fat accumulation was assessed on HepG2 cells. As showed in Fig. 1A Ole did not affect cell viability in vitro. At the concentrations of 10 and 25 μM Ole appears to be ineffective, whereas, at the concentration of 50 μM, it was able to decrease the accumulation of fat in hepatoma cell line (Fig. 1B).

3.2. Ole improves liver histology in HFD mice

We used both HFD fed male and female mice, treated with 0.03% of Ole. ND and HFD mice were treated or not with 0.03% of Ole, administered by oral gavage. Haematoxylin and eosin staining of liver tissues of ND and HFD in male and female is showed in Fig. 2A. As predictable, Ole was able to improve hepatic steatosis either in females or males, but with a higher extent in female mice (Fig. 2A, bottom panels).

Furthermore, we found, after Ole treatment, a decrease of body, liver and heart weights, highlighting a reduction that was significant only for the heart (Fig. 2B).

Biochemical parameters reported in Table 1 highlight a significant increase in transaminases, as well as in cholesterol and HDL serum levels in males and females that came back to normal values in the mice group treated with Ole. Triglycerides were significantly increased by HFD in female mice, and again Ole was able to restore their basal levels.

3.3. Anti-inflammatory effects of Ole

In order to evaluate if Ole treatment could influence the inflammatory status induced by feeding mice with HFD, we evaluated IL1-α, IL-2, TNF-α and G-CSF in the serum of mice (Fig. 3A–D). Ole was able to significantly down-regulate serum levels of IL1-α and G-CSF, in ND and HFD males and females (Fig. 3A and D). On the contrary, it seems that Ole did not significantly influence the higher levels of IL-2 and TNF-α (Fig. 3B and C), even if IL-2 was significantly lower in HFD female mice (Fig. 3B). Again, Ole effects on TNF-α in ND mice are more evident in female respect to male (Fig. 3C).

3.4. Antioxidant effects of Ole

To assess the Ole effects on the first line defense antioxidant-superoxide dismutase enzymes (SOD), the total superoxide dismutase specific activity (tSOD) was estimated. No significant differences were highlighted (Fig. 4A).

Given that 90% of the activity of tSOD is related to the SOD1 activity, we planned to evaluate the contribution of SOD1 and the mitochondrial SOD2 isoforms, individually. Very interestingly, we observed no changes in SOD1 specific activity (Fig. 4B), while a sex related increase of SOD2 enzymatic activity in ND and HFD female mice treated with Ole was detected (Fig. 4C).

The enzymatic activity of SOD detoxifies superoxide anion producing hydrogen peroxide, however, even if to a lesser extent, could also have toxic properties. For this reason, we evaluated the enzymatic activity of CAT that breaks down H_2O_2 into water and molecular oxygen, curtailing free radical-induced damage. Administration of HFD diet caused opposite effects on CAT activity, inducing in male mice a significant decrease of the enzyme activity that, on the contrary, was higher in females (Fig. 4D). Interestingly enough, as shown in Fig. 4D, Ole administration abolished the highlighted sex differences.

Transcripts and protein expression levels of the three studied enzymes were reported in supplementary data (Supplementary Figs. S1 and S2).

3.5. Ole modifies the SOD1 intracellular localization and increases SOD2 cytosol expression

To better understand the role of SODs after Ole treatment, we assessed the expression of SOD1 and SOD2 by using immuno-histochemical staining in liver tissues of ND and HFD mice (Fig. 5). Importantly, HFD induced the nuclear localization of SOD1 in both male and female mice (Fig. 5H, left and right panels, respectively). Interestingly, the liver tissues of HFD mice treated with Ole showed SOD1 back in the cytosol (Fig. 5K).

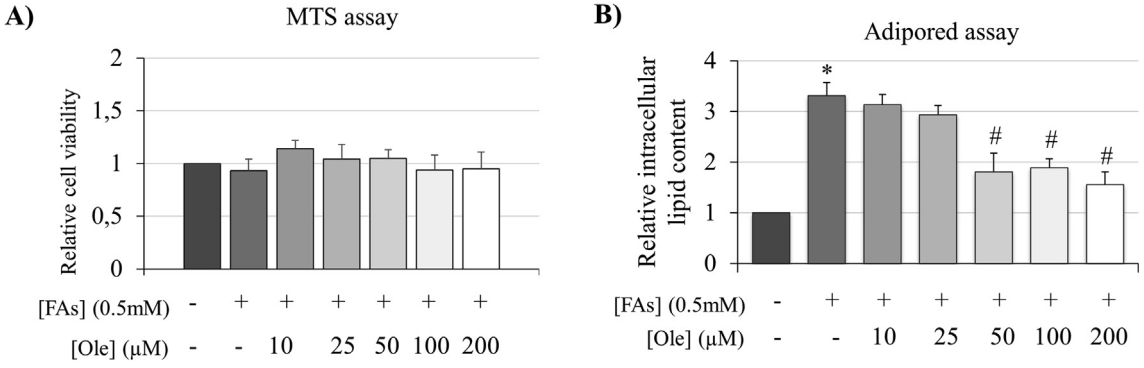

Fig. 1. Oleuropein couteracts fat accumulation in hepatoma cell lines. Cell viability (A) and lipid accumulation (B) in HepG2 cells treated for 24 hrs with FAs (0.5 mM) and increasing dosages of Ole (0–200 μM) evaluated by MTS assay and adipored assay (plating 10^4 cells in 96 black plates), respectively. HepG2 cells cultured in medium containing only an equivalent concentration (v/v) of MetOH 99%, were used as controls. The results, derived from five independent experiments, are represented as mean ± SD. (* $P < 0.05$, vs control without Ole treatment; $^{\#}$ $P < 0.05$ vs FAs without Ole treatment).

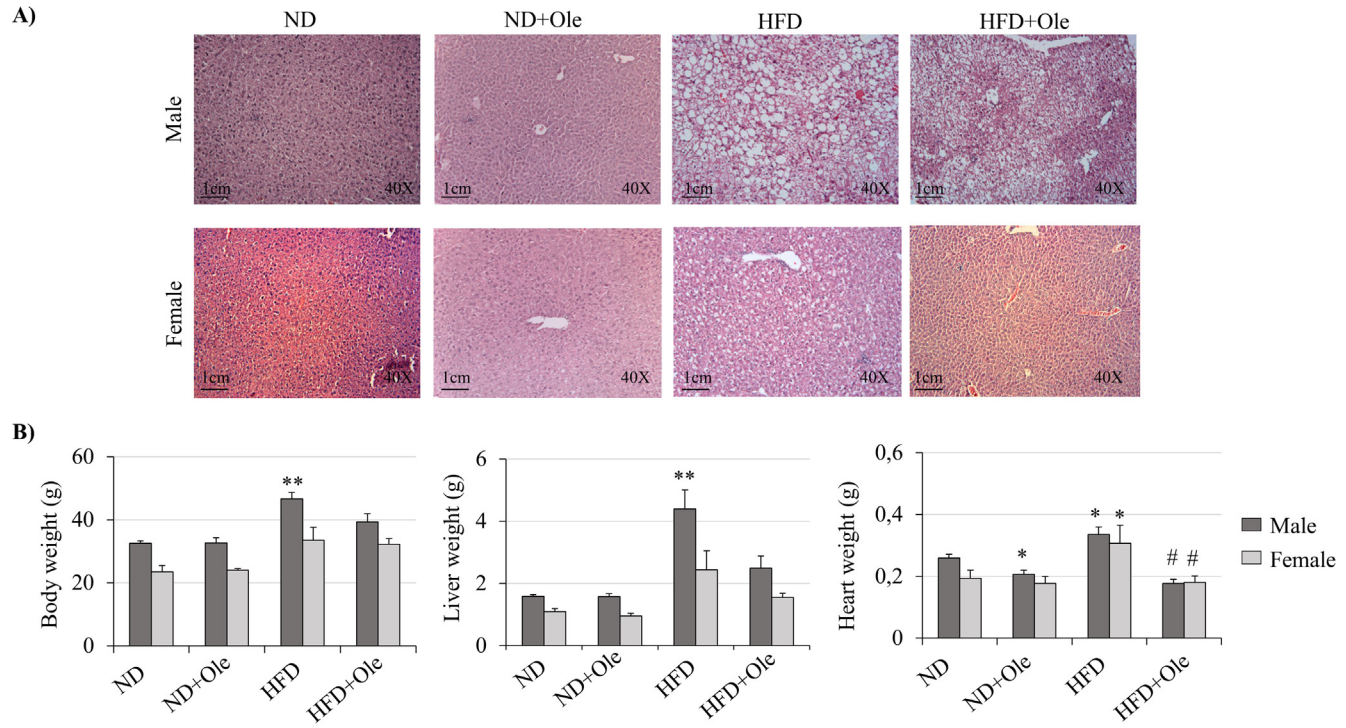

Fig. 2. Liver histology in ND and HFD mice treated or not with Ole. (A) Representative photomicrographs of Haematoxylin & Eosin stained sections of liver tissue of male (top) and female (bottom) mice fed with ND or HFD diet in presence or absence of Ole. Original magnification 40X, scale bar = 1 cm. (B) Body, liver and heart weight (gr) of ND and HFD female and male mice treated or not with Ole. The results are represented as mean values ± SD. (* $P < 0.05$, ** $P < 0.01$ vs ND mice; # $P < 0.05$ vs HFD mice).

The Ole administration was able to delocalize SOD1 in the nucleus also in ND mice, showing again a better efficacy in female respect to male mice (Fig. 5E, left and right panels, respectively).

On the other hand, as highlighted by the western blot analysis (Fig. S1), Ole induced a higher expression of SOD2 in both group of mice, without affecting its intracellular localization (Fig. 5F and L).

3.6. Effects of Ole on liver oxidative stress scavenger system and liver damage

The CAT/tSOD ratio was evaluated as it provides a better assessment of the scavenging efficiency of the antioxidant enzyme defence respect to the single enzyme activity (Park et al., 2007). In a very singular way, HFD induced an opposite behavior between males and females. In fact, while the antioxidant defence system tried to respond to HFD-induced stress in female mice, a decrease in antioxidant enzyme defence was observed in males (Fig. 6A). Very interestingly, the ratio between CAT/tSOD was significantly increased by Ole intake in the liver of both male and female HFD mice, resetting the observed sex-related differences (Fig. 6A). Moreover, a sex-related behaviour in CAT/tSOD ratio was observed in both ND and ND + Ole liver tissues of mice, in particular, HFD males showed higher level of antioxidant efficiency (Fig. 6A).

In order to evaluate if Ole protects the liver from the oxidative

Table 1
Biochemical parameters of ND and HFD mice. The results are represented as mean values ± SD. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs ND mice; # $P < 0.05$, ## $P < 0.01$; ### $P < 0.001$ vs HFD mice; § $P < 0.05$, §§ $P < 0.01$, §§§ P < 0.001 vs male mice).

	MALES			
	ND	**ND + Ole**	**HFD**	**HFD + Ole**
ALT (U/L)	50.3 ± 1.1	49 ± 2.5	125.7 ± 25.4 **	80.2 ± 7.3 #
AST (U/L)	106.6 ± 45.1	103.6 ± 22.3	208.5 ± 20.8 *	110.1 ± 10.6 ##
Total cholesterol (mg/dL)	165.3 ± 11.7	155.1 ± 6.7	202.6 ± 13.8 *	170.6 ± 10.8 #
HDL-c (mg/dL)	85.2 ± 2.75	89.3 ± 3.4	124.2 ± 2.3 ***	90 ± 1.7 ###
LDL-c (mg/dL)	11.3 ± 4.6	10.7 ± 1.3	15.3 ± 4.2	11.7 ± 2.3
TRIG (mg/dL)	120.3 ± 7.5	118.2 ± 4.2	130.2 ± 5.7	119.9 ± 6.5
	FEMALES			
	ND	**ND + Ole**	**HFD**	**HFD + Ole**
ALT (U/L)	47.6 ± 4.5	42.1 ± 19.4	77.5 ± 2.2 ***,§	50.2 ± 3.5 ###,§§
AST (U/L)	101.4 ± 22.5	90.3 ± 8.5	200.2 ± 5.2 **	109.4 ± 12.2 ###
Total Cholesterol (mg/dL)	150 ± 11.4	152.9 ± 6.3	198.1 ± 1.9 **	164.1 ± 3.2 ###
HDL-c (mg/dL)	82.6 ± 1.8	88.3 ± 2.5 *	116.5 ± 3.4 ***,§	84.2 ± 4.2 ###
LDL-c (mg/dL)	9.4 ± 2.3	8.9 ± 1.5	12.8 ± 2.3	10.1 ± 1.9
TRIG (mg/dL)	92.5 ± 4.7 §§§	88.6 ± 5.1 §§	107.8 ± 9.6 §	99.2 ± 3.6 §§

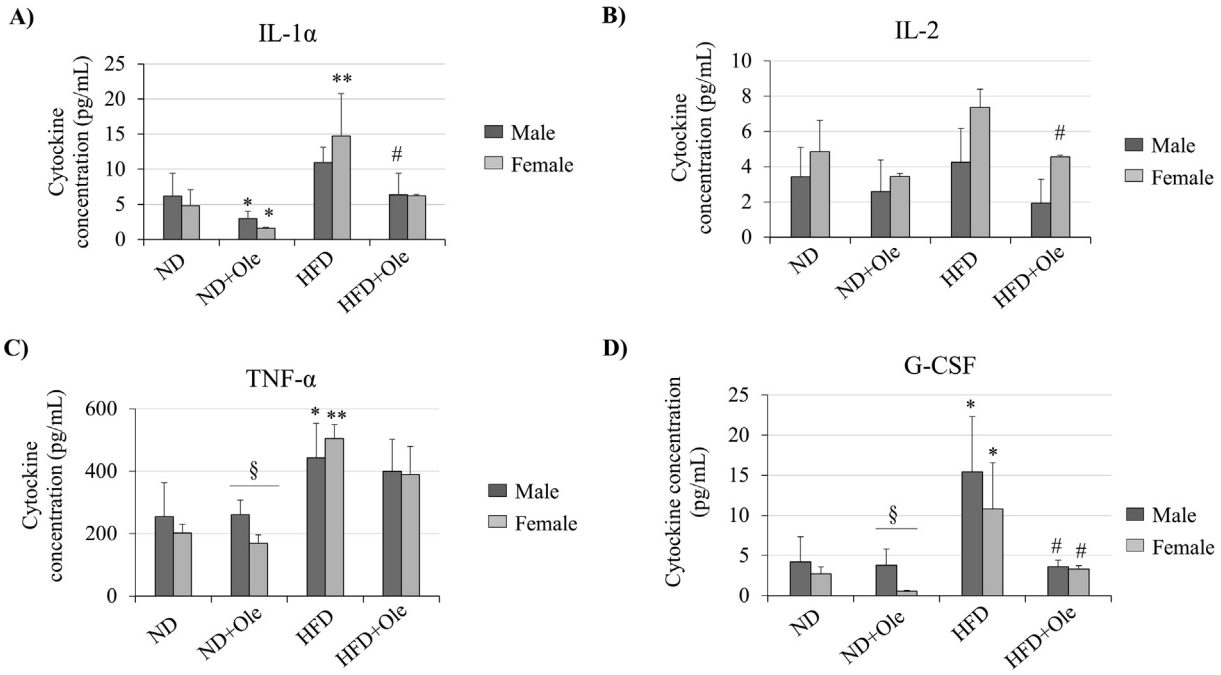

Fig. 3. Inflammatory cytokines in ND and HFD mice +/- Ole. (A) Interleukin 1 alpha (IL-alpha, (B) Interleukin 2 (IL-2), (C) Tumor necrosis factor alpha (TNF- alpha and (D) Granulocyte Colony-Stimulating Factor (G-CSF) modulation in ND and HFD male and female mice treated or not with Ole. Analyses were performed by Bio-Plex multiplex biometric ELISA-based immunoassay. The results are represented as mean values ± SD. (* $P < 0.05$, ** $P < 0.01$ vs ND mice; # $P < 0.05$ vs HFD mice; § $P < 0.05$ vs male mice).

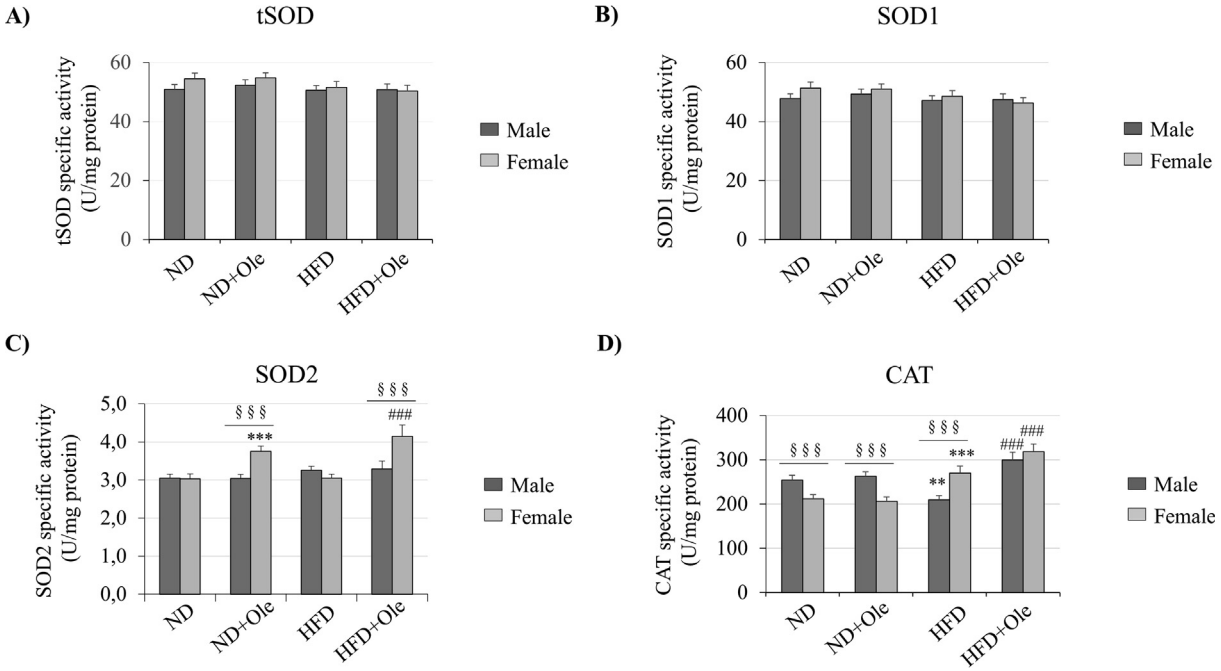

Fig. 4. Antioxidant system in ND and HFD mice treated or not with Ole. Effects of Ole on specific activity of tSOD (A), SOD1 (B), SOD2 (C) and CAT (D). All the results were expressed as means ± SD. Data were analyzed by a factorial ANOVA with post-hoc Tukey's tests for multiple comparison (*$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs ND mice; # $P < 0.05$, ## $P < 0.01$; ### $P < 0.001$ vs HFD mice; §§§ $P < 0.001$ vs male mice).

stress-induced by HFD, we assessed the liver amount of Malondialdehyde (MDA), one of the most reliable and used marker of lipid peroxidation (Fig. 6B). HFD, as expected, induced an increase in oxidative stress-related liver damage in both male and female mice (Fig. 6B), however Ole was not only able to protect the liver from the oxidative stress in both sexes, but even exceeded the liver basal damage (Fig. 6B).

Interestingly, lower basal damage was observed in ND females respect to males, besides that, Ole administration further lowered it in liver of female ND mice (Fig. 6B).

3.7. Effects of Ole on Sirtuins protein levels

To try to understand the molecular mechanisms through which Ole acts, we investigated the protein levels of two of the most important Sirtuins: SIRT1 and SIRT3. In our model, HFD induces a significant

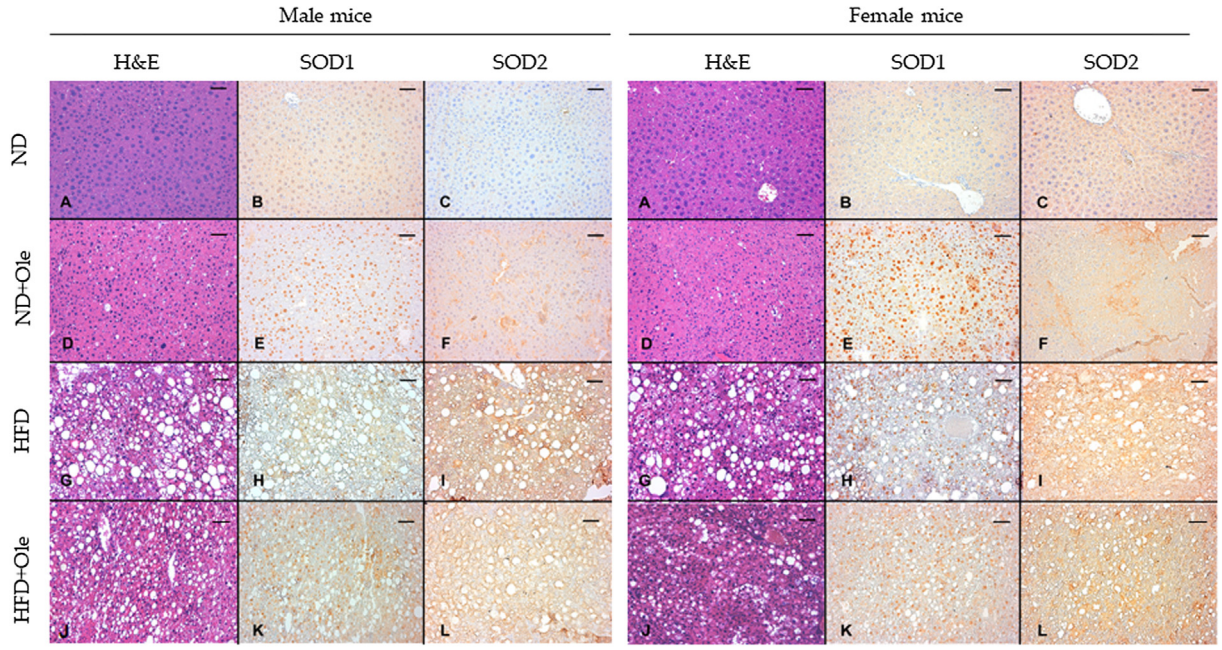

Fig. 5. Effects of Ole treatment on liver histology in ND or HFD mice. Representative liver tissue sections of immuno-histochemical analysis of SOD1 (A) and SOD2 (B) protein expression. Avidin-Biotin-Peroxidase complex method in paraffin sections lightly counterstained with ematoxylin. Original magnification 200X, scale bar = 1 cm.

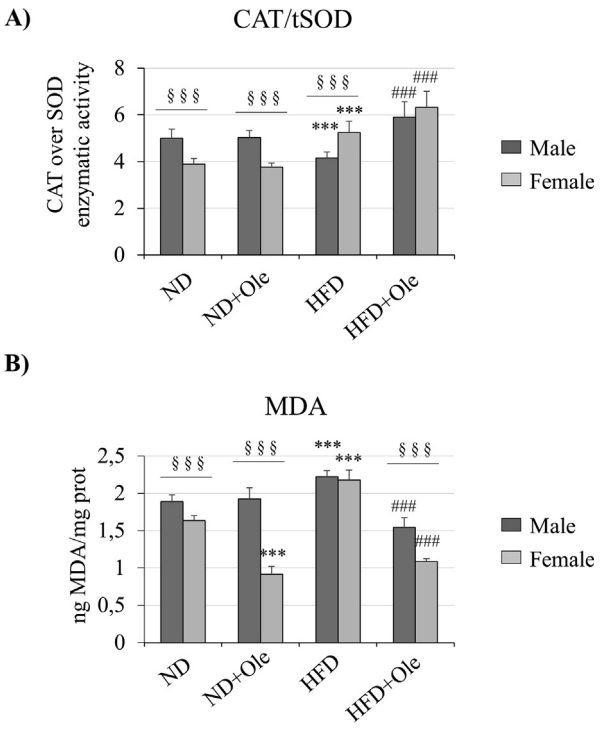

Fig. 6. Antioxidant scavenging efficiency and lipid peroxidation in ND and HFD mice +/- Ole. (A) catalase (CAT)/total superoxide dismutase (tSOD) ratios were reported. Values were expressed as mean ± SD. (B) Liver MDA concentration was assessed by TBARS assay and normalized by the amount of total protein. Values were given as mean ± SD. Data were analyzed by a factorial ANOVA with post-hoc Tukey's tests for multiple comparison (*** $P < 0.001$ vs ND mice; ### $P < 0.001$ vs HFD mice; §§§ $P < 0.001$ vs male mice).

increase of SIRT3 in HFD mice. No changes were appreciated in SIRT1 protein expression in HFD mice (Fig. 7A and B). Interestingly, in the presence of Ole, HFD male and female mice displayed a significant increase of both SIRT1 and SIRT3 protein amounts (Fig. 7A and B). Ole was also able to significantly increase protein levels of SIRT1 in both groups of mice, whereas SIRT3 was up-regulated only in ND female mice (Fig. 7B).

4. Discussion

NAFLD is a chronic disease caused by the accumulation of fat in the liver and the most severe form NASH is characterized by the presence of inflammation, oxidative stress, lipid peroxidation and mitochondrial dysfunction (Araújo et al., 2018; Czaja, 2007). Liver fat metabolism is dependent on mitochondrial activity and generates ATP but also reactive oxygen species (ROS). Considering the fundamental role of mitochondria in providing energy, it is not surprising that their function and behavior are crucial to the physiology of cellular health, and that "mitochondrial dysfunction" has been implicated in a wide range of chronic inflammatory diseases and related disorders (Begriche et al., 2013; Caldwell et al., 1999; Grattagliano et al., 2012). ROS, in the liver, are involved as key secondary messengers in numerous signaling pathways and may be generated by the mitochondrial respiratory chain dysfunction as well as by the induction of pro-inflammatory cytokines (e.g., IL-1, IL-6, TNF-α) (Stojsavljević et al., 2014). The imbalance between oxidative stress and antioxidants plays a main role in the pathogenesis and severity of NAFLD/NASH. In keeping with pieces of evidence, the intake of unhealthy diet is related to mitochondrial dysfunction and is caused by multiple mechanisms, including SOD family, CAT and Sirtuins imbalance (Ding et al., 2017; Kendrick et al., 2011; Nassir & Ibdah, 2016).

In order to evaluate the antioxidant properties of Ole we studied its effects on the activity of SOD family and CAT enzymes in C57BL/6J mice fed ND or HFD for 8 weeks, adding to their diet 0.03% of Ole for the following 8 weeks.

Interestingly enough, even if the activity of SOD1 was unchanged in both ND and HFD mice, before and after Ole treatment, it was possible to appreciate, in HFD mice by immuno-histochemical analysis, that this antioxidant enzyme delocalized in the nucleus and partially came back to the cytosol in the presence of Ole (see Fig. 5). The choice to specifically analyze SOD family and CAT was made for the reasons reported below. Although, ROS are normally kept below the toxic limit through

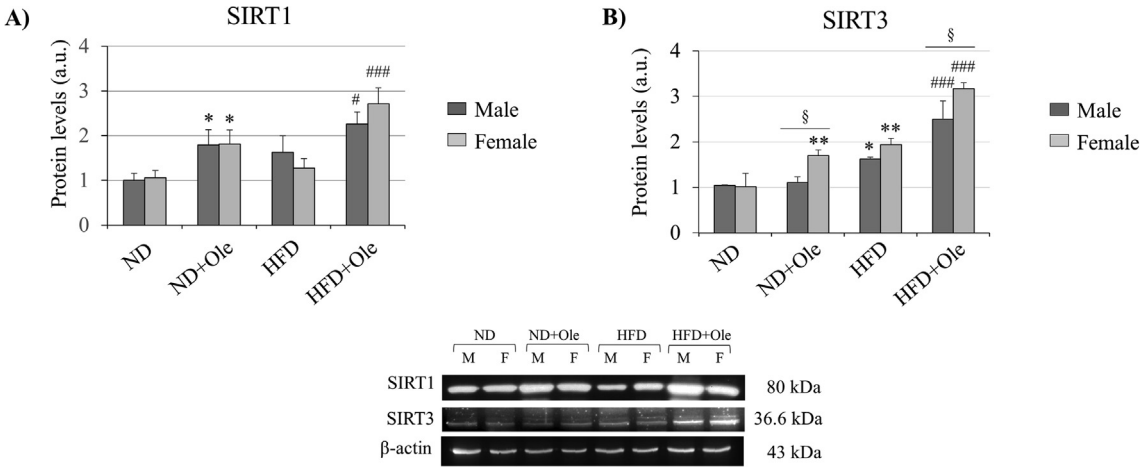

Fig. 7. Effects of Ole treatment on Sirtuins expression in ND and HFD mice in presence or absence of Ole. Western blot analysis and relative densitometry of SIRT1 (A) and SIRT3 (B) proteins. Values are expressed as fold mean ± SD. Data were analyzed by a factorial ANOVA with post-hoc Tukey's tests for multiple comparison (*P < 0.05, **P < 0.01 vs ND mice; # P < 0.05, ###P < 0.001 vs HFD mice; § P < 0.05 vs male mice).

the efficient activity of SOD, CAT and glutathione peroxidase (GPx) their role in protecting tissue is quite different. Superoxide dismutase enzyme rapidly reduces superoxide anion to H_2O_2 and O_2, while at later time GPx and CAT detoxify hydrogen peroxide preventing tissue damage even far from the ROS production site. The GPx, in particular, has a good efficiency at low concentrations of H_2O_2, but if the hydrogen peroxide reaches high levels, as happens during HFD diet intake, the peroxidase activity becomes no longer adequate, thus to get an efficient antioxidant activity is needed the action of CAT (Ighodaro & Akinloye, 2018; Tsang et al., 2014; Wang et al., 2018).

The observed nuclear localization of SOD1 is probably related to the higher ROS intracellular production after HFD diet administration for maintaining genomic stability. The intra-nuclear localization of SOD1 induces the activation of nuclear transcription factors, such as PGC1, ATM and Mec1 that in turn prompt the expression of antioxidant genes (Caldwell et al., 1999). Accordingly, Ole administration, exerting antioxidant effects, allows SOD1 to come back into the cytoplasm by lowering intracellular oxidative stress (see Fig. 5A).

In addition, has been reported that mitochondrial SOD2 activity displays a gender specific behavior. Accordingly, SOD2 protein expression and activity are significantly increased only in HFD female mice (see Fig. 4C).

Furthermore, it is quite interesting to observe the same Ole-dependent specific induction of SIRT3 protein expression in HFD female mice (see Fig. 7B). The altered protein expression of SIRT3 has been associated with unhealthy diet intake and it has been hypothesized to have a main role in starting fat related liver damage. Interestingly enough, the similar highlighted trend of SOD2 activity and SIRT3 protein expression seem to reflect a more effective activation of the antioxidant defense by female respect to male mice (see Fig. S1, 4C, 6B and 7B) (Kendrick et al., 2011; Yoshino & Imai, 2011). Moreover, it has been demonstrated that SIRT1 in response to cellular stress is able to deacetylate the transcription factor FOXO1 which, in turn, increases the expression of a huge number of proteins involved in stress resistance, including SOD2 (see Fig. 7A) (Baur, Ungvari, Minor, Le Couteur, & de Cabo, 2012; Hsu et al., 2010; Klotz et al., 2015; Kobayashi et al., 2005; Strycharz et al., 2018).

Ole, in presence of HFD, completely reverses the HFD related specific sex differences observed, suggesting that Ole, in stress conditions, has a significant antioxidant effect, making catalase able in limiting free radical-induced liver damage also in males (see Fig. 4D and 6A). Here, it is important to emphasize that the good efficiency of the antioxidant defense system depends on the cooperation of antioxidant enzymes activity. Therefore, the CAT/tSOD activity ratio is a better indicator of scavenging efficiency against ROS rather than the individual antioxidant enzymes activity (see Fig. 6A).

Finally, all the data reported above are confirmed by quantifying the lipid peroxidation status evaluated as the liver amount of MDA. Again, the level of oxidative damage was lower in HFD females, hypothesizing the presence of a higher basal resistance to oxidative damage in females (see Fig. 6B).

Fig. 3 shows that the antioxidant Ole effects improves the pro-inflammatory status of both ND and HFD mice.

Besides this, it is important to note that our work demonstrated that Ole has a sex specific activity also in ND female mice. Ole, in fact, specifically reduced liver amount of MDA, increasing SIRT3/SOD2 signaling, involved in mitochondrial oxidative repair, in ND female but not in male mice (see Fig. 4C, 6B and 7B).

However, our study has some limitations mainly related to the fact that liver damage obtained by dietary modification in mice could not be really comparable to the liver damage of subjects who usually eat unhealthy diets. In addition, the age of onset and the duration of HFD in mice are important parameters for evaluating the effects of a treatment on liver steatosis, but the experimental conditions are really far from what happen in human real life.

Our work demonstrates that Ole treatment is able to reset, in HFD fed mice, the imbalance of the three studied antioxidant enzymes, improving liver oxidative stress related damage that characterizes the progression of NAFLD toward NASH. Interestingly, Ole treatment overrides sex specific differences, restoring an effective antioxidant response in both sexes in presence of stressful stimuli such as unhealthy diet.

A graphical representation of the effects of Ole in steatotic mice is shown (Fig. 8, Supplementary Fig. S1 and Supplementary Fig. S2).

5. Funding Statement

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Ethics Statement

All animal protocols were in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at the University of Florence, Italy (178/2013B, on 16 July 2013).

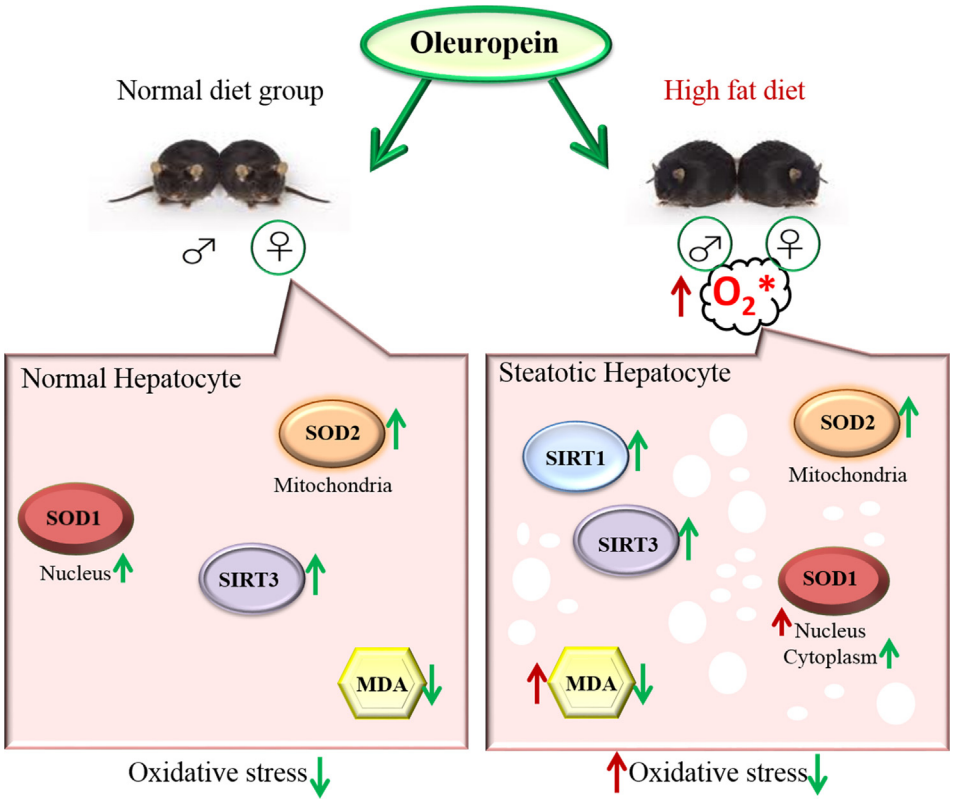

Fig. 8. Graphical representation of the effects of Ole in ND and HFD male and female mice. The red arrows indicate the effects of HFD; the green arrows the effects related to the administration of Ole nutriaceutical compound. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

CRediT authorship contribution statement

Silvano Junior Santini: Methodology, Data curation, Formal analysis, Writing - review & editing, Visualization. **Cristiana Porcu:** Methodology, Formal analysis, Visualization. **Giovanni Tarantino:** Writing - review & editing. **Fernanda Amicarelli:** Writing - review & editing, Supervision. **Clara Balsano:** Conceptualization, Data curation, Writing - original draft, Supervision, Project administration.

Declaration of Competing Interest

The research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2019.103756.

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