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Research article

Acute administration of the olive constituent, oleuropein, combined with ischemic postconditioning increases myocardial protection by modulating oxidative defense

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Abstract

Oleuropein, one of the main polyphenolic constituents of olive, is cardioprotective against ischemia reperfusion injury (IRI). We aimed to assess the cardioprotection afforded by acute administration of oleuropein and to evaluate the underlying mechanism. Importantly, since antioxidant therapies have yielded inconclusive results in attenuating IRI-induced damage on top of conditioning strategies, we investigated whether oleuropein could enhance or imbed the cardioprotective manifestation of ischemic postconditioning (PostC). Oleuropein, given during ischemia as a single intravenous bolus dose reduced the infarct size compared to the control group both in rabbits and mice subjected to myocardial IRI. None of the inhibitors of the cardioprotective pathways, L-NAME, wortmannin and AG-490, influence its infarct size limiting effects. Combined oleuropein and PostC cause further limitation of infarct size in comparison with PostC alone in both animal models. Oleuropein did not inhibit the calcium induced mitochondrial permeability transition pore opening in isolated mitochondria and did not increase cGMP production. To provide further insights to the different cardioprotective mechanism of oleuropein, we sought to characterize its anti-inflammatory potential in vivo. Oleuropein, PostC and their combination reduce inflammatory monocytes infiltration into the heart and the circulating monocyte cell population. Oleuropein's mechanism of action involves a direct protective effect on cardiomyocytes since it significantly increased their viability following simulated IRI as compared to non-treated cells. Oleuropein confers additive cardioprotection on top of PostC, via increasing the expression of the transcription factor Nrf-2 and its downstream targets *in vivo.* In conclusion, acute oleuropein administration during ischemia in combination with PostC provides robust and synergistic cardioprotection in experimental models of IRI by inducing antioxidant defense genes through Nrf-2 axis and independently of the classic cardioprotective signaling pathways (RISK, cGMP/PKG, SAFE).

Keywords: cardioprotection, inflammation, ischemia-reperfusion injury, oleuropein, postconditioning, antioxidant defense systems

1. Introduction

Acute myocardial infarction (MI) is a major cause of mortality and heart failure development worldwide [1]. Early reperfusion therapy is a critical strategy to limit the final infarct size and has improved clinical outcomes in patients with acute MI over the past 3 decades [2]. However, although early mortality after acute MI is declining in most countries and especially in those with high per capita incomes, long-term mortality and subsequent post MI heart failure remains significant. Cardioprotection on top of early reperfusion as a strategy to reduce the amount of myocardium that dies during a period of ischemia remains of significant clinical importance and an unmet clinical need [3]. Thus far, several cardioprotective pharmacological and interventional strategies directed at limiting cardiac injury acting upon reperfusion have had modest results despite their promising effects in multiple experimental animal models. The difficulty in translating the strategies developed in animal models to patients suffering from myocardial ischemia suggests that the concept of a "magic bullet" for a "magic single target" is not feasible. In this regard, for optimal cardioprotection, it is required to have a combination of additive or synergistic multitarget therapies [4].

The reperfusion of ischemic myocardium paradoxically induces cardiomyocyte death, known as myocardial ischemia-reperfusion injury (IRI). The mechanism of IRI is multifactorial in nature and involves the contribution of divergent biologic pathways, including ion channels, reactive oxygen species (ROS), inflammation, and endothelial dysfunction [5]. Intracellular calcium overload, rapid normalization of pH upon reperfusion and oxidative stress result in the opening of the mitochondrial permeability transition pore (MPTP) triggering cell death. Among the myriad of targets against IRI, a significant number, including the inhibition of MPTP opening, belong to three well-considered cardioprotective pathways, namely the Reperfusion Injury Salvage Kinase (RISK), the Survivor Activating Factor Enhancement (SAFE) and the cyclic guanosine 3′,5′-monophosphate/Protein Kinase G (cGMP/PKG). Several pharmacological agents and the induction of the endogenous phenomenon of 'ischemic conditioning' (where the heart is subjected to short periods of ischemia and reperfusion) have been shown to mediate cardioprotection through the aforementioned pathways [6]. In fact, ischemic postconditioning (PostC), performed at the onset of reperfusion, has been demonstrated to be an effective method to combat myocardial IRI and represents one feasible cardioprotective maneuver in the clinical practice [7].

In the present study, we used oleuropein, a bioactive polyphenolic compound isolated from *Olea europaea* that possesses high antioxidant capacity and diverse pharmacological properties including anti-inflammatory, anticancer, hypoglycemic, hypolipidemic and neuroprotective effects [8–10] underlying its potential potent multitarget effects against IRI. Previous studies have demonstrated that the chronic administration of oleuropein reduces myocardial infarct size, confers strong antioxidant protection and reduces circulating lipids in anesthetized rabbits [11]. Moreover, it has been demonstrated that oleuropein exerts a protective effect against the doxorubicin (DXR)-induced cardiotoxicity by inhibiting lipid peroxidation products and reducing nitro-oxidative stress in cardiomyocytes [12]. However, its cardioprotective effect after acute administration in myocardial IRI has yet to be elucidated.

The purpose of the current research work is to characterize the cardioprotective effect of acute administration of oleuropein in terms of infarct size reduction and to determine if oleuropein in combination with PostC could enhance or imbed the cardioprotective manifestation of PostC. Our main hypothesis is that the combination of ischemic PostC with oleuropein may have a different mechanism of action and could serve as an ideal multitargeted therapy against myocardial IRI.

2. Methods and Materials

2.1 Animals

All animals were treated according to the Presidential Decree 56/2013 in harmonization to the European Directive 2010/63 for the protection of animals used for scientific purposes and conformed to the Guide for the Care and Use of Laboratory Animals published by the National Research Council. The number of animals used in the study was defined after performing statistical power analysis using G*Power tool. The experimental protocols were approved by Ethical Committee of University of Athens as well as the Veterinary Authorities of Region of Athens Greece (Ref. numbers 3306/2010 and 1316/2019) and the Veterinary Authorities of Region of Central Macedonia Greece (Ref. number 399301/2966). Surgical procedures and the interventions on the animals were performed in compliance with the guidelines ‘Practical guidelines for rigor and reproducibility in preclinical and clinical studies on cardioprotection’[13].

2.2 *In vivo* experimental protocols

Dosage protocol. New Zealand white male rabbits (n=24) weighting between 2.5 and 3.5 kg were randomized into 4 groups and were subjected to a 30 min period of regional ischemia of the heart followed by a 3 h reperfusion with the following interventions: (i) Control (n=6): no additional intervention; (ii) PostC (n=6): animals were subjected to PostC consisted of 8 cycles of 30 sec ischemia/reperfusion immediately after completion of index ischemia; (iii) Oleu50 (n=6): animals were treated with oleuropein at the dose of 50 mg/kg intravenously (iv) bolus on the 20th min of ischemia; (iv) Oleu100 (n=6): animals were treated with oleuropein at the dose of 100 mg/kg iv bolus on the 20th min of ischemia. Isolation of oleuropein was attained through the extraction of olive leaves as previously described [11]. The dosages were selected based on our previous studies concerning the cardioprotective effect of oleuropein on DXR-induced cardiotoxicity [12]. For the quantification of oleuropein in plasma, whole blood was withdrawn at the 1st and at the 20th min after reperfusion. At the end of the procedures, hearts were excised for the determination of the infarct size **(Supplementary Figure 1).**

Experimental protocol in rabbits. New Zealand white male rabbits (n=51) weighting between 2.5 and 3.5 kg were randomized into 7 groups and were subjected to a 30 min period of regional ischemia of the heart followed by a 3 h reperfusion with the following interventions: (i) Control group (n=12): no further intervention; (ii) PostC group (n=6): 8 cycles of 30 sec ischemia/reperfusion at the onset of reperfusion; (iii) Oleu group (n=6): iv bolus administration of 100 mg/kg oleuropein at the 20th min of ischemia; (iv) Oleu + PostC group (n=7); (v) Oleu + L-NAME group (n=6); (vi) Oleu + Wortmannin group (n=8) and (vii) Oleu + AG490 group (n=6). The NOS inhibitor, nitro-l-arginine methyl ester (L-NAME, Cayman Chemical, Europe), at a dose of 10 mg/kg and the PI3K/Akt inhibitor, wortmannin (Sigma-Aldrich, Darmstad, Germany), at a dose of 60 μg/kg as iv bolus doses, were administered 1 min before oleuropein administration [14]. The JAK2 inhibitor, AG490, was administered at a dose of 6 μg/kg/min 10 min before ischemia [15,16] **(Supplementary Figure 2)**. It must be noted that we used the same oleuropein group as in the dosage protocol in an attempt to limit the number of laboratory animals used. Hemodynamic variables such as heart rate and arterial blood pressure were monitored throughout the procedure (Power lab 4.0, AdInstruments, Australia) and were recorded at baseline, at the 20th min of ischemia, as well as at the 1st and 180th min of reperfusion. At the end of the procedures, hearts were excised, weighted, and were stained for infarct size determination.

Experimental protocol in mice. C57BL/6J male, 12-16 weeks old mice (n=38) receiving normal diet were used for the study. Mice were subjected to a 30 min period

of regional ischemia of the heart followed by a 3 h reperfusion with the following interventions and were randomly assigned to four groups: (i) Control group (n=9): no further intervention; (ii) PostC group (n=8): 3 cycles of 10 sec ischemia/reperfusion at the onset of reperfusion; (iii) Oleu group (n=10): receiving oleuropein at a dose of 350 mg/kg iv bolus at the 20th min of ischemia. The dose was selected after extrapolation between rabbits and mice; (iv) PostC + Oleu group (n=11), receiving the combination of oleuropein at a dose of 350 mg/kg bolus at the 20th min of ischemia and applying 3 cycles of 10 sec ischemia/reperfusion at the onset of reperfusion. At the end of the experiment, hearts were excised, weighted and were stained for the infarct size determination. Experimental protocol is illustrated in **Supplementary Figure 3**.

In a second set of experiments additional C57BL/6J mice (n=31) were used in order to investigate the mobilization of leukocytes after myocardial ischemia-reperfusion under the effect of oleuropein and its combination with PostC. Animals were randomized to the same four groups (n=8 for the control group; n=6 for the PostC group; n=7 for the Oleu group; n=6 for the PostC+Oleu group) as described above and subjected to myocardial ischemia for 30 min followed by 3 h of reperfusion. Blood, heart tissue from the ischemic part of the myocardium and spleen were obtained and analyzed with FACS Canto II cytometer (BD Biosciences, San Jose, CA, USA). In order to evaluate our protocol for flow cytometry analyses, 4 mice were used as a sham operated group. Mice were anesthetized and operated as described below with the difference that although the 6-0 silk suture was ligated, the polyethylene tube was not placed and ischemia was not induced. Myocardial tissue samples were used for the flow cytometric analyses.

In a third set of experiments, 24 C57BL/6J additional mice were randomized to the same groups (n=6 per each group) and subjected to myocardial ischemia for 30 min followed by 180 min of reperfusion. At the end of reperfusion, myocardial tissue samples were obtained and snap-frozen for the molecular mechanism's analyses.

2.3. Quantification of oleuropein in rabbit plasma using UHPLC-HRMS/MS

All solvents used were of liquid chromatography-mass spectrometry (LC–MS) grade and have been purchased from Fluka/Riedel-de Haën (Buchs, Switzerland). Purified oleuropein from olive leaves [95% purity assessed using analytical reversed phased (RP)- high-performance liquid chromatography (HPLC) employing a Thermo Finnigan Spectra system (column: LiChrosorb RP-18, 250 × 4.0 mm, 5 μm, elution solvent: H_2O:

acetonitrile gradient, flow:1 mL/min, UV-detection: 254 nm)] was used in this study. Plasma levels of oleuropein were analyzed using a methodology developed in our laboratory, modified and partially validated for the analysis of rabbit plasma. Briefly an ultra-high-performance liquid chromatography (UHPLC) with high-resolution mass spectrometry (HRMS) has been employed for the analysis of the circulating levels of the oleuropein in rabbit plasma, with the samples being pre-treated by solid phase extraction [17].

2.3.1. Sample preparation

Oasis HLB® Cartridges (30 mg/mL) has been employed for the clean-up of plasma samples using the following protocol: each cartridge was washed with 500 μL of methanol and subsequently equilibrated with 1000 μL of water. Following, 65 μL of plasma spiked with 10 μL of the internal standard (2,4-dinitrophenol,1μg/mL, Sigma-Aldrich, Darmstad, Germany) have been applied to the cartridge, washed with 200 μL 0.1% aq. formic acid, dried for 1 min and eluted with 1 mL acetonitrile: methanol (1:1 v/v). The eluate was evaporated to dryness by means of a GeneVac apparatus and the eluate was reconstituted to 65 μL of the initial composition of the mobile phase.

2.3.2 UHPLC-HRMS

A hybrid LTQ Orbitrap Discovery XL mass spectrometer (Thermo Scientific, Bremen, Germany), coupled to an Accela HPLC system (Thermo Scientific, Bremen, Germany) was used for the analyses. The selected reaction monitoring (SRM) methodology has been employed using the transitions referred to **Table S1**. An isolation width of 2 amu was used for the precursor ion, whereas the mass accuracy for the product ions has been set to $5x10^{-4}$ amu. An RP-C18 Hypersil Gold column (50 x 2.1 mm, 1.9 μm; Supelco, Darmstadt, Germany) was used for the separation of the analyte from the plasma substances preceded by an in-line filter. The chromatographic program used is tabulated in **Table S2.** A representative LC-HRMS chromatogram is shown in **Supplementary Figure 4.**

2.3.3. Method validation

The methodology has been partially validated for calibration model, sensitivity, matrix effect, accuracy precision and dilution integrity. Briefly, the linearity has been assessed by analyzing a spiked rabbit plasma ranging from 1-3000 ng/mL. A quadratic approximation has been applied using 1/x weighting. The calibration curve equation was the following: $\mathbf{y=0.021(\pm0.001)*X-3.3*10^{-6}(\pm4.5*10^{-7})*X^2-0.02(\pm0.02)}$ with a correlation coefficient of 0.9991.

Precision (inter- and intra-day), accuracy and the matrix effect have been evaluated at four quality control (QC) levels, namely low QC (13 ng/mL), middle QC1 (200 ng/mL), middle QC2 (750 ng/mL) and high QC (2500 ng/mL) and evaluated as relative standard deviation (RSD, %) and relative percentage error (%Er). The respective values did not exceed 12% for accuracy and 9% for precision with the exception of low QC for which it was found to be 15.4%. No matrix effect has been calculated, whereas the mean recovery was found to be 95%. The dilution integrity study did not show any statistically significant difference for the target concentrations after dilution.

2.4. Models of IRI

2.4.1 Surgical procedure of IRI in rabbits and infarct size measurement

The surgical procedure of IRI in rabbits has been previously described [11,18]. Briefly, all rabbits were anesthetized with sodium thiopental (30mg/kg, iv, Pentothal, Abbott) and orally intubated. Arterial pressure was measured via a catheter inserted in the carotid artery and a bipolar chest lead was used for continuous electrocardiographic recording. Blood pressure and heart rate were continuously monitored (Power lab 4.0, AdInstruments, Australia). The heart was exposed and a 3–0 silk suture was passed around a prominent coronary artery. Ischemia was induced by securing the suture over the artery with the aid of a small piece of soft tubing. The successful induction of ischemia was verified by visual inspection (cyanosis) and by ST elevation on the electrocardiogram. Reperfusion was achieved by unclamping the tube. For the study of infarct size, rabbits were euthanized 3h after reperfusion with a high-dose injection of sodium thiopentone (150 mg/kg, iv) and explanted hearts were cannulated and perfused with normal saline for blood removal. Then, the coronary ligature was retightened at the same site and 10 mL of green fluorescent microspheres (Duke Scientific Corp, Palo Alto, CA, USA) were infused for the separation of the normally perfused area from the area at risk. Hearts were cut into 3 mm thick sections and stained with triphenyltetrazolium chloride (TTC, Sigma-Aldrich, Darmstad, Germany) at 37°C and immersed in formaldehyde. With a wavelength of 366 nm UV light, we identified the risk zone of the infarcted myocardium. The infarcted, the risk, and the normal areas were traced onto a transparent sheet, scanned, and measured with IMAGE J software. The areas of myocardial tissue at risk and infarcted were automatically transformed into

volumes and expressed in cm^3. The percentage of infarct-to-risk area ratio (I/R, %) as well as risk-to-all area ratio (R/A, %) were calculated [19].

2.4.2 Surgical procedure of IRI in mice

The IRI model of mice was established as previously described [20]. After anesthetized by the use of an intraperitoneal (ip) combination of ketamine (100 mg/kg), xylazine (20 mg/kg), and atropine (0.6 mg/kg) [13] all mice were tracheotomized and subjected to artificial respiration using MiniVent Ventilator for Mice (Model 845, Harvard Apparatus). Briefly, after left sided thoracotomy, the heart was visualized, the pericardium was incised and the left anterior descending coronary (LAD) of mice was ligated using a 6-0 silk suture. For the reperfusion of myocardium, the ligature was released and hearts were reperfused for 3 h since at this timepoint, the recruitment of leukocytes to the heart is evident [21]. At the end of reperfusion, hearts were gently excised, while animals were under surgical anesthesia, cannulated and perfused with normal saline for blood removal. Evans Blue (Sigma-Aldrich, Darmstad, Germany) and TTC staining was performed as previously described for infarct size measurement [20]. For each heart, the percentages of I/R and R/A were calculated.

2.5. Calcium retention capacity (CRC) assay

In order to conduct the CRC assay, 10 additional C57BL/6 mice (8-10 weeks old) were used in the study. The CRC assay in the isolated murine mitochondria (0.25 mg/mL) was conducted as previously described [22–24] in order to challenge mitochondria with spikes of calcium ions to undergo permeability transition. Cyclosporine A (CsA) (1 μg/mL) was used as a positive control. Mitochondria were exposed to different concentrations of oleuropein (500 μM, 100 μM, 50 μM and 10 μM) and their CRC was determined. All experiments were performed in duplicate in three repetitions.

2.6. Determination of intracellular cyclic GMP (cGMP) levels under the effect of oleuropein

Rat aortic smooth muscle cells (RASMC) were isolated from 12-14 weeks old, male Wistar rats, five rats per isolation, as previously described [25]. Briefly, once animals were fully anesthetized with pentobarbital sodium (40 mg/kg, ip), they were exsanguinated and the thoracic aortas were removed. More than 95% of isolated cells stained positive for smooth muscle α-actin. RASMC (between passages 2 and 5) were routinely cultured in DMEM containing 4.5 g/L glucose and supplemented with 10%

fetal bovine serum and antibiotics. Confluent cells were incubated in HBSS in the presence of isobutyl methyl xanthine (IBMX;1 mM) for 5 min. Cells were then treated with 1 μM of the nitric oxide (NO) donor, sodium nitroprusside (SNP) (positive control, Sigma-Aldrich, Darmstad, Germany), and with oleuropein at various concentrations from 1μM to 100 μM. After the 15 min incubation with SNP or oleuropein, medium was rapidly aspirated, and the extraction of the cGMP was achieved by adding 200 μL of 0.1 N HCl. After 30 min, HCl extracts were collected and centrifuged at 600 g for 10 min to remove debris. The supernatants were directly analyzed for cGMP by enzyme immunoassay according to manufacturer's instructions [25,26].

2.7. Flow cytometric analysis

Single cell solutions of mice blood, spleens and hearts were prepared to be stained with fluorophore-coupled antibodies for flow cytometric analyses. Blood was drawn via cardiac puncture and collected in tubes containing ACD (citrate-dextrose solution) as anticoagulant. The red blood cells were osmotically lysed with lysing solution containing NH_4Cl for 5 min at room temperature. The cell suspension was then centrifuged at 300 g for 5 min at 4°C, the supernatant was discarded, and the pellet was washed with cold PBS supplemented with 0.5% (w/v) BSA.

Extracted spleens were placed on a 70μm cell strainer and were crushed by mechanical pressure using a syringe's plastic plunger. Collected cells were washed with PBS supplemented with 2% FCS, and cell suspension was centrifuged at 300 g for 5 min at 4°C. The pellet was then lysed with lysing solution for 3 min at room temperature, centrifuged at 300 g for 6 min at 4°C, and was washed with PBS containing 2% FCS. The hearts were excised and digested with a cocktail of collagenase type I 1 mg/mL (Sigma-Aldrich, Darmstad, Germany), DNase I 50 U/mL (ABM, Canada) and hyaluronidase 300 μg/ mL (Sigma-Aldrich, Darmstad, Germany), in PBS containing 20 mM HEPES at 37°C for 60 min. The cell suspension was passed through a 70 μm strainer (Corning Inc., New York, USA) and lysed with lysing solution for 3 min. The cell suspension was centrifuged at 400 g for 10 min. After discarding the supernatant, the pellet was resuspended with cold PBS 2% FCS.

The single cell suspension obtained from any tissue were then evaluated for cell density and cell viability with Trypan-Blue staining on a hemocytometer. The minimum cut-off point set for cell viability was 90% fur further processing. Cells were then incubated with Fc-block anti-CD16/32 mAb (BD Pharmingen, San Diego, CA, USA) for 10 min at 4°C to prevent unspecific binding and then stained with anti-CD11b-APC

(clone M1/70, BD), anti-Ly-6G-PE (clone 1A8, BD) and anti-Ly-6C-FITC (clone AL-21, BD) for 30 min at 4°C. Stained cells were centrifuged at 300 g for 5 min at 4°C, washed twice with PBS 0.5% FCS and stained with 7-AAD (Biolegend, San Diego, CA, USA) for the exclusion of nonviable cells. The acquisition of all samples was performed on a BD FACSCantoII cytometer with a FSC threshold set at 10.000 and data analysis was conducted with BD FACSDiva software. A minimum of 10^6 total events were acquired for each heart sample and a total of 10^5 events were acquired for all blood and spleen samples. The monocytes/macrophages were identified as $CD11b^+$/ $Ly\text{-}6C^{high/low}$/LyG^- and the neutrophils were identified as $CD11b^+$ $Ly\text{-}6G^+$ following the gating strategy illustrated in **Supplementary Figure 5.**

2.8. Simulated ischemia (SI)/ reoxygenation protocol

Male Wistar rats (n=4) weighing between 250 and 300 g were used in this assay. Animals were anesthetized with sodium pentothione (100 mg/kg, ip) and 30 min after dissociation, ventricular cardiac myocytes were isolated by cardiac retrograde aortic perfusion and collagenase treatment as previously described [27]. Cells were finally resuspended in Krebs incubation medium. Preparations were considered successful when the yield of rod-shaped cells was more than 70%. To simulate the internal environment during local ischemia, a modified Krebs buffer (SI buffer) consisting of 137 mM NaCl, 3.8 mM KCl, 0.49 mM $MgCl_2$, 0.9 mM $CaCl_2$ and 4.0 mM Hepes pH 6.5, supplemented with 10 mM 2-deoxyglucose, 20 mM sodium lactate, 1 mM sodium dithionite and 12 mM KCl, was used. Ischemia was induced by exposing cardiomyocytes to SI buffer (under N_2, 37 °C) for 30 min. Following ischemia, SI buffer was washed off and the cells were reperfused with Krebs medium for 2 h. Oleuropein at various concentrations from 50 μM to 200 μM was added at the 20^{th} min of SI and was also present through re-oxygenation. The doses of the *in vitro* assay were chosen based on our results for the plasma concentration of oleuropein which reaches up to 140 μM. Cell viability was determined after 2 h of re-oxygenation using the 3-(4,5-dimethylthiazol2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay which assesses mitochondrial activity in living cells [27].

2.9. Real-Time (RT) PCR

RT PCR performed with the CFX96 Real-Time PCR Detection System (Bio-Rad, Munich, Germany) was conducted in murine pulverized hearts. RNA, isolated by the standardized Trizol protocol, was reverse-transcribed to cDNA using high-capacity cDNA reverse transcription kit (Takara). The primer pairs presented in **Table S3** were designed (Primer-Blast, NCBI, NIH) and used in order to detect mRNA expression of C-C Motif Chemokine Ligand 2 (CCL2), interleukin (IL)-1β, IL-8, IL-6, E-selectin, intercellular adhesion molecule-1 (ICAM-1), macrophage colony-stimulating factor (M-CSF), granulocyte colony-stimulating factor (G-CSF), stromal cell-derived factor-1 (SDF-1), NADPH oxidase-2 (NOX-2) (Eurofins Genomics AT, GmbH). Samples were analyzed by the standardized SYBR®Green method (Kapa Biosystems) according to the manufacturer's instructions. The comparative delta CT method was used for relative mRNA quantification. Gene expression was normalized to the endogenous control (GAPDH mRNA), and the expression of the target gene mRNA of each sample was expressed relative to that of the control [28].

2.10 Western Blot analysis in myocardial tissue

Western Blot analysis in myocardial tissue samples obtained from the ischemic part at 3 h of reperfusion was performed as described previously [20,29]. Samples were pulverized in liquid nitrogen and dry ice and were homogenized using lysis solution (25 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% NP- 40, 5% glycerol, pH 7.4). Lowry method was employed for the determination of the protein content. An equal amount of protein (40 or 50 μg) was used to prepare the western blot samples by mixing with Dave's buffer**.** The samples were boiled at 100°C for 5 min and stored at −80ºC. The samples were then separated by SDS-polyacrylamide gel electrophoresis 7.5–15% and transferred onto a polyvinylidene difluoride membrane (PVDF). After blocking with 5% non-fat dry milk, membranes were incubated overnight at 4°C with the following primary antibodies, heme oxygenase 1 (HO-1) (dilution 1:1000, Rabbit mAb #5853) superoxide dismutase-2 (SOD-2) (dilution 1:4000, Rabbit mAb #13141), nuclear factor (erythroid-derived 2)-like 2 (Nrf-2) (dilution 1:1000, Rabbit mAb #12721), gp91-phox (dilution 1:500 #130543, Santa Cruz Biotechnology, USA), phospho-Akt (Ser473) (dilution 1:1000, Rabbit mAb #4060), Akt (40D4) (dilution 1:1000, Mouse mAb #2920), phospho-Stat3 (Tyr705) (3E2) (dilution 1:1000, Mouse mAb #9138), Stat3 (124H6) (dilution 1:1000, Mouse mAb #2920), Phospho-p44/42 MAPK (Erk1/2)

(Thr202/Tyr204) (E10) (dilution 1:2000, Mouse mAb #9106), p44/42 MAPK (Erk1/2) (L34F12) (dilution 1:1000, Mouse mAb #4696), GAPDH (14C10) (dilution 1:2500 Rabbit mAb #2118), α-Actinin (D6F6) (dilution 1:4000, Rabbit mAb #6487) and β-tubulin (dilution 1:1000, Rabbit mAb #2146)**.** Unless otherwise indicated**,** all antibodies were purchased from Cell Signaling Technology (CST, USA). Membranes were then incubated with secondary antibodies for 2 h at room temperature (Anti-rabbit IgG, HRP-linked Antibody #7074 and Anti-mouse IgG, HRP-linked Antibody #7076) and developed using the GE Healthcare ECL Western Blotting Detection Reagents (Thermo Scientific Technologies, Thermo Fisher Scientific Inc., Waltham). The proteins β–tubulin, a-actinin and GAPDH were used as loading controls [20,22].

2.11 Statistical analysis

Statistical analysis was performed using the GraphPad Prism 8 software (Graph Pad Software, Inc.). All the results were plotted in graphs as mean ± standard error (SEM) of the mean values. Comparisons of numeric variables between the groups (more than two) were conducted using One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Comparisons of numeric variables between two groups were conducted using unpaired two-tailed Student's t-test. Hemodynamic data were analyzed using a two-way repeated measures ANOVA with Tukey's post-test. The cut-off for statistical significance was set at $P< 0.05$.

Results

Acute administration of oleuropein 10 min prior to reperfusion reduced infarct size in a rabbit model of myocardial IRI

We examined the effect of oleuropein on reducing the myocardial infarct size after administration at the 20th min of ischemia at two different doses, 50 mg/kg and 100 mg/kg. Oleuropein at the dose of 50 mg/kg caused no significant change in infarct size compared to the control group. At the dose of 100 mg/kg, oleuropein significantly reduced the infarct size from 48.6±2.4 % to 20.0±2.8 % (***P<0.001) in comparison with the control group. The application of PostC was as potent as oleuropein treatment in terms of infarct size reduction (26.7±2.7%, ***P<0.001 vs. Control group), indicating the strong cardioprotective effect of oleuropein **(Supplementary Figure 6)**. A highly sensitive, rapid and specific UHPLC-HRMS/MS, method was developed and validated in order to quantify the oleuropein levels in plasma rabbits' samples during reperfusion. The results presented in the **Table S4** indicate a fast elimination of oleuropein after its administration in comparable rates for both doses tested. Interestingly, by doubling the dose there is a striking 20-24-fold increase at the circulating levels of oleuropein indicating a non-linear pharmacokinetic profile [30]. This non-linear effect could be attributed to the degradation of oleuropein from corresponding enzymes e.g. esterases. In high administered dose (100 mg/kg), the enzyme systems might be saturated and oleuropein could not be metabolized, assuming Michaelis-Menten kinetics of oleuropein degradation, leaving a high amount of the substance intact and thus circulating. It should be noted that oleuropein does not degrade chemically even to the stomach pH [31] and it metabolizes extensively affording more than 10 degradation products [32]. Given that the infarct limiting effect was observed in the higher dose that also resulted in high concentration of oleuropein in plasma, we chose the dose of 100 mg/kg for further evaluation of the cardioprotective effects of oleuropein. In addition, the plasma concentration of oleuropein reached approximately 140 μM at the 1st min of reperfusion, a factor that was taken into consideration for our further *in vitro* assays with oleuropein.

Oleuropein administration enhances the beneficial effect of PostC on limiting the infarct size in rabbits independently of the classic cardioprotective pathways

In order to explore if the cardioprotective effect of oleuropein is mediated through the classic cardioprotective pathways such as the RISK, cGMP/PKG and SAFE pathways, we measured infarct size in animals administered with oleuropein in combination with the inhibitors wortmannin, L-NAME and AG-490. We found that none of the inhibitors had an influence on the infarct-limiting effect of oleuropein **(Figure 1A)**. Pharmacological inhibition of PI3K with wortmannin, did not abolish the cardioprotective effect of oleuropein (I/R%: 23.1±2.3% for the Oleu+Wort group vs. 20.0±2.8% for Oleu group). Similarly, the pharmacological inhibition of NOS with L-NAME, did not alter the effect of Oleu on infarct size (I/R%: 22.4±3.4 % for the Oleu+L-NAME group). Last, the administration of JAK-2 inhibitor, AG490, had no effect on the cardioprotective properties of oleuropein (I/R%: 16.5±0.7% for the Oleu+AG490) **(Figure 1A)**. The doses of the inhibitors were selected according to the literature and according to our previous works, showing that at these doses' inhibitors specifically abrogated infarct size reduction [14,15,33]. R/A percentage did not differ between groups verifying the reproducibility of our surgical maneuvers **(Supplementary Figure 7)**. These results indicate that oleuropein has potential cardioprotective effects by reducing the infarct size with a mechanism independent of the RISK, SAFE and cGMP/PKG pathways. These findings urged us to investigate whether oleuropein could further protect the ischemic heart on top of the application of PostC. Therefore, we included an additional group in our experimental protocol in which oleuropein was administered in combination with PostC maneuver. Importantly, the combination of PostC and oleuropein significantly decreased the infarct size in comparison with PostC alone (I/R%: 14.6±0.9% vs. 26.7±2.7 %, #P<0.01), indicating that this combination represents a potent multitarget therapy against IRI **(Figure 1A)**. We must point out that the I/R% was similar between Oleu and PostC+ Oleu groups. The effect of PostC, oleuropein alone or in combination with the inhibitors as well as PostC+ Oleu on heart rate and on mean arterial blood pressure (MAP) in rabbits is shown on **Table S5**. Recordings of MAP at the 20th min of ischemia, at the 1st min of reperfusion and at the end of the reperfusion period showed no significant changes in all groups, whereas heart rate was significantly decreased in Oleu+L-NAME and Oleu+AG-490 group in comparison with the oleuropein group only at the 20th min of ischemia (258±17 in Oleu+ L-NAME group (**P<0.01) and 266±9 in Oleu+AG-490 group (*P<0.05) vs. 346±42 for the Oleu group) probably due to an acute effect of the administration of the inhibitors.

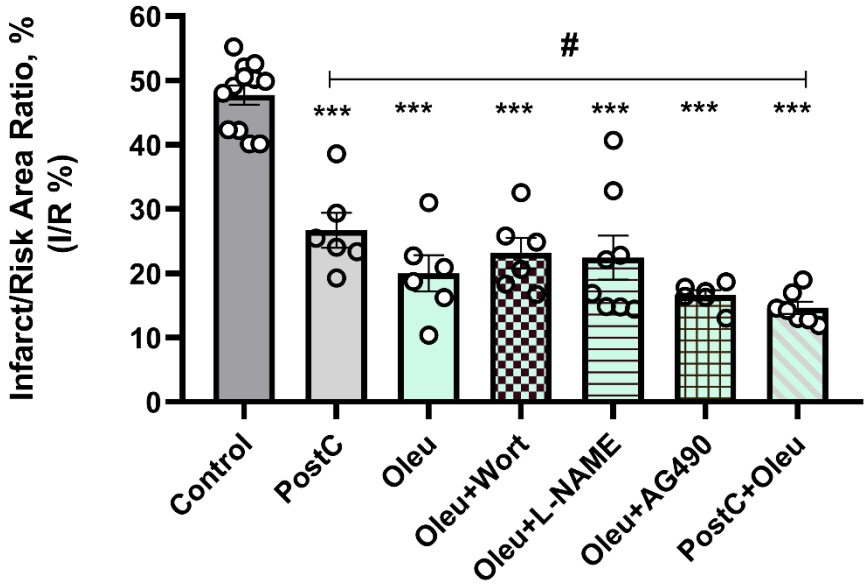
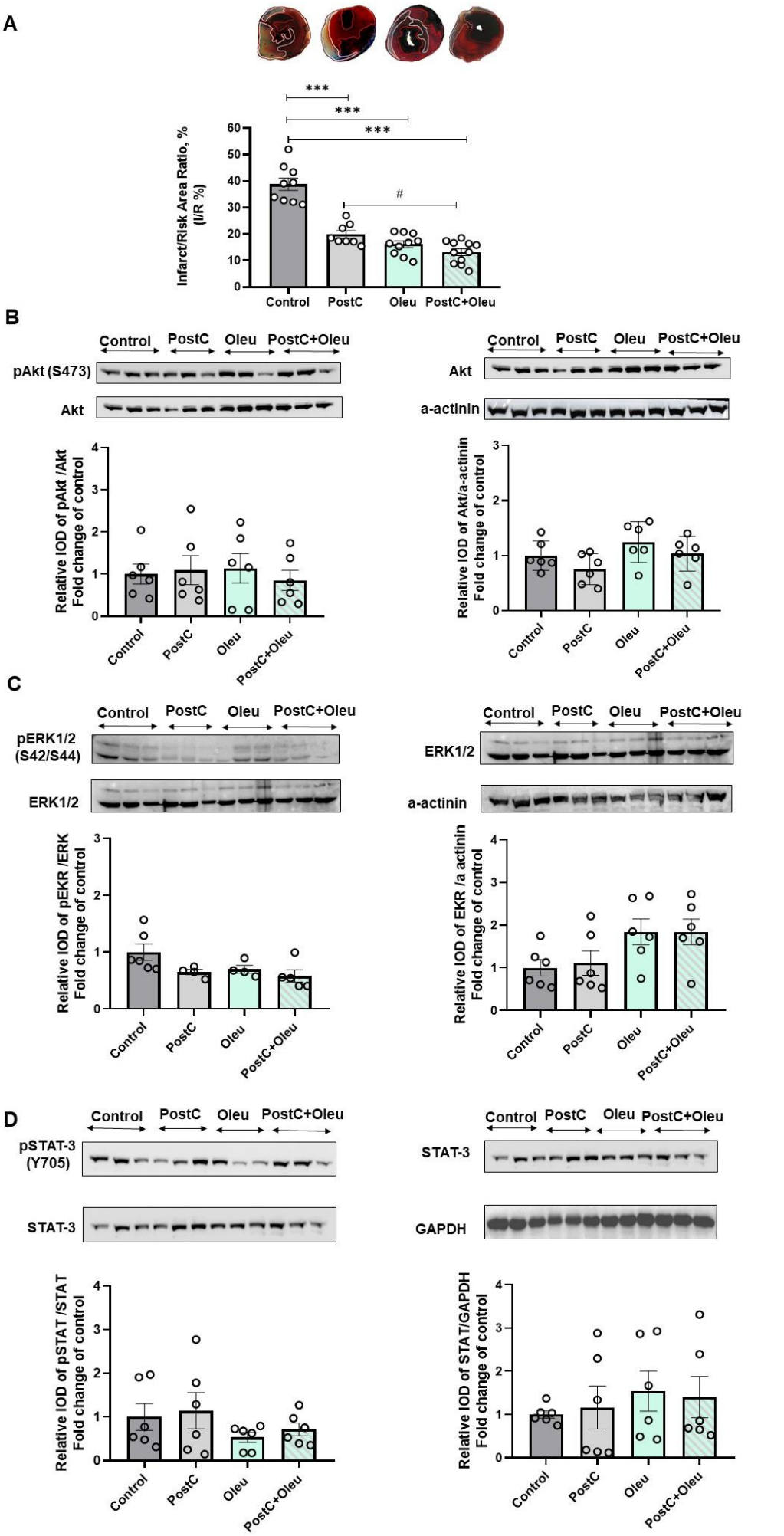


Figure 1. Effect of oleuropein and PostC on limiting the infarct size in a rabbit model of IRI. The role of the classic cardioprotective pathways in oleuropein's cardioprotection. Bars of percentage of I/R in different study groups of rabbits subjected to myocardial IRI. Control (n=12); PostC (n=6); Oleu (n=6); Oleu + PostC (n=7); Oleu + L-NAME (n=6); Oleu + Wortmannin (n=8); Oleu + AG490 (n=6). The values for each animal are presented as a scatter plot. Data are expressed as means ± SEM. Comparison between groups were performed using one-way ANOVA and Tukey post hoc test. All groups vs. Control group, ***P<0.001 and PostC+ Oleu group vs. PostC group, $^{\#}$P<0.01.

Oleuropein administration in combination with PostC is the most effective strategy in reducing infarct size in mice subjected to myocardial IRI independently of RISK and SAFE pathways

To further confirm our previous finding, we evaluated the infarct size limiting effects of oleuropein and its combination with PostC in mice. Oleuropein was found to have a similar cardioprotective effect against myocardial IRI in mice as it was shown in rabbits, since we found a significant reduction of 58% in infarct size of the Oleu group when compared to controls (I/R%: 16.1±1.2% vs. 38.8±2.3%, ***P < 0.001, **Figure 2A)**. Similar to the rabbit's protocol, importantly, oleuropein treatment combined with PostC significantly reduced the infarct size in a more effective way compared to PostC

alone (I/R%: 13.1±1.28% vs. 19.9± 1.4%, #P<0.05, **Figure 2A)**. All groups had similar R/A, % myocardium areas (59.9 ± 1.6% in Control, 54.9 ± 3.4% in PostC, 59.5±2.9% in Oleu and 55.0 ± 2.4% in PostC + Oleu, P=NS, **Supplementary Figure 8)**. Therefore, our results indicate that the acute administration of oleuropein shows an enhanced effect on the top of PostC in limiting infarct size in mice. Moreover, in order to investigate whether oleuropein's, PostC and their combination cardioprotective effect is dependent on RISK and SAFE pathways activation in mice, we investigated the phosphorylation and expression of Akt and ERK1/2 as significant signaling molecules of the RISK pathway and STAT-3 as a significant signaling molecule of the SAFE pathway [34] at 3h of reperfusion. All proteins expression and phosphorylation were similar between the treatment and control groups (P=NS, **Figure 2B, 2C, 2D).**


A
***
***
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#
Infarct/Risk Area Ratio, % (I/R %)
Control
PostC
Oleu
PostC+Oleu
B
pAkt (S473)
Akt
Relative IOD of pAkt /Akt Fold change of control
Akt
a-actinin
Relative IOD of Akt/a-actinin Fold change of control
C
pERK1/2 (S42/S44)
ERK1/2
Relative IOD of pEKR /ERK Fold change of control
ERK1/2
a-actinin
Relative IOD of EKR /a actinin Fold change of control
D
pSTAT-3 (Y705)
STAT-3
Relative IOD of pSTAT /STAT Fold change of control
STAT-3
GAPDH
Relative IOD of STAT/GAPDH Fold change of control

Figure 2. Oleuropein, PostC and their combination limit the infarct size in a murine model of IRI independently of RISK and SAFE pathways at 3 h of reperfusion. A. Bars of percentage of I/R in different study groups of mice subjected to myocardial IRI. Control (n=9); PostC (n=8); Oleu (n=10); PostC + Oleu (n=11). The values for each animal are presented as a scatter plot. All groups vs. Control group, ***P<0.001 and PostC+Oleu group vs. PostC group, $^{\#}$P<0.05. Representative images of the myocardial infarcts of the murine model of IRI are depicted (blue/dark is due to Evans Blue staining; the red line accounts for the area at risk; the white line is denoted as the infarcted tissue). **B.** Representative Western Blots of p(S473)Akt/Akt, and Akt/a-actinin. Relative densitometric graphs of p(S473)Akt/Akt and Akt/a-actinin at 3h of reperfusion after normalization to total protein (P=NS). **C.** Representative Western Blots of pERK1/2 (p-p42/44) and ERK1/2 (p-p42/44) and relative densitometric graphs at 3 h of reperfusion after normalization to total protein are presented (P=NS). **D.** Representative Western Blots of p(Y705)STAT-3/STAT-3 and STAT-3/GAPDH and relative densitometric graphs at 3 h of reperfusion after normalization to total protein are presented (P=NS). Dots represent biological replicates (n=6 per group). Data are expressed as means ± SEM. Comparison between groups were performed using one-way ANOVA and Tukey post hoc test.

Oleuropein does not suppress calcium induced MPTP opening on mice mitochondria

The inhibition of the MPTP opening at the onset of reperfusion has been shown to underlie the cardioprotection elicited by PostC [35]. The cardioprotective RISK, SAFE and cGMP/PKG pathways seem to converge to mitochondria and prevent MPTP opening [36]. Therefore, subsequently we investigated the effect of oleuropein on calcium induced MPTP opening. We measured CRC in isolated mice heart mitochondria treated with oleuropein in various concentrations ranging between 500 μM-10 μM and cyclosporin A CsA (1 μg/mL), used as a positive control. As expected, we found that CsA increased mitochondrial CRC (**P<0.01 vs. all other study groups) whereas oleuropein had no effect on mitochondrial susceptibility to permeability transition (P=NS) **(Figure 3).** These findings suggest that oleuropein does not act as MPTP desensitizer.

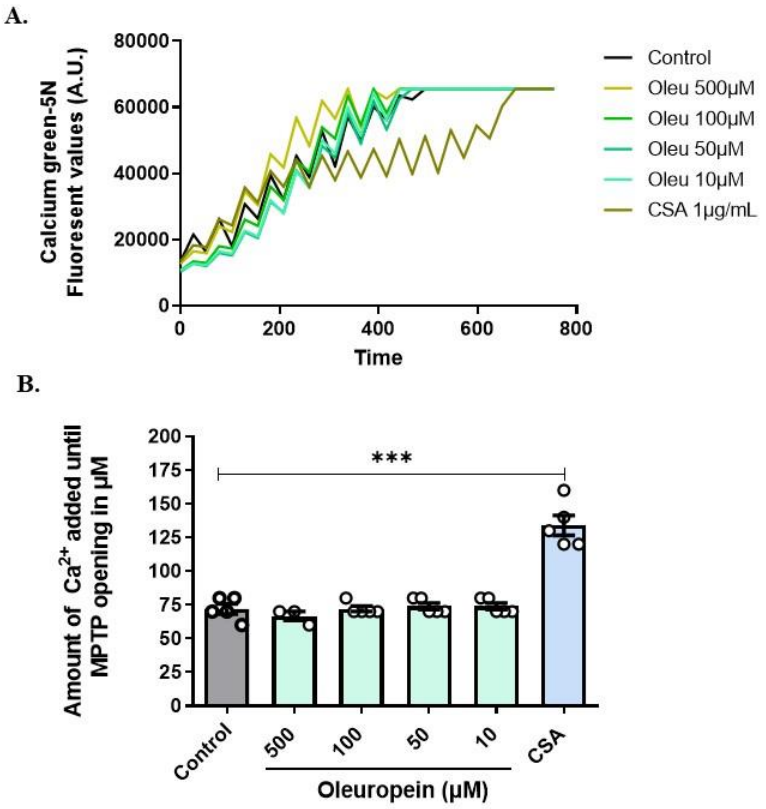

Figure 3. **Effects of oleuropein on MPTP opening**. **A.** Fluorescence tracing of the extra-mitochondrial calcium. **B.** Calcium uptake (µM) graph in the isolated mitochondria. Bars present means ± SEM. Comparison between groups were performed using one-way ANOVA and Tukey post hoc test. CSA group vs. Control group, ***P<0.001. CsA; Cyclosporin A

Oleuropein does not induce cGMP accumulation

In order to confirm that the cardioprotective mechanism of oleuropein is independent of the cGMP/PKG pathway, we determined the effects of oleuropein on cGMP production. RASMC were treated with different concentrations of oleuropein (from 1 µM to 100 µM) in the presence of phosphodiesterase (PDE) inhibition (IBMX). The exposure of cells to 1 µM SNP in the presence of IBMX significantly increased the baseline levels of cyclic GMP. However, intracellular cGMP levels, measured in cell lysates, did not increase in response to oleuropein at concentrations ranged from 1-100 µM **(Figure 4).**

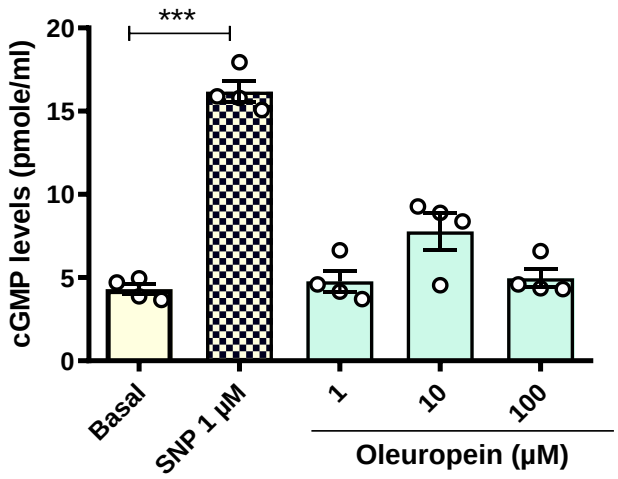

Figure 4. Effect of different concentrations of oleuropein on cGMP accumulation. Bars present mean ±SEM. Comparison between groups were performed using one-way ANOVA and Tukey post hoc test. SNP vs. basal levels, ***P<0.001 SNP: sodium nitroprusside

Oleuropein, PostC and their combination reduce inflammatory monocyte infiltration into the heart

Myocardial IRI elicits a strong inflammatory response and profound recruitment of innate immune cells, particularly neutrophils and monocytes, while oleuropein has been proposed to possess immunomodulatory potency [37–39]. Taking into account, that efficient reduction of immune cells and decreased accumulation in the infarct area can reduce the infarct size [20,23,40], we explored the possible synergistic role of oleuropein and PostC on the early phase of inflammation. In particular, we examined monocyte infiltration which bears a pronounced role in IRI in the murine models of 3-24h reperfusion [21,41].

We induced IRI in C57BL/6 mice and analyzed single-cell suspensions of digested IRI heart at 3 h of reperfusion. Using flow cytometry analysis, we showed that the recruitment of myeloid cells ($CD11b^{+}$) was significantly elevated after IRI in comparison with sham-operated animals (*P<0.05) **(Figure 5A)**. After confirming the cell recruitment in our model, we investigated the effect of oleuropein treatment and PostC on the recruitment of inflammatory monocytes ($Ly6C^{high}$) and neutrophils. After 3h of reperfusion, hearts' infiltration with $Ly6C^{high}$ inflammatory monocytes was significantly decreased in all intervention groups in comparison with control group (PostC: 2.9±0.8 %, Oleu: 3.1±0.6 %, PostC+Oleu: 2.9±0.5 % vs. 6.8±1.0% for Control

group, *P<0.01). Neutrophils levels were similar between groups suggesting that Ly6C^{high} inflammatory monocytes are the dominated leukocyte population in 3h of reperfusion **(Figure 5B)**. Oleuropein, PostC and their combination reduced significantly the number of Ly6C^{high} inflammatory monocytes in blood in comparison with control group [PostC: 2.7±0.5 % (*P<0.01), Oleu: 3.2±0.5 % (*P<0.05), PostC+Oleu: 2.2±0.7 % (**P<0.01) vs. 5.6±0.4 % for Control group] whereas neutrophils were found similar between groups **(Figure 5C)**. Spleen leukocyte populations remained unaffected (P=NS) **(Figure 5D)**. This is the first study showing that oleuropein, PostC and their combination have a direct effect on modulating inflammatory monocyte population to the infarcted area indicating a correlation between infarct size and monocyte infiltration. The decreased number of inflammatory monocytes to the infarcted area is mostly attributed to the decreased number in blood.

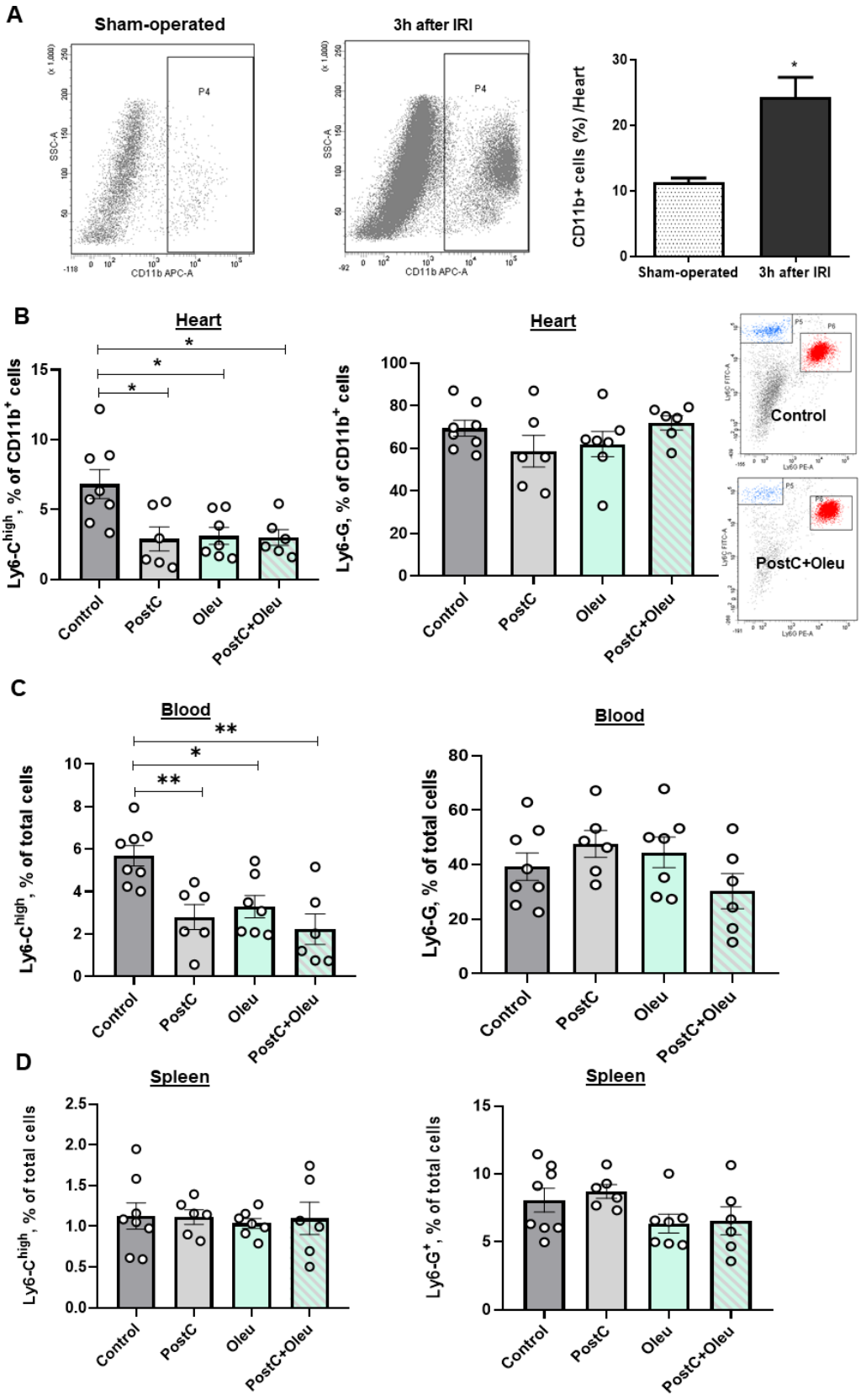

Figure 5. Effect of oleuropein and PostC on myeloid cells, inflammatory monocytes and neutrophils in heart, blood and splenic tissue determined with flow cytometry analyses. A. Flow cytometric illustrations and graphs of the percentage of myeloid cells ($CD11b^+$) to the infarcted myocardium in sham-operated group and in

control group of mice subjected to myocardial IRI. Comparisons of numeric variables between the two groups were conducted using unpaired two-tailed Student's t-test. *P<0.05 vs. sham-operated group. **B.** Bars of the percentage of Ly6C^{high} monocytes/ macrophages and the percentage of neutrophils in heart after 3h of reperfusion. Representative flow cytometric illustrations of neutrophils and monocytes/macrophages. All groups vs. control group, *P<0.05. **C.** Bars of the percentage of Ly6C^{high} monocytes/ macrophages and the percentage of neutrophils in blood after 3h of reperfusion. PostC and PostC+Oleu vs. Control group, **P<0.01 and Oleu vs. Control group, *P<0.05. **D.** Bars of the percentage of Ly6C^{high} monocytes/ macrophages and the percentage of neutrophils in spleen after 3h of reperfusion. Control (n=6); PostC (n=6); Oleu (n=6); PostC + Oleu (n=6). The values for each animal are presented as a scatter plot. Data are expressed as the mean ± SEM. Comparison between groups were performed using one-way ANOVA and Tukey post hoc test. IRI: Ischemia-reperfusion injury

Effect of oleuropein and PostC on gene expression levels of pro-inflammatory mediators after myocardial IRI in mice

Next, we investigated if the suppression of monocyte infiltration by oleuropein and PostC is associated with the decrease of different pro-inflammatory mediators. To this end, we observed the gene expression of several cytokines, chemokines and adhesion molecules known to play critical role in increasing the pro-inflammatory response to acute myocardial IRI by mediating the recruitment of inflammatory cells into the infarct zone [42]. From the pro-inflammatory mediators tested in this study, only myocardial mRNA levels of IL-6 and E-selectin were significantly decreased in the oleuropein group compared to Control and PostC groups (*P<0.05) **(Figure 6)**.

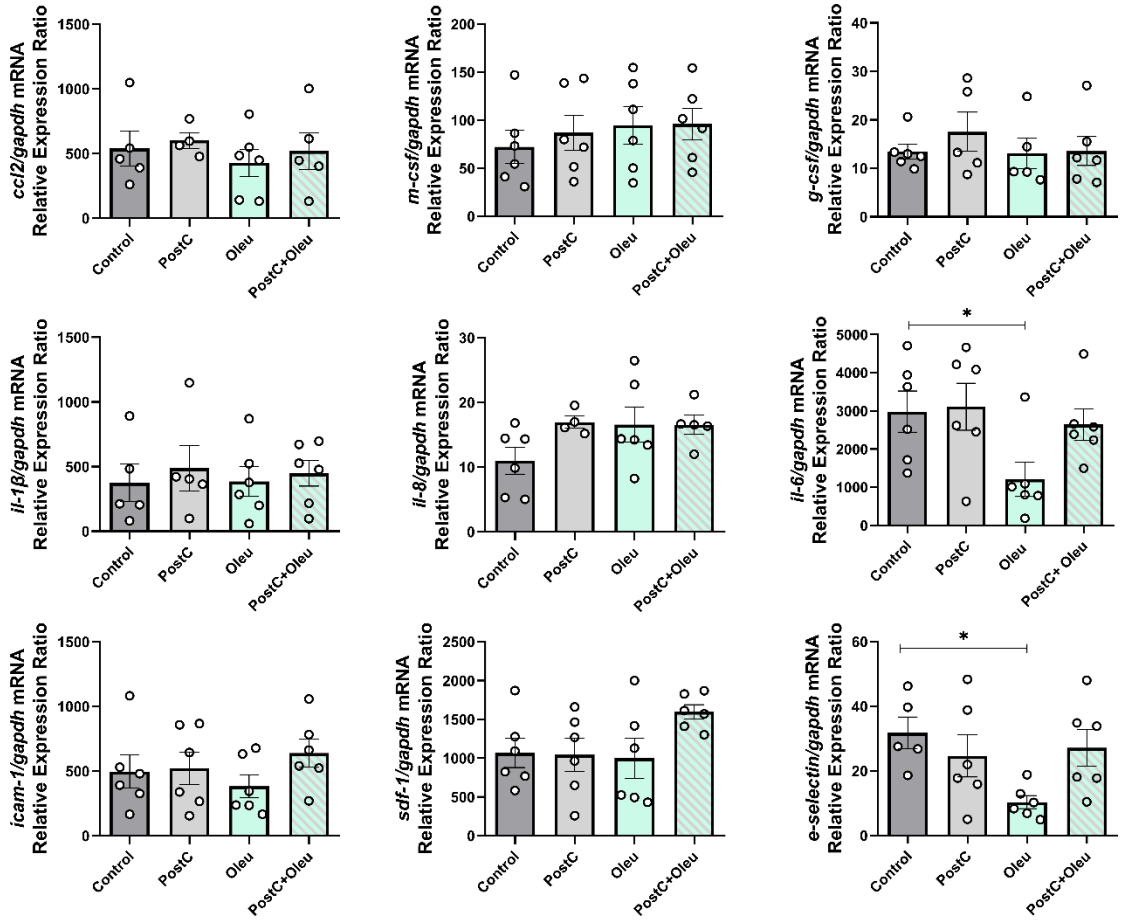

Figure 6. Effect of oleuropein and PostC on the mRNA expression of pro-inflammatory mediators' genes (*ccl2, m-csf, g-csf, il1-β, il-8, il-6, icam-1, sdf-1, e-selectin*) in the myocardium determined using real-time PCR. All results were normalized against GAPDH. The values for each animal are presented as a scatter plot. Data are expressed as the mean ± SEM. Comparison between groups were performed using one-way ANOVA and Tukey post hoc test. Oleuropein vs. control group, *P<0.05. *ccl2*: C-C motif chemokine ligand 2; *gapdh*: glyceraldehyde 3-phosphate dehydrogenase, *g-csf*: granulocyte colony-stimulating factor; *icam-1*: intercellular adhesion molecule-1; *il*: interleukin; *m-csf*: macrophage colony-stimulating factor; *sdf-1*: stromal cell-derived factor-1

Oleuropein ameliorates cell death *in vitro* after SI/reoxygenation

Concerning the enhanced effect of oleuropein on top of PostC in reducing MI, we hypothesized that these therapies reduce cardiomyocyte death via different intracellular cascades. Therefore, we examined the effect of oleuropein in adult rat cardiomyocytes *in vitro* using MTT assay, in order to investigate if the limitation of infarct size is mainly due to oleuropein's protective effect on cardiomyocytes. As expected, cell viability was reduced after cardiomyocytes were subjected to SI/R injury. Treatment with oleuropein

(50-200 μM) significantly increased the cell viability in a dose dependent manner, as shown in **Figure 7**. It must be noted that the lowest dose of oleuropein (50μM) had significantly decreased viability compared to control group ($^{\$}P<0.01$, **Figure 7**). The results clearly illustrate the ability of oleuropein to protect adult rat cardiomyocytes against SI/reoxygenation injury and indicate that oleuropein has a direct effect on cardiomyocytes.

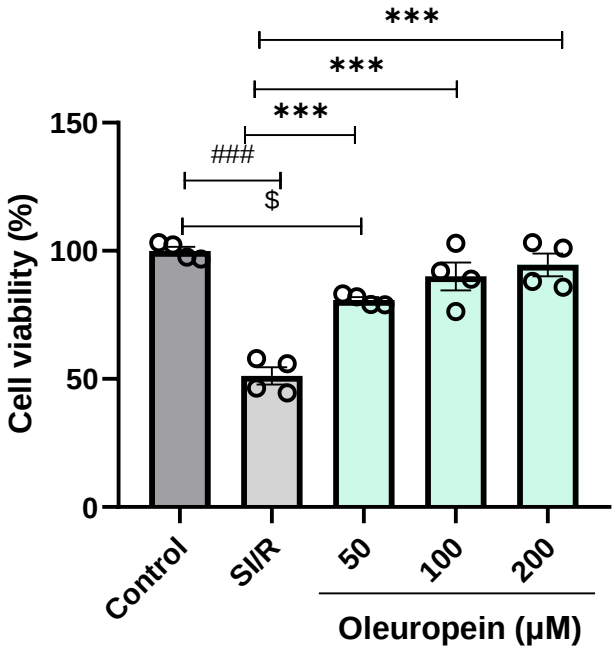

Figure 7. Oleuropein ameliorates adult rat cardiomyocytes death *in vitro* after SI/reoxygenation. Cellular viability after SI/reoxygenation was determined by MTT dye reduction. Data are expressed as the mean ± SEM (n=4). Comparison between groups were performed using one-way ANOVA and Tukey post hoc test. SI/R vs. control, $^{\#\#\#}P<0.001$, oleuropein at 50μM vs. control, $^{\$}P<0.01$ and oleuropein (50, 100, 200 μM) vs. SI/R, ***P<0.001. SI/R: simulated ischemia/reoxygenation.

The Nrf-2 cytoprotective signaling plays key role in the cardioprotection induced by oleuropein in combination with PostC

Nrf-2 is a crucial regulator for the upregulation of genes affecting cytoprotective systems and Nrf-2-mediated signaling plays a key role in the innate immune/inflammatory pathway regulating pro-inflammatory biomarkers, including IL-6 and E-selectin [43,44]. Due to the importance of Nrf-2 signaling in combating myocardial damage from IRI and its consequences, the protein expression of Nrf-2 and its downstream targets SOD-2 and HO-1 were evaluated. Oleuropein and the

combination of oleuropein with PostC upregulated the expression of Nrf-2 (*P<0.05 vs. Control group). Also, the expression of Nrf-2 is significantly increased in oleuropein group in comparison with PostC (P<0.05) indicating the additive cardioprotective effect of oleuropein over PostC **(Figure 8A)**. We also observed significant increased expression of HO-1 in mice treated with both oleuropein and PostC (*P<0.05 vs. Control group) **(Figure 8B)**. Importantly, oleuropein as well as oleuropein in combination with PostC significantly increased the protein expression of SOD-2 compared to the control group (*P<0.05 vs. Control group) **(Figure 8C)**. Nrf-2 depletion has been reported to upregulate Nox-2 and Nox-4 isoform of the NADPH oxidase system which is widely recognized as a major contributor in intracellular ROS homeostasis [45]. Since abrogation of Nox-2 but not Nox-4 improves myocardial IRI [46], we evaluated the mRNA and protein expression of Nox-2. In fact, our results demonstrate that both oleuropein and the combination of oleuropein with PostC significantly reduced the expression of *nox-2* gene (**P<0.001, *P<0.05 vs. Control group) and NOX-2 protein levels **(Figure 8D and 8E)**. PostC alone did not decrease any of the above-mentioned redox molecules after 3h of reperfusion. These results suggest that oleuropein attenuates cardiac IRI via the Nrf-2 cytoprotective signaling and the downstream regulation of the cellular and mitochondrial antioxidant defense.

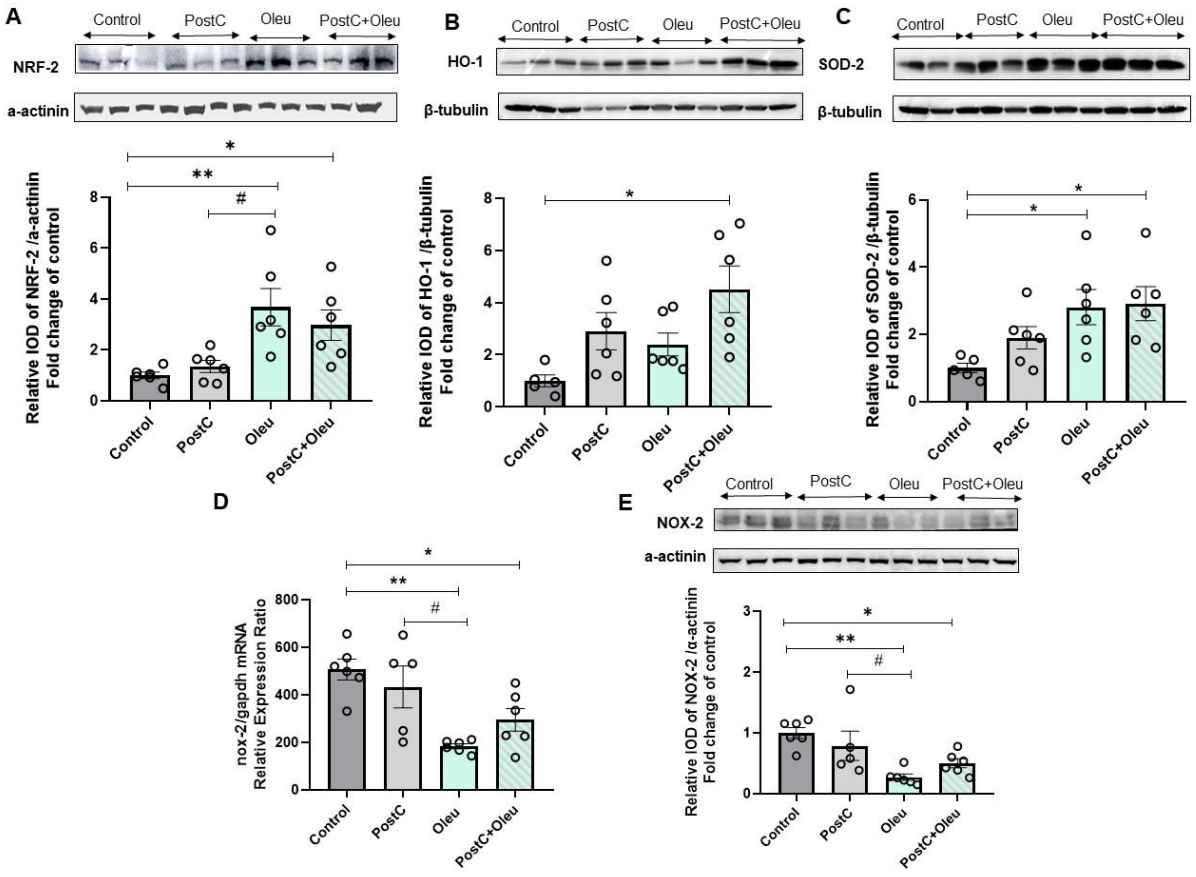

Figure 8. Effect of oleuropein, PostC and their combination on redox signaling molecules. A. Representative western blot and bar graph of Nrf-2/α-actinin of mice subjected to myocardial IRI (Oleu group vs. Control, **P<0.01, PostC+Oleu group vs. Control, *P<0.05) and Oleu group vs. PostC, $^{\#}$P<0.05, **B.** Representative western blot and bar graph of HO-1/ β-tubulin of mice subjected to myocardial IRI (PostC+Oleu group vs. Control, *P<0.05), **C.** Representative western blot and bar graph of SOD-2/β-tubulin of mice subjected to myocardial IRI (Oleu group and PostC+Oleu group vs. Control, *P<0.05) **D.** mRNA expression of *nox-2* gene (Oleu group vs. Control, *P<0.001 and PostC+Oleu group vs. Control, *P<0.05, PostC group vs. Oleu, $^{\#}$P<0.05) **E.** Representative western blot and bar graph of Nrf-2/α-actinin of mice subjected to myocardial IRI (Oleu group vs. Control, *P<0.001 and PostC+Oleu group vs. Control, *P<0.05, PostC group vs. Oleu, $^{\#}$P<0.05). Control (n=6); PostC (n=6); Oleu (n=6); PostC + Oleu (n=6). Data are expressed as the mean ± SEM. Comparison between groups were performed using one-way ANOVA and Tukey post hoc test.

Discussion

In the present study, using *in vivo* rabbit and murine models of IRI, we evaluated the cardioprotective effects of oleuropein and explored the mechanisms involved. Our findings demonstrate that acute administration of oleuropein reduces infarct size in rabbits and mice independently of RISK, SAFE and cGMP/PKG pathways and it conferred additional protection when combined with PostC. Oleuropein prevented cardiomyocyte cell death without inhibiting directly the MPTP opening, which implies that novel intracellular pathways are key mediators for the cardioprotective effect of this bioactive natural product. Oleuropein and its combination with PostC significantly attenuated IRI induced pro-inflammatory monocyte infiltration concomitantly with an increase in Nrf-2 transcriptional factor and SOD-2 expression. To the best of our knowledge, this is the first study demonstrating that acute oleuropein treatment *in vivo* is cardioprotective on top of PostC by modulating the oxidative defense in cardiomyocytes via Nrf-2 pathway.

Over the last decades, olive oil has been exploited not only as a nutritional but also as a pharmacological agent [47]. The beneficial effects of virgin olive oil are attributed to the content of polyphenols and especially oleuropein which is present in *Olea europaea* products in a high concentration exerting antioxidant, anti-thrombotic, anti-atherogenic, and anti-inflammatory effects. Oleuropein shows beneficial effects on several aspects of cardiovascular disease. Evidence from experimental studies renders oleuropein as an ideal compound for preventing myocardial damage and more importantly as a promising complimentary therapeutic intervention against IRI [48].

Chronic oleuropein administration for 3 or 6 weeks in rabbits reduced infarct size [11] and oleuropein administration in rats for 2 consecutive days significantly inhibited the extent of myocardial infarct size compared to the controls [43]. Herein we demonstrate for first time the acute cardioprotective effects of oleuropein against myocardial IRI when administered prior to reperfusion which matches the clinical setting. Importantly, the anti-infarct effect of a single dose of oleuropein was confirmed in two different animal models (rabbits and mice) of myocardial IRI. Our results are in agreement with a previous *ex-vivo* study which demonstrated that treatment of rats with a single dose of ip oleuropein is capable of protecting the heart against IRI for at least 3 h [49]. It seems that administration of the oleuropein before the onset of reperfusion is an effective way to treat myocardial IRI. Our experimental approach proves that oleuropein offers therapeutic value to the actual clinical settings versus the pre-treatment strategies.

To gain further insight into the underlying mechanisms responsible for oleuropein-mediated cardioprotection, we evaluated whether it displays anti-infarct effects through the known cardioprotective pathways. The signal transduction by the RISK, the cGMP/PKG and the SAFE pathways play a crucial role in preventing IRI in the myocardium [50,51]. Therefore, as a first step, we tested whether oleuropein's protection is abolished by blocking these endogenous cardioprotective pathways. Cardioprotection was preserved, demonstrating that oleuropein's cardioprotection in not mediated through these classic cardioprotective pathways. In addition, in order to demonstrate if oleuropein regulate key proteins in RISK and SAFE pathways, we explored the phosphorylation and expression of Akt, ERK1/2 and STAT-3. We found no differences in the phosphorylation and expression of Akt, ERK1/2 and STAT-3 among the different study groups indicating that oleuropeins' cardioprotective effect against IRI in mice is not associated with the classic cardioprotective pathways. We must mention that we did not observed any differences in the above mentioned signaling molecules under the effect of PostC after 3 h of reperfusion, a finding that is in line with previous reports describing that the phosphorylation of proteins such as AKT, ERK1/2 and STAT-3 increased up to 30 minutes of reperfusion (maximum expression) and tended to decrease thereafter [52,53]. Moreover, phosphorylation at 10 min of reperfusion has been identified as the causal signaling step for STAT-3 phosphorylation when IRI versus ischemic PostC is compared [52]. Furthermore, in order to confirm if the cardioprotective mechanism of oleuropein depends on cGMP/PKG pathway, we determined the effects of oleuropein on cGMP production and we found that it did not induce cGMP accumulation confirming that it acts independently of the cGMP/PKG pathway.

The above signaling cascades are complex and include activation of sarcolemmal receptors and cytosolic kinases, as well as reduced MPTP opening, Ca^{2+}overload and proteolysis [54]. Moreover, RISK, SAFE and cGMP/PKG pathways are triggered by ischemic conditioning, so we hypothesized that oleuropein on top of PostC could exert an additive cardioprotective effect with a different mechanism of action from PostC. Indeed, a further reduction in infarct size was observed when oleuropein was combined with PostC. It has been reported that redox signaling at the time of myocardial reperfusion is also required to mediate the cardioprotection triggered by ischemic conditioning. There is evidence that treatment with large-spectrum antioxidants prior to the conditioning stimulus can abrogate conditioning-induced cardioprotection [55]. In fact, N-acetyl-cysteine (NAC) or mercaptopropionyl

glycine (MPG) which are ROS scavengers prevent the protective effect of PostC [56–58]. In the light of the above data, we suggest, that the cardioprotective mechanism of oleuropein goes beyond radical scavenging and classic intrinsic pathways of cardioprotection.

Several reports indicate that polyphenols are possible modulators of MPTP opening [59]. The cardioprotective effect of resveratrol in isolated rat hearts was correlated with prevention of MPTP opening [60]. Importantly, hydroxytyrosol, which is the phenolic chemical part of oleuropein, given at 100 μM increased the resistance to Ca^{2+} induced MPTP opening in isolated mitochondria [61]. In the present study by performing the CRC assay on mice heart mitochondria we first demonstrate that oleuropein does not alter the susceptibility of mitochondria to calcium by directly inhibiting the MPTP. Although, our experimental set up cannot exclude the oleuropein's effect on reperfusion-induced MPTP opening *in vivo*, we found that oleuropein's mechanism of action is distinct than other polyphenolic antioxidants.

Thereafter, we sought to explore the mechanisms by which oleuropein enhances protection on top of PostC and we evaluated the documented in the literature anti-inflammatory properties of this bioactive olive compound [37]. Myocardial IRI induces cardiac tissue damage by significant leukocyte infiltration, particularly of monocytes [62] and neutrophils [63], and the subsequent release of pro-inflammatory mediators. The first pool of cell population to infiltrate the infarct consists of monocytes. Monocytes play an early role in attracting neutrophils to the infarct and amplifying the initial inflammatory response. Monocyte recruitment to the ischemic myocardium follows a biphasic response, the earlier one includes the $Ly6C^{high}$ monocytes migration to the infarct (early inflammatory phase) and the latter one is the reparative phase where $Ly\text{-}6C^{low}$ monocytes dominate during the resolution of inflammation and propagate repair [64]. Our results indicated high level of $Ly6C^{high}$ monocytes and neutrophils infiltration in the area of myocardial IRI at 3h reperfusion compared with the sham operation group, a finding which is in agreement with previous reports [21]. At the same study, the depletion of monocytes, but not neutrophils, reduces myocardial IRI in a murine model suggesting that neutrophil may not be primary therapeutic target of IRI in this model [21].

Our findings demonstrate that oleuropein, PostC and their combination reduce the infiltration of the pro-inflammatory monocytes to the heart and decrease their population in blood whereas they have no effect on neutrophils mobilization to infiltrate the heart. To the best of our knowledge, there is no data regarding the effect of PostC

on Ly6C^{high} inflammatory monocytes whereas it seems that direct attenuation of neutrophil accumulation within the area at risk of the myocardium is not a primary mechanism of PostC protection [65]. Inhibition of inflammatory monocytes infiltration and leucocyte migration is an attractive strategy against IRI and the novel combined therapy, oleuropein and PostC, modulates the monocyte early response leading to a reduced infarct size. However, in our study this mechanism fails to explain the enhanced protection conferred by oleuropein on the top of the PostC strategy.

To this end, we assumed that the reduction in monocyte infiltration by oleuropein administration and the combination therapy can be attributed to increased cardiomyocyte viability and reduced cardiac damage. Our *in vitro* results in isolated cardiomyocytes, revealed that oleuropein exhibits a direct protective effect on cardiomyocytes since it significantly increased cardiomyocyte viability following SI/reoxygenation. Our finding is consistent with a published study suggesting that oleuropein treatment increased the MTT dye reduction in a dose-dependent manner in adult rat cardiomyocytes accompanied with a dose-dependent reduction in lactate dehydrogenase (LDH) activity [9]. Therefore, we prove that oleuropein has a direct effect on cardiomyocytes and the infarct size limitation is mainly due to oleuropein and not to its metabolites. The reason why oleuropein and PostC exert synergistic cardioprotective effect is that the two strategies attenuate myocardial cell death via different pathways.

The inflammatory response also involves the activation of cytokine and chemokines cascades and oxidative stress after reperfusion in the infarcted myocardium which have been established in numerous studies [66,67]. Thus, we aimed to explore a series of pro-inflammatory and adhesion related molecules that are well-known to play an important role in myocardial IRI and may explain a distinct mechanism of action of oleuropein to PostC. In the present study, PostC, oleuropein and their combination exhibited no effect on genes that are related to the CCL2/ C-C chemokine receptor type 2 (CCR2) axis that have reported to play a dominant role in recruiting the inflammatory monocytes to the ischemic myocardium. Although, clinical studies have demonstrated a correlation between increased levels of CCL2/monocyte chemoattractant protein-1 (MCP-1) and worse outcomes following cardiac injury, experimental studies have shown that MCP-1 deficiency and MCP-1 antibody inhibition do not change the infarct size [68].

IL-6 is one important pleiotropic inflammatory mediator that is released quickly upon myocardial IRI. It contributes to the cascade of the inflammatory events in the

infarcted region during the early period (<1 day) of reperfusion, an effect that is not mediated through changes in IL-1β, TNFα, neutrophil influx or an altered activation of the tissue factor-thrombin pathway [69]. Of note, the E-selectin which is secreted from injured myocytes surrounding the infarct core initiates the recruitment of circulating granulocytes and monocytes within hours after reflow [70]. Our results showed that oleuropein decreases the mRNA levels of IL-6 and E-selectin in myocardial tissue whereas other reports have shown that oleuropein decreases IL-6 at a transcriptional level in liver and kidney tissues [71]. It has also been demonstrated that oleuropein, when administered for two consecutive days, inhibited serum levels of IL-6 in rats subjected to myocardial IRI [72]. To the best of our knowledge, there is no data regarding the effect of PostC on mRNA levels of IL-6. IL-6 and E-selectin gene transcription can be induced via multiple pathways, depending on the initiating stimulus [73,74]. We speculated that PostC as a mechanical stimulus that acts through potential mechanosensors (integrins, cytoskeleton, and sarcolemmal proteins) activates major intracellular cross-talking signal transduction pathways that result in the induction of cytokine genes [75], is as robust that diminishes the effect observed by oleuropein. Thus, the combination therapy with Oleuropein and PostC shows a recovery in IL-6 and E-selectin mRNA levels. The effect of oleuropein on *il-6* and *e-selectin* levels may partially explain its effect on lesser inflammatory monocyte recruitment to the infarcted myocardium. Although oleuropein ‘s role as a pro-inflammatory mediator inhibitor is shown in our work, this needs to be confirmed in future studies.

Among the myriad of signaling cascades that have been reported to exert cardioprotection, one distinct player within the cellular redox balance is Nrf-2 which controls a long list of genes to regulate various cellular responses. Nrf-2 plays a key role in protecting cardiomyocytes from oxidative stress by binding antioxidant-response elements (ARE) of gene promoters to induce expression of numerous target genes, e.g. glutathione peroxidase (*GPx1*), *ho-1*, *sod-2* and thioredoxin (*txn*). In fact, a growing number of experimental studies have illustrated that Nrf-2 can attenuate IRI and effectuate cardioprotective effects [76]. The expression of Nrf-2 and its downstream enzymes such as HO-1 has been shown elevated during reperfusion after administration of either cardioprotective drugs [77–79] or natural products [80]. Of note, polyphenols from the extra virgin olive oil like hydroxytyrosol, tyrosol, and oleuropein can activate Nrf-2 signaling and its dependent genes expression, inducing a cellular defense response against oxidative injuries and pro-inflammatory stimuli [43]. More specifically, oleuropein considerably increased the levels of Nrf-2 dependent

phase II enzymes, such as HO-1 in the hypothalamic paraventricular nucleus in spontaneously hypertensive rats (SHR) in comparison with the saline-treated SHR rats [81]. Importantly, we herein demonstrate that the combination of oleuropein with PostC, markedly increases the expression of Nrf-2 and its antioxidant defense proteins HO-1 and SOD-2. Oleuropein in combination to PostC downregulates *nox-2* gene expression and protein levels after 3 h of reperfusion in a similar extend to the oleuropein group alone. Previous reports have demonstrated that oleuropein reduces Nox-2 mRNA levels in spontaneously hypertensive rats resulting in elimination of the increased aortic superoxide levels [82]. However, no conspicuous changes of mRNA and protein expression of Nox-2 were found after 3h of reperfusion in the PostC group compared to the control group similar to a previous report, showing that sevoflurane postconditioning does not affect Nox-2 protein expression evaluated 24 h after reperfusion [83]. In parallel our study demonstrates that Nox-2 is involved at late reperfusion (3h) indicating that reactive oxygen species can contribute to reperfusion injury not only at the early stages of reperfusion. Similar reports have been made in other models of ischemia reperfusion and specifically the transient focal cerebral ischemia model, indicating that Nox2 does not participate in the early burst of reperfusion-related ROS production, but it contributes to the ROS generation process during prolonged reperfusion [84]. We would expect that decreased Nox-2 by oleuropein will lead to less cardiac fibrosis and hypertrophy at the remodeling phase after IRI [85,86] and our future research projects are welcome to verify this favorable outcome. These findings stand as the backbone of the cardioprotective effect of oleuropein. We must point out that our results showed a paradoxical significant increase of HO-1 expression only in the combination group indicating that both PostC and Nrf-2 stimuli by oleuropein are required for HO-1 translation, however this needs to be proved by further investigation with gene depletion studies.

Although, the mechanism by which Nrf-2 regulates NADPH oxidase is unidentified and several regulating signaling loops may exist [45,87], our findings clearly point out that oleuropein shifts the cells towards the antioxidant protection. To the best of our knowledge this is the first study shows that oleuropein increases the expression of Nrf-2 in myocardium subjected to ischemia/reperfusion and additional studies should be performed in order to further confirm this finding.

In conclusion, this study demonstrates that oleuropein treatment administered during ischemia provides cardioprotective effects in animal models of myocardial IRI. When oleuropein is combined with PostC, an enhanced effect on the top of Post C in

limiting the infarct size was observed, a finding that can be attributed to the upregulation of Nrf-2 mediated expression of antioxidant defense genes which lead to the fine tuning of the oxidative state of the cell. Therefore, the upregulation of antioxidant defense systems in the myocardium may represent an ideal aim for multitarget treatments against myocardial IRI.

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