Olive mill wastewater and hydroxytyrosol inhibits atherogenesis in apolipoprotein E-deficient mice

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Abstract

Background and Aims

The Mediterranean diet, which is characterized by high consumption of olive oil, prevents cardiovascular disease. Meanwhile, olive mill wastewater (OMWW), which is obtained as a byproduct during olive oil production, contains various promising bioactive components such as water-soluble polyphenols. Hydroxytyrosol (HT), the major polyphenol in OMWW, has anti-oxidative and anti-inflammatory properties; however, the atheroprotective effects of OMWW and HT remain to be fully understood. Here, we investigated the effect of OMWW and HT on atherogenesis.

Methods and Results

Male 8-week-old apolipoprotein E-deficient mice were fed a western-type diet supplemented with OMWW (0.30%w/w) or HT (0.02%w/w) for 20 weeks. The control group was fed a non-supplemented diet. OMWW and HT attenuated the development of atherosclerosis in the aortic arch as determined by Sudan IV staining ($P<0.01$, respectively) without alteration of body weight, plasma lipid levels, and blood pressure. OMWW and HT also decreased the production of oxidative stress ($P<0.01$, respectively) and the expression of NADPH oxidase subunits (e.g., NOX2 and p22phox) and inflammatory molecules (e.g., IL-1β and MCP-1) in the aorta. The results of in vitro experiments demonstrated that HT inhibited the expression of these molecules that were stimulated with LPS in RAW264.7 cells, murine macrophage-like cells.

Conclusion

OMWW and HT similarly attenuated atherogenesis. HT is a major component of water-soluble polyphenols in OMWW, and it inhibited inflammatory activation of macrophages. Therefore, our results suggest that the atheroprotective effects of OMWW are at least partially attributable to the anti-inflammatory effects of HT.

Key Words: atherosclerosis, inflammation, macrophage, olive polyphenol, hydroxytyrosol

Abbreviations

ApoE$^{-/-}$; apolipoprotein E-deficient

Ctrl; control

EVOO; extra-virgin olive oil

HT; hydroxytyrosol

ICAM-1; intercellular adhesion molecule-1

IL; interleukin

MCP-1; monocyte chemoattractant protein-1

OMWW; olive mill wastewater

PAI-1; plasminogen activator inhibitor-1

qPCR; quantitative real-time PCR

VCAM-1; vascular cell adhesion molecule-1

WTD; western-type diet

Introduction

Cardiovascular events based on atherosclerotic disease remain the leading cause of morbidity and mortality globally[1]. There have been substantial achievements in the prevention or risk reduction of atherosclerotic disease by employing clinical interventions against risk factors, although those are not enough[2]. In addition to these clinical interventions, one of the most important strategies to prevent cardiovascular events is the promotion of a healthy lifestyle throughout life[3,4]. Recently, there has been growing interest in the link between dietary patterns and atherosclerotic disease. Among them, the Mediterranean diet, which is characterized by high consumption of olive oil, especially extra-virgin olive oil (EVOO), has attracted much attention[5-9]. In fact, several studies indicated that better conformity with the traditional Mediterranean diet is associated with better cardiovascular outcomes, including reductions in rates of coronary heart disease, ischemic stroke, and total cardiovascular disease. Furthermore, in the PREDIMED trial and more recent CORDIOPREV trial, a Mediterranean diet supplemented with EVOO showed a reduction in cardiovascular events compared with the control diet[8,10]. Previous clinical and basic research has suggested that the anti-inflammatory effects of monounsaturated fatty acids (MUFAs) such as oleic acid, which is a major nutritional component of EVOO, on vascular cells are associated with their cardioprotective effects[11-13].

In addition to MUFAs, olives contain various attractive biological compounds[14,15]. Especially, a byproduct obtained during olive oil production, olive mill wastewater (OMWW), contains water-soluble polyphenols such as hydroxytyrosol (HT)[16]. Previous studies have demonstrated that water-soluble polyphenols in OMWW have anti-inflammatory and/or anti-oxidative properties, implying their cardioprotective effects[17-23]. However, the results of previous studies, in which HT was administered to atherosclerotic animal models, are controversial[24]. Therefore, in this study, we administered OMWW, which contains water-soluble polyphenols including HT or HT, to apolipoprotein E-deficient ($ApoE^{-/-}$) mice and examined whether OMWW prevents atherogenesis in this mouse model. We also investigated the effects of HT on atherogenesis and the pro-inflammatory activation of macrophages to examine the mechanism.

Materials and Methods

Chemicals

OMWW is obtained during the process of the extraction of olive oil. In this study, OLIVEX HT6® (GROUPE GRAP'SUD) was used. OLIVEX HT6® is characterized by a higher polyphenol content (30%), and it includes HT (6%) and tyrosol (1%). HT was purchased from Tokyo Chemical Industry Co., Ltd. Compositional data provided by the supplier is shown in Table1.

Animals and drug administration

ApoE$^{-/-}$ mice (C57BL/6J background), a hypercholesterolemic mouse model of atherosclerosis, were originally purchased from The Jackson Laboratory. Male ApoE$^{-/-}$ were fed a western-type diet (WTD) supplemented with OMWW (0.30%w/w) or HT (0.02%w/w), from 8 weeks old for 20 weeks. Both foods include a similar amount of HT. The control group received a non-supplemented WTD. Mice were maintained under controlled lighting (12 h light/dark) and temperature (24°C) conditions. All animal experimental procedures conformed to the guidelines for animal experimentation of Tokushima University. The protocol was reviewed and approved by our institutional ethics committee.

Analysis of blood pressure and serum lipid levels

Blood pressure was measured by a tail-cuff system (BP-98A、Softron) as we described previously[25]. At the time of sacrifice, blood was collected from the heart, and plasma was separated and stored at −80°C until required. Plasma total cholesterol, low-density lipoprotein-cholesterol, high-density lipoprotein-cholesterol, and triglyceride levels were measured using a 7180 Clinical Analyzer (Hitachi High-Technologies GLOBAL).

Analysis of atherosclerotic lesions

The development of atherosclerotic lesions in the aorta was assessed as we previously described[26]. In brief, after sacrifice and perfusion with 0.9% sodium chloride solution, both the heart and whole aorta were immediately dissected from the body. The upper part of the thoracic aorta was opened longitudinally and fixed with 10% neutral buffered formalin. Quantification of atherosclerotic lesions in the aortic arch was performed by en face Sudan IV staining. The lower part of the thoracic aorta and the abdominal aorta were removed and snap-frozen in liquid nitrogen for further analyses.

Measurement of aortic O_2^- production

Production of O_2^- in the aorta was measured using a lucigenin-enhanced chemiluminescence assay as described previously[27]. The thoracic aorta with endothelium was cut into small pieces and placed in modified Krebs/HEPES buffer (20 mM HEPES, 119 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, 0.4 mM NaH_2PO_4, 0.15 mM Na_2HPO_4, 1 mM $MgSO_4$, 5 mM, $NaHCO_3$, and 5.5 mM glucose) in wells of a 96-well plate. After applying lucigenin at a final concentration of 5 μM, chemiluminescence was recorded for 10 minutes at 37°C continuously. Then, NADPH was added at a final concentration of 0.5 μM, and chemiluminescence was recorded for 10 minutes continuously. The vessels were then dried by placing them in a 90°C oven for 24 h for determination of dry weight. The production of superoxide was determined by calculating area under the curve.

Cell culture

RAW264.7, a murine macrophage cell line, was purchased from KAC Co., Ltd. RAW264.7 was cultured in DMEM (Sigma-Aldrich) supplemented with 10% FBS and 1% penicillin G/streptomycin in a CO_2 incubator with 5% CO_2 at 37°C. RAW264.7 cells were pre-treated with HT (3, 10, or 30 μM) for 1 hour in serum-starved conditions, and then stimulated with 50 ng/ml LPS for 24 hours.

Quantitative real-time PCR

Total RNA was extracted from the aorta and RAW264.7 cells using a RNeazy Mini Kit (Qiagen) according to the manufacturer's instructions. A quality check of RNA in all samples was performed in a Bioanalyzer 2100 (Agilent Technologies) and samples with RIN>8.0 was used for further analysis. cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). To quantify the difference in gene expression, quantitative real-time PCR (qPCR) was performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems) using TaqMan gene expression assay probes (Thermo Fisher Scientific) by the ΔΔCt method. Gene specific primers were as follows; Cybb (NOX2, Mm01287743_m1), NOX4 (NOX4, Mm00479246_m1), Cyba (p22phox, Mm00514478_m1), Ncf1 (p47phox, Mm00447921_m1), Rac2 (Rac2, Mm00485472_m1), Tnf (Tnfα, Mm00443258_m1), Ccl2 (MCP1, Mm00441242_m1), Vcam1 (VCAM1, Mm01320970_m1), Icam1 (ICAM1, Mm00516023_m1), and Serpine1 (PAI1, Mm00435858_m1). Data are expressed in arbitrary units by 18S rRNA (18S, 18sRNA; Hs99999901_s1).

Statistical analysis

All results are expressed as mean ± SEM. Comparisons of multiple groups were performed using one-way analysis of variance followed by Dunnett's multiple comparison test. A value of $P<0.05$ was considered significant.

Results

OMWW and HT attenuated atherogenesis in ApoE$^{-/-}$ mice

Administration of OMWW or HT for 20 weeks did not affect body weight, plasma lipid levels, or blood pressure in ApoE$^{-/-}$ mice (Table 2). Both OMWW and HT reduced the development of atherosclerosis in the aortic arch ($P<0.01$, respectively); however, there was no statistically significant difference between the two groups (Figure 1).

OMWW and HT reduced oxidative stress

O_2^- measurement in the thoracic aorta demonstrated that administration of OMWW or HT significantly reduced the production of superoxide compared with the control group ($P<0.01$, respectively) (Figure 2A). Both treatments also suppressed the expression of NADPH oxidase subunits in the abdominal aorta (Figure 2B). In particular, significant suppression of NOX2 and p22phox by OMWW ($P<0.05$) or HT ($P<0.05$), of p47phox by HT ($P<0.05$), and of Rac2 by OMWW ($P<0.01$) was observed.

OMWW and HT reduced vascular inflammation

Administration of OMWW or HT decreased the expression of inflammatory molecules in the abdominal aorta (Figure 3). OMWW significantly reduced the expression of interleukin (IL)-1β, monocyte chemoattractant protein-1 (MCP-1), intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) ($P<0.05$, respectively). Similarly, HT significantly reduced the expression of IL-1β, MCP-1, ICAM-1, VCAM-1, and plasminogen activator inhibitor-1 (PAI-1) ($P<0.05$, respectively).

HT attenuated inflammatory activation of macrophages induced by LPS

To investigate the anti-inflammatory effects of HT, a major polyphenol contained in OMWW, in vitro experiments using RAW264.7 cells were performed. LPS promoted the expression of inflammatory

molecules such as IL-1β, MCP-1, iNOS, and ICAM-1 in this cell-type, while pre-treatment with HT attenuated their expression in a dose-dependent manner (Figure 4).

Discussion

This study investigated the effects of OMWW and HT on atherogenesis in an atherosclerotic mouse model. HT is known to be a major component of water-soluble polyphenols in OMWW. The results of in vivo experiments demonstrated that OMWW and HT significantly inhibited the development of atherosclerotic lesions and the expression of inflammatory molecules in the aorta, without alteration of metabolic parameters and blood pressure. Both treatments also reduced the production of superoxide and the expression of NADPH oxidase subunits in the aorta. In this study, both treatment groups showed similar atheroprotective effects. The dosage of HT in the OMWW group and HT group was equal (0.02% (w/w)), and HT is a major component of water-soluble polyphenols in OMWW. These dose was similar to a previous study[28]. The dosage closely approximated human intake[16,28] and was a very low one once body surface area is taken into account[29]. Considering the case of actual administration to humans, according to the previous human studies of HT administration[30,31], it seems easier to take HT as a supplement than from a regular diet. Furthermore, the results of in vitro experiments showed that HT suppressed inflammatory activation of macrophages. These results suggested that OMWW attenuated atherogenesis by reduction of oxidative stress and inflammation, at least partially, and that HT contributed to the atheroprotective effects of OMWW.

Numerous studies have indicated that the Mediterranean diet reduces cardiovascular events. EVOO and MUFAs are a major component of the Mediterranean diet. Besides, OMWW obtained during the process of extraction of olive oil also contains various bioactive components[14]. Previous studies have shown that water-soluble polyphenols in OMWW potentially have atheroprotective effects[21-23]. HT is a major component of olive-derived water-soluble polyphenols, and it has various effects; including anti-inflammatory effects[18-20,32], protective effects of endothelial function[33,34], inhibitory effects of vascular smooth muscle cells migration[35,36], antihypertensive effect[37,38], and epigenetic effect via microRNAs[39,40];

however, it is still unclear whether HT attenuates the development of atherosclerosis, especially in vivo[17,24]. Tyrosol, a dehydroxylated form of HT, is a second major water-soluble polyphenol in OMWW. Tyrosol also has anti-inflammatory properties, though several studies suggested its effect is weaker than that of HT[41,42]. Several previous studies suggested that OMWW has potential atheroprotective effects, associated with the effects of these bioactive components. An in vitro study demonstrated that olive extraxt suppressed inflammatory responses in in vitro experiments[43]. Also, another study demonstrated that OMWW improved glucose and lipid profile in rats and humans in vivo[15]. Furthermore, a previous study demonstrated that consumption of OMWW increased glutathione level in healthy volunteers[16]. However, few studies have examined whether OMWW inhibits atherogenesis in vivo.

Our present study demonstrated that both OMWW and HT reduced the expression of NADPH oxidase subunits and superoxide production in the aorta of treated mice, suggesting the suppression of oxidative stress. Numerous studies have indicated that oxidative stress promotes inflammatory responses in various cell types in the vasculature, leading to the development of atherosclerosis[44-46]. In this study, both treatments reduced the expression of inflammatory molecules (e.g., IL-1β and MCP-1) in the aorta. We also examined the effects of HT on macrophages, a key player in atherogenesis[47]. In our in vitro experiments, HT clearly attenuated inflammatory molecule expression in murine macrophage-like cells stimulated with LPS. Previous studies have demonstrated anti-oxidative stress effects of HT[32]. In addition, one study reported that HT attenuated oxidative stress, suppressing the production of oxidized LDL, one of the proinflammatory activators of macrophages[19,20]. Thus, our results, taken together with those of previous studies, suggest that OMWW and HT suppress oxidative stress and pro-inflammatory activation of macrophages, leading to the inhibition of atherogenesis.

The results of our present study demonstrated that OMWW and HT attenuated atherogenesis in WTD-fed ApoE$^{-/-}$ mice without alteration of the lipid profile. Similarly, a previous study showed that HT reduced atherosclerosis development in high-fat-fed rabbits[17], while HT improved the lipid profile in their study. On the other hand, there is a report demonstrating that HT increased atherosclerosis development in ApoE$^{-/-}$ mice fed normal chow[24]. In that study, total

cholesterol level was lower than that in our study; however, it increased after HT administration. There is a possibility that HT effectively inhibits inflammatory responses caused by exogenous cholesterol included in the diet. Also, the profile of gene expression including adhesion molecules and inflammatory molecules associated with atherogenesis is different between the aorta in normal chow-fed ApoE$^{-/-}$ mice and that in western-type diet-fed ApoE$^{-/-}$ mice[48]. Therefore, the difference in diet and administration period might explain these discrepancies. Further studies are required to establish the atheroprotective effects of OMWW and HT.

EVOO and bioactive compounds derived from olives have been gathering interests in promoting a healthy lifestyle[39]. Accumulating evidence has proven that the Mediterranean diet is associated with the decrease in metabolic disorders, cardiovascular diseases, cancer risk, neurological disorders, and inflammatory disease. In addition, for over 25 years, to reveal underlying mechanisms or develop new drugs, clinical and basic researchers have been using isolated olive components such as OMWW and other polyphenols[49]. Many of these studies have been performed in experimental animals and reported that olive-derived polyphenols including HT abrogate the expression of inflammatory cytokines, bioactive lipids, and reactive oxygen species, suggesting anti-inflammatory and anti-oxidative effects. Some human studies have been done, however its effect is still controversial[39,50], however, of note, several studies have demonstrated that OMWW increased the plasma concentration of glutathione, an anti-oxidative molecule[28,51], decreased TXB_2[52], and reduced inflammation[53]. Further studies are needed to have better understanding beneficial effects of olive extracts and isolated olive components.

This study has several limitations. First, we used OLIVEX HT6® (GROUPE GRAP'SUD) as OMWW. Various olive-derived phenol products which contain different percentage of phenols are commercially available and several studies have reported their favorable effects[54-56]. We did not aim to compare them in this study, but we need to be careful for cytotoxicity and maximum intake/upper limits of them to establish health promotion with these bioactive nutrients[57-59]. Second, the dosage of our study was similar to previous study, however we did not examine the dose dependency in in vivo study. Third, although there was no significant difference in body

weight between groups, detailed dietary intake, stability of OMWW in the diet, and blood concentration of HT were not be verified in this study.

Conclusions

Our results demonstrated that OMWW reduced oxidative stress and inflammation in the vasculature and attenuated the development of atherosclerosis in WTD-fed ApoE$^{-/-}$ mice. These atheroprotective effects of OMWW are attributable, at least partially, to the anti-oxidative and anti-inflammatory properties of HT, a major component of olive-derived water-soluble polyphenols. The results of our study may be useful for understanding the vascular protective effects of the olive and its derivatives and promoting a healthy lifestyle.

Competing interests

The Department of Cardio-Diabetes Medicine, Tokushima University Graduate School is supported in part by unrestricted research grants from Boehringer Ingelheim. The authors declare that they have no conflict of interest.

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References

1. Libby P (2012) Inflammation in atherosclerosis. Arterioscler Thromb Vasc Biol 32(9):2045-2051
2. Libby P, Everett BM (2019) Novel Antiatherosclerotic Therapies. Arterioscler Thromb Vasc Biol 39(4):538-545
3. Benjamin EJ, Larson MG, Keyes MJ, Mitchell GF, Vasan RS, Keaney JF, Jr., Lehman BT, Fan S, Osypiuk E, Vita JA (2004) Clinical correlates and heritability of flow-mediated dilation in the community: the Framingham Heart Study. Circulation 109(5):613-619
4. Esposito K, Pontillo A, Di Palo C, Giugliano G, Masella M, Marfella R, Giugliano D (2003) Effect of weight loss and lifestyle changes on vascular inflammatory markers in obese women: a randomized trial. JAMA 289(14):1799-1804
5. Esposito K, Maiorino MI, Ciotola M, Di Palo C, Scognamiglio P, Gicchino M, Petrizzo M, Saccomanno F, Beneduce F, Ceriello A, Giugliano D (2009) Effects of a Mediterranean-style diet on the need for antihyperglycemic drug therapy in patients with newly diagnosed type 2 diabetes: a randomized trial. Ann Intern Med 151(5):306-314
6. Esposito K, Marfella R, Ciotola M, Di Palo C, Giugliano F, Giugliano G, D'Armiento M, D'Andrea F, Giugliano D (2004) Effect of a mediterranean-style diet on endothelial dysfunction and markers of vascular inflammation in the metabolic syndrome: a randomized trial. JAMA 292(12):1440-1446
7. Estruch R, Martinez-Gonzalez MA, Corella D, Salas-Salvado J, Ruiz-Gutierrez V, Covas MI, Fiol M, Gomez-Gracia E, Lopez-Sabater MC, Vinyoles E, Aros F, Conde M, Lahoz C, Lapetra J, Saez G, Ros E, Investigators PS (2006) Effects of a Mediterranean-style diet on cardiovascular risk factors: a randomized trial. Ann Intern Med 145(1):1-11
8. Estruch R, Ros E, Salas-Salvado J, Covas MI, Corella D, Aros F, Gomez-Gracia E, Ruiz-Gutierrez V, Fiol M, Lapetra J, Lamuela-Raventos RM, Serra-Majem L, Pinto X, Basora J, Munoz MA, Sorli JV, Martinez JA, Fito M, Gea A, Hernan MA, Martinez-Gonzalez MA, Investigators PS (2018) Primary Prevention of Cardiovascular Disease with a Mediterranean

Diet Supplemented with Extra-Virgin Olive Oil or Nuts. N Engl J Med 378(25):e34

9. Estruch R, Ros E, Salas-Salvado J, Covas MI, Corella D, Aros F, Gomez-Gracia E, Ruiz-Gutierrez V, Fiol M, Lapetra J, Lamuela-Raventos RM, Serra-Majem L, Pinto X, Basora J, Munoz MA, Sorli JV, Martinez JA, Martinez-Gonzalez MA, Investigators PS (2013) Primary prevention of cardiovascular disease with a Mediterranean diet. N Engl J Med 368(14):1279-1290

10. Delgado-Lista J, Alcala-Diaz JF, Torres-Peña JD, Quintana-Navarro GM, Fuentes F, Garcia-Rios A, Ortiz-Morales AM, Gonzalez-Requero AI, Perez-Caballero AI, Yubero-Serrano EM, Rangel-Zuñiga OA, Camargo A, Rodriguez-Cantalejo F, Lopez-Segura F, Badimon L, Ordovas JM, Perez-Jimenez F, Perez-Martinez P, Lopez-Miranda J (2022) Long-term secondary prevention of cardiovascular disease with a Mediterranean diet and a low-fat diet (CORDIOPREV): a randomised controlled trial. Lancet 399(10338):1876-1885

11. Gillingham LG, Harris-Janz S, Jones PJ (2011) Dietary monounsaturated fatty acids are protective against metabolic syndrome and cardiovascular disease risk factors. Lipids 46(3):209-228

12. Schwingshackl L, Hoffmann G (2014) Monounsaturated fatty acids, olive oil and health status: a systematic review and meta-analysis of cohort studies. Lipids Health Dis 13:154

13. Tierney AC, Roche HM (2007) The potential role of olive oil-derived MUFA in insulin sensitivity. Mol Nutr Food Res 51(10):1235-1248

14. Antonia Nunes M, Costa ASG, Bessada S, Santos J, Puga H, Alves RC, Freitas V, Oliveira M (2018) Olive pomace as a valuable source of bioactive compounds: A study regarding its lipid- and water-soluble components. Sci Total Environ 644:229-236

15. Peroulis N, Androutsopoulos VP, Notas G, Koinaki S, Giakoumaki E, Spyros A, Manolopoulou E, Kargaki S, Tzardi M, Moustou E, Stephanou EG, Bakogeorgou E, Malliaraki N, Niniraki M, Lionis C, Castanas E, Kampa M (2019) Significant metabolic improvement by a water extract of olives: animal and human evidence. Eur J Nutr 58(6):2545-2560

16. Visioli F, Bernardini E (2011) Extra virgin olive oil's polyphenols: biological activities. Curr Pharm Des 17(8):786-804

17. Gonzalez-Santiago M, Martin-Bautista E, Carrero JJ, Fonolla J, Baro L, Bartolome MV, Gil-Loyzaga P, Lopez-Huertas E (2006) One-month administration of hydroxytyrosol, a phenolic antioxidant present in olive oil, to hyperlipemic rabbits improves blood lipid profile, antioxidant status and reduces atherosclerosis development. Atherosclerosis 188(1):35-42

18. Karkovic Markovic A, Toric J, Barbaric M, Jakobusic Brala C (2019) Hydroxytyrosol, Tyrosol and Derivatives and Their Potential Effects on Human Health. Molecules 24(10)

19. Mateos R, Martinez-Lopez S, Baeza Arevalo G, Amigo-Benavent M, Sarria B, Bravo-Clemente L (2016) Hydroxytyrosol in functional hydroxytyrosol-enriched biscuits is highly bioavailable and decreases oxidised low density lipoprotein levels in humans. Food Chem 205:248-256

20. Perrone MA, Gualtieri P, Gratteri S, Ali W, Sergi D, Muscoli S, Cammarano A, Bernardini S, Di Renzo L, Romeo F (2019) Effects of postprandial hydroxytyrosol and derivates on oxidation of LDL, cardiometabolic state and gene expression: a nutrigenomic approach for cardiovascular prevention. J Cardiovasc Med (Hagerstown) 20(7):419-426

21. Tresserra-Rimbau A, Rimm EB, Medina-Remon A, Martinez-Gonzalez MA, Lopez-Sabater MC, Covas MI, Corella D, Salas-Salvado J, Gomez-Gracia E, Lapetra J, Aros F, Fiol M, Ros E, Serra-Majem L, Pinto X, Munoz MA, Gea A, Ruiz-Gutierrez V, Estruch R, Lamuela-Raventos RM, Investigators PS (2014) Polyphenol intake and mortality risk: a re-analysis of the PREDIMED trial. BMC Med 12:77

22. Medina-Remon A, Casas R, Tressserra-Rimbau A, Ros E, Martinez-Gonzalez MA, Fito M, Corella D, Salas-Salvado J, Lamuela-Raventos RM, Estruch R, Investigators PS (2017) Polyphenol intake from a Mediterranean diet decreases inflammatory biomarkers related to atherosclerosis: a substudy of the PREDIMED trial. Br J Clin Pharmacol 83(1):114-128

23. Hernaez A, Remaley AT, Farras M, Fernandez-Castillejo S, Subirana I, Schroder H, Fernandez-Mampel M, Munoz-Aguayo D, Sampson M, Sola R, Farre M, de la Torre R,

Lopez-Sabater MC, Nyyssonen K, Zunft HJ, Covas MI, Fito M (2015) Olive Oil Polyphenols Decrease LDL Concentrations and LDL Atherogenicity in Men in a Randomized Controlled Trial. J Nutr 145(8):1692-1697

24. Acin S, Navarro MA, Arbones-Mainar JM, Guillen N, Sarria AJ, Carnicer R, Surra JC, Orman I, Segovia JC, Torre Rde L, Covas MI, Fernandez-Bolanos J, Ruiz-Gutierrez V, Osada J (2006) Hydroxytyrosol administration enhances atherosclerotic lesion development in apo E deficient mice. J Biochem 140(3):383-391

25. Salim HM, Fukuda D, Higashikuni Y, Tanaka K, Hirata Y, Yagi S, Soeki T, Shimabukuro M, Sata M (2016) Dipeptidyl peptidase-4 inhibitor, linagliptin, ameliorates endothelial dysfunction and atherogenesis in normoglycemic apolipoprotein-E deficient mice. Vascul Pharmacol 79:16-23

26. Fukuda D, Nishimoto S, Aini K, Tanaka A, Nishiguchi T, Kim-Kaneyama JR, Lei XF, Masuda K, Naruto T, Tanaka K, Higashikuni Y, Hirata Y, Yagi S, Kusunose K, Yamada H, Soeki T, Imoto I, Akasaka T, Shimabukuro M, Sata M (2019) Toll-Like Receptor 9 Plays a Pivotal Role in Angiotensin II-Induced Atherosclerosis. J Am Heart Assoc 8(7):e010860

27. Rajagopalan S, Kurz S, Munzel T, Tarpey M, Freeman BA, Griendling KK, Harrison DG (1996) Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation. Contribution to alterations of vasomotor tone. J Clin Invest 97(8):1916-1923

28. Giordano E, Dávalos A, Visioli F (2014) Chronic hydroxytyrosol feeding modulates glutathione-mediated oxido-reduction pathways in adipose tissue: a nutrigenomic study. Nutr Metab Cardiovasc Dis 24(10):1144-1150

29. Reagan-Shaw S, Nihal M, Ahmad N (2008) Dose translation from animal to human studies revisited. Faseb j 22(3):659-661

30. González-Santiago M, Fonollá J, Lopez-Huertas E (2010) Human absorption of a supplement containing purified hydroxytyrosol, a natural antioxidant from olive oil, and evidence for its transient association with low-density lipoproteins. Pharmacol Res 61(4):364-370

31. Lopez-Huertas E, Fonolla J (2017) Hydroxytyrosol supplementation increases vitamin C levels in vivo. A human volunteer trial. Redox Biol 11:384-389

32. Yonezawa Y, Miyashita T, Nejishima H, Takeda Y, Imai K, Ogawa H (2018) Anti-inflammatory effects of olive-derived hydroxytyrosol on lipopolysaccharide-induced inflammation in RAW264.7 cells. J Vet Med Sci 80(12):1801-1807

33. Yao F, Jin Z, Lv X, Zheng Z, Gao H, Deng Y, Liu Y, Chen L, Wang W, He J, Gu J, Lin R (2021) Hydroxytyrosol Acetate Inhibits Vascular Endothelial Cell Pyroptosis via the HDAC11 Signaling Pathway in Atherosclerosis. Front Pharmacol 12:656272

34. Vijakumaran U, Shanmugam J, Heng JW, Azman SS, Yazid MD, Haizum Abdullah NA, Sulaiman N (2023) Effects of Hydroxytyrosol in Endothelial Functioning: A Comprehensive Review. Molecules 28(4)

35. Vijakumaran U, Yazid MD, Hj Idrus RB, Abdul Rahman MR, Sulaiman N (2021) Molecular Action of Hydroxytyrosol in Attenuation of Intimal Hyperplasia: A Scoping Review. Front Pharmacol 12:663266

36. Zrelli H, Matsuka M, Araki M, Zarrouk M, Miyazaki H (2011) Hydroxytyrosol induces vascular smooth muscle cells apoptosis through NO production and PP2A activation with subsequent inactivation of Akt. Planta Med 77(15):1680-1686

37. Hermans MP, Lempereur P, Salembier JP, Maes N, Albert A, Jansen O, Pincemail J (2020) Supplementation Effect of a Combination of Olive (Olea europea L.) Leaf and Fruit Extracts in the Clinical Management of Hypertension and Metabolic Syndrome. Antioxidants (Basel) 9(9)

38. Quirós-Fernández R, López-Plaza B, Bermejo LM, Palma-Milla S, Gómez-Candela C (2019) Supplementation with Hydroxytyrosol and Punicalagin Improves Early Atherosclerosis Markers Involved in the Asymptomatic Phase of Atherosclerosis in the Adult Population: A Randomized, Placebo-Controlled, Crossover Trial. Nutrients 11(3)

39. Visioli F, Davalos A, López de Las Hazas MC, Crespo MC, Tomé-Carneiro J (2020) An overview of the pharmacology of olive oil and its active ingredients. Br J Pharmacol

177(6):1316-1330

40. Del Saz-Lara A, López de Las Hazas MC, Visioli F, Dávalos A (2022) Nutri-Epigenetic Effects of Phenolic Compounds from Extra Virgin Olive Oil: A Systematic Review. Adv Nutr 13(5):2039-2060

41. Gutierrez VR, de la Puerta R, Catala A (2001) The effect of tyrosol, hydroxytyrosol and oleuropein on the non-enzymatic lipid peroxidation of rat liver microsomes. Mol Cell Biochem 217(1-2):35-41

42. Rosignoli P, Fuccelli R, Fabiani R, Servili M, Morozzi G (2013) Effect of olive oil phenols on the production of inflammatory mediators in freshly isolated human monocytes. J Nutr Biochem 24(8):1513-1519

43. Baci D, Gallazzi M, Cascini C, Tramacere M, De Stefano D, Bruno A, Noonan DM, Albini A (2019) Downregulation of Pro-Inflammatory and Pro-Angiogenic Pathways in Prostate Cancer Cells by a Polyphenol-Rich Extract from Olive Mill Wastewater. Int J Mol Sci 20(2)

44. Kim SY, Lee JG, Cho WS, Cho KH, Sakong J, Kim JR, Chin BR, Baek SH (2010) Role of NADPH oxidase-2 in lipopolysaccharide-induced matrix metalloproteinase expression and cell migration. Immunol Cell Biol 88(2):197-204

45. Griendling KK, Sorescu D, Ushio-Fukai M (2000) NAD(P)H oxidase: role in cardiovascular biology and disease. Circ Res 86(5):494-501

46. Vendrov AE, Hakim ZS, Madamanchi NR, Rojas M, Madamanchi C, Runge MS (2007) Atherosclerosis is attenuated by limiting superoxide generation in both macrophages and vessel wall cells. Arterioscler Thromb Vasc Biol 27(12):2714-2721

47. Shirai T, Hilhorst M, Harrison DG, Goronzy JJ, Weyand CM (2015) Macrophages in vascular inflammation--From atherosclerosis to vasculitis. Autoimmunity 48(3):139-151

48. Castro C, Campistol JM, Barettino D, Andres V (2005) Transcriptional profiling of early onset diet-induced atherosclerosis in apolipoprotein E-deficient mice. Front Biosci 10:1932-1945

49. Pastor A, Rodríguez-Morató J, Olesti E, Pujadas M, Pérez-Mañá C, Khymenets O, Fitó M,

Covas MI, Solá R, Motilva MJ, Farré M, de la Torre R (2016) Analysis of free hydroxytyrosol in human plasma following the administration of olive oil. J Chromatogr A 1437:183-190

50. Crespo MC, Tomé-Carneiro J, Dávalos A, Visioli F (2018) Pharma-Nutritional Properties of Olive Oil Phenols. Transfer of New Findings to Human Nutrition. Foods 7(6)

51. Visioli F, Wolfram R, Richard D, Abdullah MI, Crea R (2009) Olive phenolics increase glutathione levels in healthy volunteers. J Agric Food Chem 57(5):1793-1796

52. Léger CL, Carbonneau MA, Michel F, Mas E, Monnier L, Cristol JP, Descomps B (2005) A thromboxane effect of a hydroxytyrosol-rich olive oil wastewater extract in patients with uncomplicated type I diabetes. Eur J Clin Nutr 59(5):727-730

53. Martínez N, Herrera M, Frías L, Provencio M, Pérez-Carrión R, Díaz V, Morse M, Crespo MC (2019) A combination of hydroxytyrosol, omega-3 fatty acids and curcumin improves pain and inflammation among early stage breast cancer patients receiving adjuvant hormonal therapy: results of a pilot study. Clin Transl Oncol 21(4):489-498

54. Albini A, Festa MMG, Ring N, Baci D, Rehman M, Finzi G, Sessa F, Zacchigna S, Bruno A, Noonan DM (2021) A Polyphenol-Rich Extract of Olive Mill Wastewater Enhances Cancer Chemotherapy Effects, While Mitigating Cardiac Toxicity. Front Pharmacol 12:694762

55. Cappelli K, Ferlisi F, Mecocci S, Maranesi M, Trabalza-Marinucci M, Zerani M, Dal Bosco A, Acuti G (2021) Dietary Supplementation of Olive Mill Waste Water Polyphenols in Rabbits: Evaluation of the Potential Effects on Hepatic Apoptosis, Inflammation and Metabolism through RT-qPCR Approach. Animals (Basel) 11(10):2932.

56. Tundis R, Conidi C, Loizzo MR, Sicari V, Romeo R, Cassano A (2021) Concentration of Bioactive Phenolic Compounds in Olive Mill Wastewater by Direct Contact Membrane Distillation. Molecules 26(6)

57. Yates AA, Erdman JW, Jr., Shao A, Dolan LC, Griffiths JC (2017) Bioactive nutrients - Time for tolerable upper intake levels to address safety. Regul Toxicol Pharmacol 84:94-101

58. Babich H, Visioli F (2003) In vitro cytotoxicity to human cells in culture of some phenolics from olive oil. Farmaco 58(5):403-407

59. Auñon-Calles D, Canut L, Visioli F (2013) Toxicological evaluation of pure hydroxytyrosol. Food Chem Toxicol 55:498-504

Table 1. Compositinal data of OMWW in this study.

Polyphenols	Content
Total polyphenols	≥ 30%
Hydroxytyrosol	> 6%
Tyrosol	> 1%

Table 2. Effects of OMWW and HT on metabolic parameters and blood pressure.

	Control	OMWW	HT	P-value
Body weight, g	39.0±1.6	37.6±1.5	35.5±1.3	0.27
Total cholesterol, mg/dl	1374±46.6	1355±60.9	1422±106.7	0.82
Triglyceride, mg/dl	176.1±17.3	137.5±12.5	157.4±22.9	0.34
HDL-cholesterol, mg/dl	77.4±4.9	79.6±4.1	81.4±2.6	0.79
LDL-cholesterol, mg/dl	1241±48.5	1219±37.7	1242±49.1	0.92
Blood glucose, mg/dl	157.6±9.7	145.5±6.3	155.3±11.2	0.61
Heart rate, bpm	664.4±18.7	676.3±16.9	648.9±18.8	0.54
Systolic BP, mmHg	114.9±5.6	114.3±1.4	110.9±3.6	0.69
Diastolic BP, mmHg	78.5±3.8	72.8±3.3	68.1±1.9	0.08

HDL, high-density lipoprotein; LDL, low-density lipoprotein; BP, blood pressure. All values are mean ± SEM.

Figure legends

Figure 1. Attenuation of atherogenesis by OMWW and HT.

En face Sudan IV staining demonstrated that administration of OMWW or HT to WTD-fed ApoE$^{-/-}$ mice for 20 weeks significantly reduced the development of atherosclerosis in the aortic arch (n=8-10, per group). There was no difference between the two treatment groups. Scale bar: 1 mm. **; P<0.01 and ***; P<0.001. All values are mean ± SEM.

Figure 2. Attenuation of oxidative stress in the aorta by OMWW and HT.

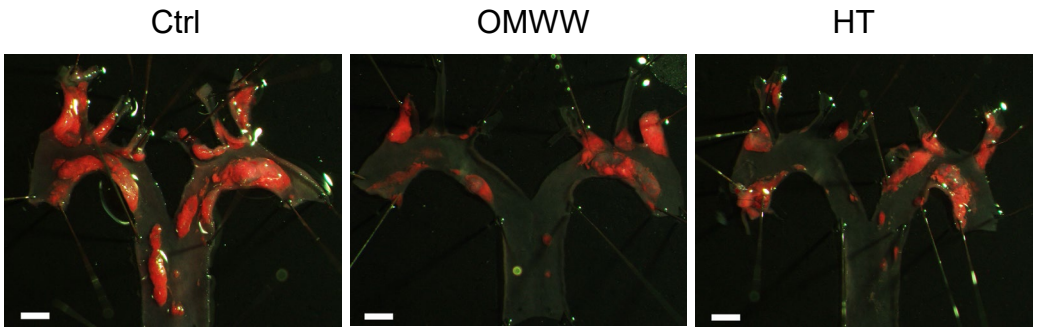
(A) Administration of OMWW or HT to WTD-fed ApoE$^{-/-}$ mice for 20 weeks decreased the production of O_2^- in the aorta compared with the control group (n=8-10, per group). There was no difference between the two treatment groups. **(B)** Administration of OMWW or HT to WTD-fed ApoE$^{-/-}$ mice for 20 weeks decreased the expression of NADPH oxidase subunits in the aorta (n=8-10, per group). *; P<0.05, **; P<0.01, and ***; P<0.001. All values are mean ± SEM.

Figure 3. Attenuation of expression of inflammatory molecules in the aorta by OMWW and HT.

Administration of OMWW or HT to WTD-fed ApoE$^{-/-}$ mice for 20 weeks decreased the expression of inflammatory molecules in the aorta. (n=8-10, per group). *; P<0.05, **; P<0.01, and ***; P<0.001. All values are mean ± SEM.

Figure 4. Attenuation of pro-inflammatory activation of macrophages by HT.

qPCR demonstrated that LPS promoted the expression of inflammatory molecules in a murine macrophage cell line, RAW264.7, which was attenuated by pre-incubation with HT. (n=12, per group). *; P<0.05, **; P<0.01, and ***; P<0.001. All values are mean ± SEM.



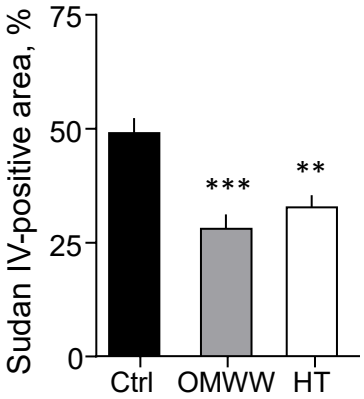
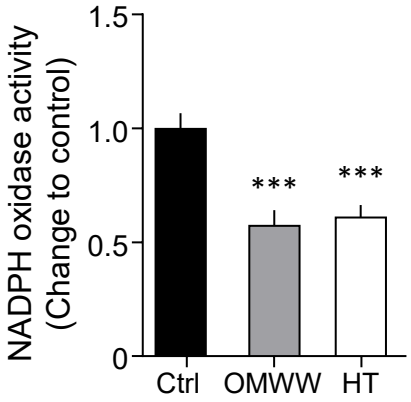
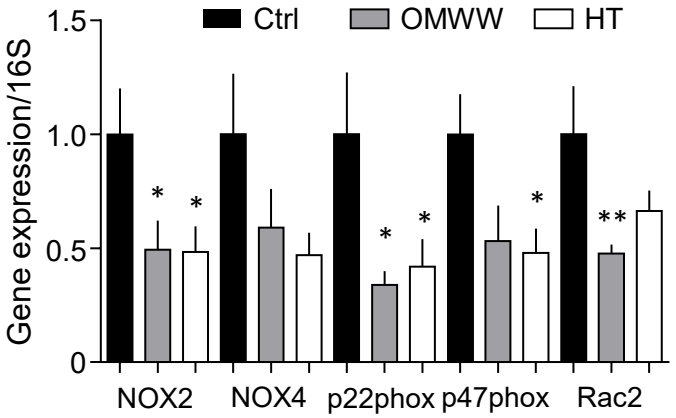


Figure 1

A


NADPH oxidase activity
(Change to control)
1.5
1.0
0.5
0
***
***
Ctrl
OMWW
HT

B


Ctrl
OMWW
HT
Gene expression/16S
1.5
1.0
0.5
0
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NOX2
NOX4
p22phox
p47phox
Rac2

Figure 2

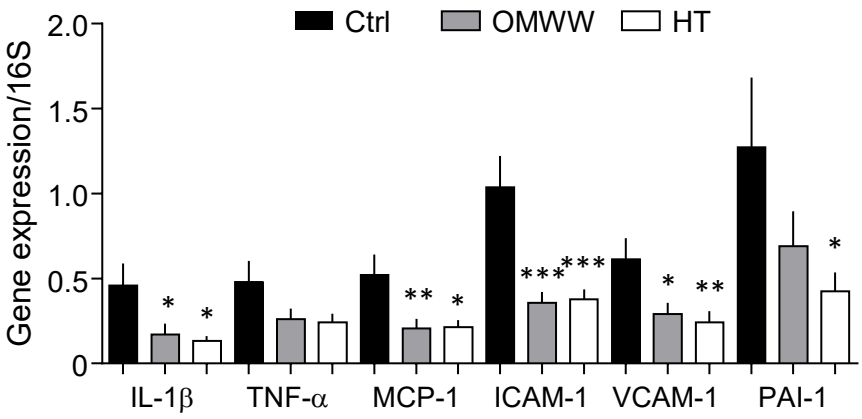
Ctrl
OMWW
HT
Gene expression/16S
2.0
1.5
1.0
0.5
0
IL-1β
TNF-α
MCP-1
ICAM-1
VCAM-1
PAI-1

Figure 3

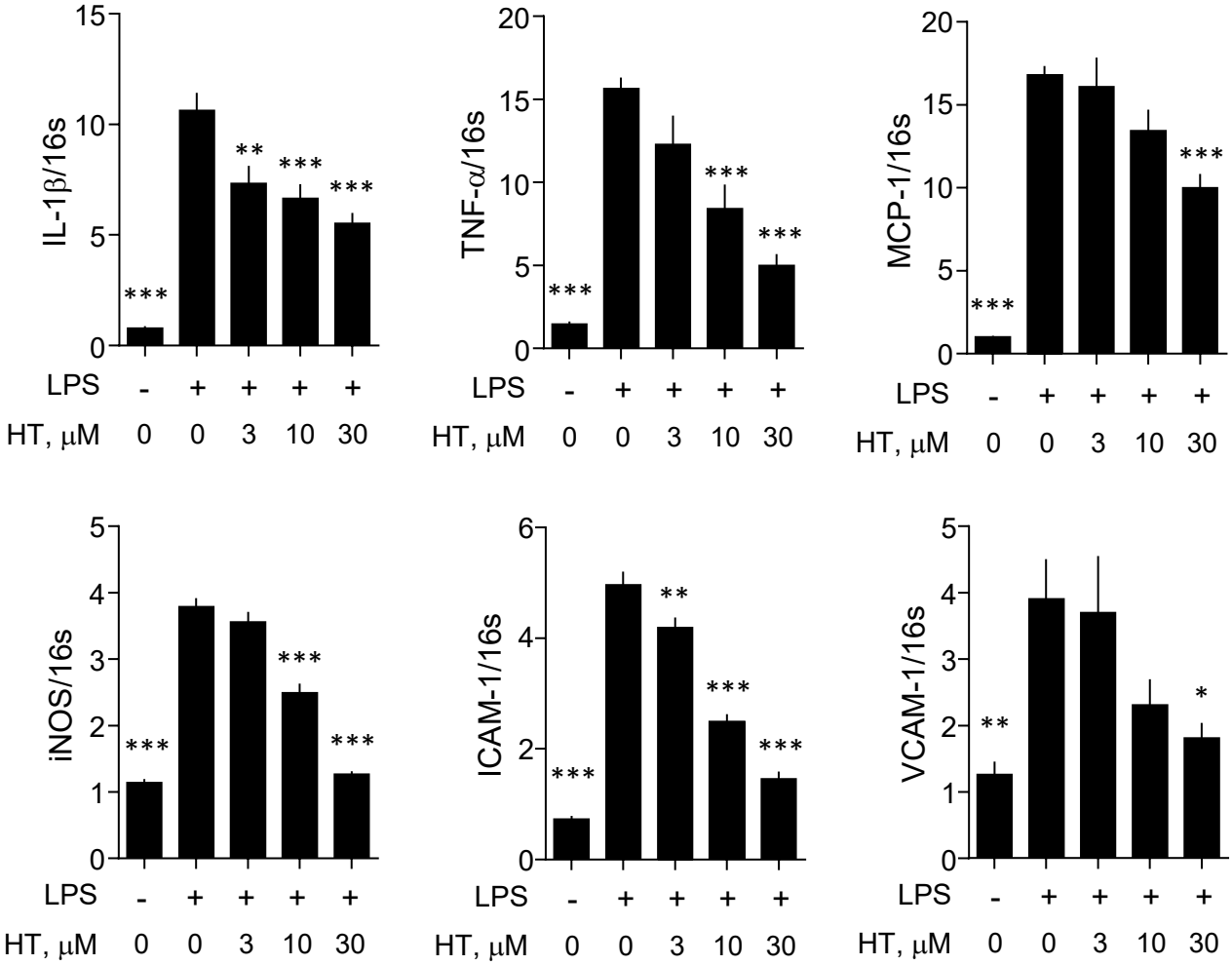
IL-1β/16s
TNF-α/16s
MCP-1/16s
iNOS/16s
ICAM-1/16s
VCAM-1/16s
LPS - + + + +
HT, μM 0 0 3 10 30

Figure 4