Effect of Olive Oil Phenolics on Lipidemic Profile and Oxidative Stress in Mice

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Introduction

The World Health Organization (WHO) cites Cardiovascular Diseases (CVD) as one of the main causes of death worldwide [1,2]. Free radicals are known to play a catalytic role in the etiopathogenesis of CVD, resulting in oxidative stress that initiates lipid oxidative reactions [3]. Major modifications of Low Density Lipoprotein (LDL) and High Density Lipoprotein (HDL) are due to lipid peroxidation [4]. The resulting oxidized products are principal contributors to atherosclerotic plaque formation [5–7]. For example, Atherosclerosis is an inflammatory disease that affects the arterial wall [8] and is characterized by high lipid concentration and inflammatory cells in the intima of large arteries [9,10]. Hypercholesterolemia is characterized by elevated LDL cholesterol that triggers its deposition to the arterial wall and then to the intima [11,12] where it is easily oxidized by the abundant free radicals in the intercellular environment. Oxidized LDL (oxLDL) induces pro-inflammatory reactions via various regulators, e.g. TNF-α, IL-1 and macrophage colony-stimulating factor (MCSF), maintaining activation and relocation of inflammatory cells to the arterial intima [13,14], and contributes to the formation of the fibrous cap of atherosclerotic plaque [15,16].

Metabolic Syndrome (MS) represents an important public health challenge worldwide [17]. MS is a group of closely related to CVD risk factors that include insulin resistance, hyperglycaemia, low HDL cholesterol and elevated Very Low Density Lipoprotein (VLDL) triglycerides. Type II Diabetes, also a common worldwide health disorder is characterized by insulin resistance, hyperglycaemia, and hyperlipidaemia, with the resultant contribution to CVD. Indeed, Diabetes and CVD are two major risk factors for MS and thus, their metabolisms, particularly aberrant lipid metabolism [18], are inextricably linked. Diabetes increases the risk for CVD approximately two-fold.

Lifestyle modifications are routinely recommended to reduce CVD risk. Nutritional factors, in particular, play an important role in the prevention of CVD, diabetes, and MS. Nutrients, rich in antioxidants and poly-/mono- unsaturated fatty acids, such as olive oil, reduce the risk of CVD development [9]. Olive oil, a main component of the Mediterranean Diet, is one of the best energy sources that protect health [19,20]. Its prophylactic role against CVD is due to its multiple beneficial effects. It improves glucose metabolism [21], regulates the lipemic profile and blood pressure, preserves the endothelium normal function and reduces oxidation of LDL cholesterol by suppressing inflammatory phenomena.

The protective role of olive oil is based on its high concentration in ω-9 monounsaturated fatty acids (MUFA), mainly the oxidation-resistant oleic acid [9,22]. Additionally it contains high levels of phenolic antioxidants such as hydroxytyrosol and its derivatives which protect significantly against blood lipid oxidation according to EFSA [23] and Commission Regulation (EEC) [24]. If an olive oil contains more than 5 mg of hydroxytyrosol derivatives per 20 g it can have an officially recognized health claim in the European Union. Compared to other sources of oleic acid like sunflower oil, olive oil is more effective in decreasing the levels of cholesterol and inhibiting platelet activation, demonstrating the importance of its phenolic composition [25,26]. MUFAs, in combination with phenolics, are able to significantly decrease the levels of the oxidized lipoproteins and thus CVD risk [26].

Abstract

Free radical mediated oxidative stress initiates lipid oxidative reactions that play a major role in the pathogenesis of cardiovascular disease (CVD). CVD and diabetes are major risk factors in Metabolic Syndrome (MS) and their metabolisms are inextricably linked. Diabetes increases the risk of CVD approximately two-fold. One major and effective therapy for MS is lifestyle modifications including adoption of the Mediterranean diet. The latter is rich in olive oil, a monounsaturated fat that may contain phenols which prevents lipid oxidation. Thus, the protective effect on oxidative stress and lipemic profile of four olive oils was examined in mice, in relation to phenolic content (oleocanthal and oleacein) and in comparison to sunflower oil, a monounsaturated fat containing about 3–4 fold less monounsaturated fatty acids (MUFA) than olive oil. Mice were fed a high fat diet (50 days) followed by streptozotocin administration (40 days) to induce a diabetic state. All mice were hyperlipidemic by day 50. Total cholesterol (TC) LDL cholesterol, triglycerides, uric acid, TBARS, and oxidized LDL (oxLDL) were analyzed. After streptozotocin administration, TC and LDL were significantly elevated in control groups, while oils rich in phenols restricted further elevation. Triglycerides remained stable until day 50, followed by a 3-fold reduction. Regarding sunflower oil, uric acid and oxLDL were elevated but olive oils kept uric acid and oxLDL unchanged while TBARS were decreased by day 50. In conclusion, after induction of oxidative stress, olive oils rich in phenols, especially oleocanthal, significantly protected against further oxidative stress on lipidemic parameters compared to sunflower oil.

Keywords: Cardiovascular disease; Olive oil; Oleocanthal; Oleacein; Oxidative stress; Lipidemic profile
There is a variety of phenolics identified in olive oil, with antioxidant and anti-inflammatory properties, mainly tyrosol, hydroxytyrosol and their esterified secoiridoid derivatives. The most important secoiridoids are the aglycone of oleuropein and ligstroside and their respective decarboxylated dialdehydic structures [27].

Oleocanthal, the dialdehydic form of decarboxylated aglycone of ligstroside, shows mainly antioxidant and anti-inflammatory action, imitating ibuprofen’s action due to its ability to inhibit the cyclooxygenases COX-1 and COX-2 activity [28]. These qualities contribute to therapeutic actions of olive oil, as an anti-inflammatory. Another important feature of oleocanthal is its ability to remain stable in gastric fluids, and exhibiting antimicrobial activity [29]. In addition, it seems to reduce aberrant metabolism occurring from MS [30].

Oleacein is the dialdehydic form of decarboxylated aglycone of oleuropein and its characteristics are very similar to oleocanthal. Oleacein displays a notable antioxidant capacity [31].

Although the protective role of olive oil phenolics has been officially recognised by EFSA in the EU, the unanswered question is if the effect is related to the total phenolic content or specific phenolic ingredients. The aim of this study was to examine the contribution of specific phenolic compounds like oleocanthal and/or oleacein to the protection of cardiac function by preventing blood cholesterolemia and oxidative stress.

**Materials and Methods**

**Animals and Experimental Groups**

Animal care was performed per the guidelines established by the European Council Directive 2010/63/EU. The experimental procedure was approved by the National Peripheral Veterinary Authority Animal Ethics Committee (721/08-02-2016). Fifty-four female SKH-2 brown hairless mice (25.69 ± 1.87 g) aged 13–14 weeks old were used for this study. All mice originated from the breeding stock of the School of Pharmacy Small Animal Laboratory (license EL 25 BIO 06). The animal room was kept at 24 ± 1°C, 45% humidity and illuminated by yellow fluorescent tubes in a 12 h cycle of light and dark. These lamps do not emit any measurable UV radiation. The mice were housed in 12 cages and had unrestricted continuous access to a high fat diet and fresh water. The basis of the diet (w/w) was 60% standard chow (Nuevo – Farma Efyra, Greece), and 40% fat (30% pure porcine fat and 10% of the oil under investigation). Sunflower oil was used as control. The composition of the olive oils under investigation is displayed in table 1. The exact concentration of the phenolics was determined with quantitative NMR spectroscopy as described previously [32] (Figure 1). This is the most accurate method to measure the phenolic content of olive oil where specific peaks correspond to clearly observable protons of the target compounds and that can be quantified using an integration tool and an internal standard.

**Dietary Groups and Treatments**

The mice were divided into 6 groups (Table 1) as follows: mice fed with sunflower oil and receiving streptozotocin (STZ) injection (group S1); mice fed with sunflower oil without STZ injection (group S2); mice fed with olive oil without phenolics (group WP), mice fed with olive oil containing only oleocanthal (group O); mice fed with olive oil that contained equal amounts of oleacein and oleocanthal (group OO); mice fed with olive oil that contained a double amount of phenolics in comparison to O and OO including both oleocanthal and oleacein (group SOO). All olive oils were examined for acidity, peroxide value, K indices and were found to belong to the extra virgin category. Acidity ranged from 0.2–0.4, peroxide values ranged from 6–8, and K232 indexes ranged from 1.650–1.820. Sunflower oil was of commercial origin and was not tested.

On day 50 of the experiment diabetes was induced in all mice except subgroup S2 Diabetes induction was achieved by five

![Figure 1: 1H-NMR profile of the aldehydic region of each of the olive oils employed in the study. The profiles reflect the levels and proportions of oleocanthal (1) and oleacein (2) in comparison to the internal standard (I.S.). Trace WP is that for olive oil showing no measurable phenolic content. Trace O, olive oil showing high oleocanthal content. Trace OO, olive oil showing near equal levels of oleocanthal and oleacein levels. Trace SOO, olive oil showing highest levels of both phenols.](image)

<table>
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<th>Lipid Source</th>
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<th>Oleacein (mg kg⁻¹)</th>
<th>Total Phenolics *</th>
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<td>S2</td>
<td>Non-stressed Control</td>
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Table 1: Phenolic content of the oils examined in this study. * Free tyrosol, free hydroxytyrosol, oleuropein aglycones and ligstroside aglycones were measured at less than 10 mg kg⁻¹.
intraperitoneal injections (one per day) of STZ (35 mg kg⁻¹) solution in sodium citrate buffer (0.1M, pH 3.5–4.5). Blood was collected from the tail for blood glucose determination levels on days 5 and 8 after the STZ injection using a glucose monitoring system with electrochemical detector and strips (ABBOTT Precision Xtra Plus, USA). Mice were considered as diabetic when glucose levels were above 180 mg dl⁻¹ and in the presence of polydipsia and polyuria symptoms. In total, the experiment lasted 90 days.

During the experiment the monitored parameters were: total cholesterol (TC), triglycerides (TG), low density lipoprotein (LDL), oxidized LDL (oxLDL), uric acid and substances reacting with thiobarbituric acid (TBARS). The time-points for measurements were: 0, 50 and 90 days of the trial.

Biochemical Markers

Commercial kits provided by BIOSIS (Biotechnology Applications Ltd, Greece) were used to identify the biochemical markers. These kits use the cholesterol oxidase phenol 4-aminoantipyrine peroxidase (CHOD-PAP) and glycerol phosphate oxidase-p-aminophenazone (GPO-PAP) methods to determine total cholesterol and triglyceride levels respectively. Both of these methods use 4-aminophenazone to develop a colored complex that absorbs at 510 nm.

The levels of LDL cholesterol on day 50 and 90 were calculated according to the Friedewald equation \[33\]:

\[
LDL = TC - HDL - TG/5
\]

The BIOSYS (Greece) commercial kit was used at baseline as the parameter values were below those required for the Friedewald equation calculations. The assay is divided into two stages. First, all other cholesterol types are degraded with the CHOD-PAP enzymatic system and then LDL cholesterol is determined using the same method.

Oxidative Stress Markers

Uric Acid

The determination of uric acid was made by a kit provided by BIOSIS (Biotechnology Applications Ltd, Greece) based on the Fossati method \[34\].

TBARS

The TBARS are derivatives of lipid peroxidation. To determine the levels of lipid peroxidation (as malonyldialdehyde equivalents, MDA), a slight modification of Keles. et al. method was followed \[35\]. 20 µl aliquots of sample or distilled water was placed in test tubes. 100 µl of trichloroacetic acid (TCA) 35% (Sigma-Aldrich, USA) and 100 µl Trizma (Tris-HCl 50mM, pH 7.4) (Sigma-Aldrich, USA) were added and the solution was rigorously vortexed, incubated for 10 minutes at room temperature and then 200 µl Na2SO4/TBA (0.75% thiobarbituric acid in 2 M Na2SO4) (Sigma-Aldrich, USA) were added to the tubes. The solution was incubated at 95°C in a water bath followed by transfer on to ice for five minutes. Finally, the tubes were centrifuged at 4,450 x g at room temperature. 200 µL of 75 % TCA were added and absorbance of the supernatant was determined at 530 nm for 8 minutes (Shimadzu UV-1700 Spectrophotometer, Japan) and quantities of TBARS determined from a reference standard.

OxLDL

OxLDL was assessed using an ELISA method provided by my BIOsource (San Diego, USA). All the data were collected from a Fluostar plate reader (Fluostar Galaxy BMG, Germany).

Statistics

Statistical analysis was performed in order to determine the effect of the respective dietary treatments on the biochemical and oxidative stress markers. Initially, the normality assumption, regarding the distribution of the data (i.e. the blood levels, the markers), were assessed. Since, the hypothesis of normal distribution was verified, the data were further analyzed using ANOVA and paired t-tests. Analysis of variance was utilized to explore the impact of the different treatments (namely, food effect) on the biomarkers. The homoscedasticity of data was evaluated by the Levene’s test. Since, in all cases the variances were found to be equal, two post-hoc criteria were applied to make comparisons between the treatments: the least significant difference (LSD) and the Bonferroni criterion. The paired t-test was applied to identify any differences between different time points (days 0, 50, and 90) of the experiment.

Figure 2: Average mouse weights throughout the experimental period. At day 0 the average mouse weight was 25.69 ± 1.87 g. The average weight had increased by at least 2 g at the final day of measurements (90 days).
Results

NMR provided the phenolic profile of the different olive oils used in the experiment (Figure 1, Table 1). Groups S and WP oils did not contain any oleacein and oleocanthal. Group O oil contained 520 mg kg⁻¹ oleocanthal and 22 mg kg⁻¹ oleacein. Group OO oil contained a similar proportion of oleacein (215 mg kg⁻¹) and oleocanthal, (217 mg kg⁻¹). Group SOO contained 697 mg kg⁻¹ oleocanthal and 241 mg kg⁻¹ oleacein.

All mice gained weight, on average two grams, throughout the experimental period (Figure 2). Food consumption was equal in all groups with a reported average intake of 3 g/day/mouse (Figure 3). In the first day of experimentation (baseline), all animals exhibited mean cholesterol levels of 46.33 mg dl⁻¹. Cholesterol in each group increased from the first to day 50, on average 119.6 mg dl⁻¹ (Figure 4). The elevated levels are statistically significant, as paired t-test indicated from the statistical analysis. In fact, there is a statistical difference between group O and groups OO and SOO, according to the ANOVA analysis, using the Bonferroni post-hoc criterion. On day 90 the levels of total cholesterol vastly increased in the cases of S1 (163.32 mg dl⁻¹), WP (158.41 mg dl⁻¹) and OO (144.58 mg dl⁻¹). However, the elevation recorded in the cases of S2 (114.86 mg dl⁻¹), O (118.88 mg dl⁻¹) and SOO (120.04 mg dl⁻¹) was diminished.

It is worth noting that the differences in cholesterol levels between S1, WP and S2, O, SOO are statistically significant (p < 0.05) per application of the Bonferroni post-hoc criterion of the ANOVA analysis.

Triglyceride results are shown in figure 5. The levels on day 50 remained stable at around 138.48 mg dl⁻¹ in comparison to baseline as per application of the paired t-test. However, these levels dropped to 53.32 mg dl⁻¹ on day 90 in all treated groups with the exception of S1 (125.5 mg dl⁻¹).

Figure 3: Average daily food intake. The average daily food intake was 3 gr/day/mouse. This rate was stable throughout the experimental period, allowing comparison of the biological effect of the constituents of the different diets.

Figure 4: Cholesterol levels. By day 50 of the study the levels of total cholesterol were doubled in all groups. Left bar-day 0; mid bar-day 50; right bar-day 90. At day 90, the end of the experimental period, oils O and SOO maintained cholesterol at the same levels as group S, the non-oxidative stressed control *. Statistical significance compared to O group (p < 0.05) at day 50 and S, group (p < 0.01) at day 90. Significance determined by Bonferroni criterion of ANOVA analysis.
Figure 5: Triglyceride levels. Left bar-day 0; mid bar-day 50; right bar-day 90. Triglyceride levels remained unchanged in all groups between day 0 and 50. However, between day 50 and day 90 clearance occurred and final levels were statistically significant* \((p < 0.05)\) for all groups compared to group S1. Significance was determined according to Bonferroni criterion of ANOVA analysis.

Figure 6: LDL Cholesterol levels. Left bar-day 0; mid bar-day 50; right bar-day 90. Levels were increased in all groups by day 50. Oils of groups O and SOO restricted increases in levels by day 90. Statistical significance* \((p < 0.05)\) compares groups to group WP. Significance was determined according to Bonferroni criterion of ANOVA analysis.

The trend of LDL cholesterol was quite similar to that of total cholesterol (Figure 6). There was a significant increase between day 1 and day 50 in all groups. Variations in the levels on days 50 and 90 were dependent on phenolic content.

The S1 group showed a significant increase in uric acid up to 7.84 mg dl\(^{-1}\) on day 90. However, the levels of uric acid remained stable in all other groups throughout the experimental period (Figure 7).

The levels of oxLDL on day 50 (27.24 \(\mu\)g ml\(^{-1}\)) are generally similar when compared to day one of experimentation (31.84 \(\mu\)g ml\(^{-1}\)) (Figure 8). On day 90 there was an increase in all groups, which is indicative of significant differences between groups S1 (48.12 \(\mu\)g ml\(^{-1}\)) and SOO (43.82 \(\mu\)g ml\(^{-1}\)) by using the LSD post hoc criterion during ANOVA analysis.

On day 50 the control S1 group showed an average TBARS level of 6.76 \(\mu\)molL\(^{-1}\) while the phenol rich oils in groups O (2.13 \(\mu\)molL\(^{-1}\)) and SOO (2.27 \(\mu\)molL\(^{-1}\)) were statistically different \((p < 0.05)\) according to LSD post hoc criterion for the ANOVA analysis. On day 90, the mean levels of all groups were back to baseline values (6.85 \(\mu\)molL\(^{-1}\)) (Figure 9).

**Discussion**

Food consumption was equal in all groups and independent of the type of the fat intake, a fact that facilitated the comparison of the biological effect of the constituents of the different diets. A significant increase of total cholesterol is observed in all groups on day 50 (Figure 4). Significant differences between the controls S1, S2, WP and the oleacein and/or oleocanthal containing oils (O, OO, SOO) were not observed, an observation that may indicate the beneficial effect of oils, rather than their antioxidant content. This is in accordance with the observations of other studies where phenolics do not seem to play an important role in the reduction of the cholesterol plasma levels [36,37]. However, the lower cholesterol levels of groups OO and SOO, when compared to group O, indicate a beneficial action in the presence of oleacein.

On day 90 the STZ injection changes the above trend, indicating also the close relation to oxidative stress and hyperlipidemia.
[17]. The lowest total cholesterol levels are reported for groups O and SOO and are very close to the control group S2, in which the mice are not treated with STZ. This is logical as the cytotoxicity of STZ is due to a free radical mechanism [38,39] and the phenolic antioxidants are known to drastically reduce oxidative stress [40]. It appears that the oils in groups O and SOO, rich in phenolics,
especially in oleocanthal, effectively prevent cholesterol elevation. Oleocanthal exhibits a great potential in health protection by its anti-inflammatory, antiplatelet, amylol - β clearance and antioxidant properties [41–43]. In the case of SOO oil a part of the activity could also be due to oleocanthen content which also possesses significant anti-inflammatory and antioxidant properties [43]. The non-phenolic content oils WP and S1 show the highest levels of cholesterol, much higher than before the STZ administration. According to other studies total cholesterol, in the presence of oxidative stress, appears to decrease in relation to the antioxidant capacity of the administered substances, in the presence of the oxidative stress [40].

The high fat diet did not influence the levels of triglycerides for the relatively long 50 day study period (Figure 5). This is in accord with previous observations that serum triglyceride levels do not benefit from olive oils [45]. However, for the longer period of 90 days there is an important decrease of triglycerides (on average 1/3 of the baseline levels in all groups except for the sunflower oil group S1) (Figure 5). This effect has previously been observed and described as “triglyceride clearance” [41]. This effect is likely due to overexpression of lipoprotein lipase (LPL), although in the case of sunflower oil, STZ toxicity probably overwhelms the organism’s homeostasis to the extent that this clearance is unsuccessful.

LDL cholesterol exhibited similar results as that of total cholesterol at both days 50 and 90 (Figure 6 and 2, respectively). At day 50, a significant increase in LDL levels occurred in all groups except for SOO. On day 90, LDL cholesterol showed a significant elevation in groups S1, WP and O but not in S2, O and SOO. This is indicative that LDL levels are oxidative stress dependent, the latter resulting from STZ injection. The relative characteristic of oils O and SOO, compared to the others, is their high oleocanthal content. Thus, this effect could be attributed to high antioxidant activity. In fact, this constituent is recognized as conveying general health benefits [46–48].

The role of uric acid in the etiopathogenesis of CVD remains unknown but in type II diabetes high cholesterol levels are associated with high uric acid content [49]. Under our experimental setup, uric acid levels are stable up to day 50 (Figure 7). This finding is in accordance with other reports whereby hypercholesterolaemia is independent of uric acid content [50]. On day 90 there is a significant increase in uric acid levels in group S1 while the levels in S2 remained stable. It is known that STZ administration leads to oxidative stress. In the case of S1 and WP, more uric acid is synthesized as a protective reaction to oxidative stress. This would not be required in group S2 where no oxidative stress is induced or in groups O, O and SOO which contain significant levels of phenolic antioxidants.

Lipid peroxidation, as estimated by TBARS level, remained stable between day 1 and day 50 for the sunflower oil groups (S1 and S2) and decreased for the olive oil groups, especially in cases of O and SOO with high oleocanthal content (Figure 9). TBARS showed an increase on day 90 because of induced oxidative stress by STZ injection.

On day 50 the oxLDL remained stable or decreased in all the groups (Figure 8), although differences between SOO and S1 approached statistical significance (p < 0.06). On day 90 the oxLDL levels were mostly elevated indicating STZ-induced oxidative stress had overwhelmed the antioxidant capacity to prevent the oxidation of LDL.

In toto, these data clearly demonstrate that olive oils, particularly those containing significant levels of phenolic antioxidants, e.g. oleocanthal, are responsible for the protective health effects attributed to these oils. The data presented in this study also provide the rationale for recommendations of the Mediterranean diet to lower CVD and MS risks. As noted, the protective effects of olive oil reside not only in its antioxidant capacity but in its fatty acid composition. Olive oil contains almost four times the level of MUFA as sunflower oil and nearly eight times less PUFA. The latter are prime targets for free radical initiated lipid oxidation. Both antioxidant capacity and fatty acid composition act in concert to convey olive oil’s protective health benefits including potentially significant protection against CVD.

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Conflict of Interest

All the authors declared that they have no conflict of interest

References


