Comparative evaluation of the cardio-metabolic effects of hydroxytyrosol and its lipophilic derivatives (hydroxytyrosyl acetate and ethyl hydroxytyrosyl ether) in hypercholesterolemic rats

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<th>Journal:</th>
<th>Food &amp; Function</th>
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<td>Manuscript ID:</td>
<td>FO-ART-12-2013-060677.R1</td>
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<tr>
<td>Article Type:</td>
<td>Paper</td>
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<td>Date Submitted by the Author:</td>
<td>15-Mar-2014</td>
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Comparative evaluation of the metabolic effects of hydroxytyrosol and its lipophilic derivatives (hydroxytyrosyl acetate and ethyl hydroxytyrosyl ether) in hypercholesterolemic rats

María Taberneró, Beatriz Sarriá, Carlota Largo, Sara Martínez-López, Andrés Madrona, José Luis Espartero, Laura Bravo, Raquel Mateos.

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Running title: Metabolic effects of hydroxytyrosol and its lipophilic derivatives

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Abstract

Hydroxytyrosol (HT), a virgin olive oil phenolic phytochemical with proven health benefits, has been used to generate new lipophilic antioxidants to preserve fats and oils against autoxidation. The aim of this work is to comparatively evaluate the physiological effects of HT and the lipophilic derivatives, hydroxytyrosyl acetate (HT-Ac) and ethyl hydroxytyrosyl ether (HT-Et) in a high-cholesterol fed animals. Male Wistar rats (n=8) were fed a standard diet (C group), a cholesterol-rich diet (Chol group) or cholesterol-rich diets supplemented with the phenolic compounds (HT group, HT-Ac group and HT-Et group) for 8 weeks. Body and tissue weights, lipid profile, redox status, and biochemical, hormonal, and inflammatory biomarkers were evaluated. Plasma levels of total, LDL-cholesterol, glucose, insulin and leptin, as well as malondialdehyde in serum increased in Chol compared to C (p<0.05). Rats fed the test diets improved their glucose, insulin, leptin and MDA levels and antioxidant capacity status, being HT-Ac the most effective compound. The studied phenolic compounds also modulated TNF-α and IL-1β plasma levels compared to Chol. HT-Ac and HT-Et improved adipose tissue distribution and adipokine production, decreasing MCP-1 and IL-1β levels. Our results confirm the metabolic effects of HT, which are maintained and even improved by the hydrophobic derivatives, particularly in HT-Ac.

Keywords: Ethyl hydroxytyrosyl ether; hydroxytyrosol; hydroxytyrosyl acetate; hypercholesterolemic rats; metabolic stress.
1. Introduction

The most representative phenolic compound in virgin olive oil, hydroxytyrosol (HT), plays an important role in the prevention of degenerative diseases\(^1\)\(^2\). Explicitly, it is involved in the reduction of LDL oxidation, a well-known cardiovascular risk factor\(^3\), and it is transiently associates with LDL lipoproteins\(^4\). Moreover, HT is involved in the inhibition of lipid and protein oxidation in human plasma\(^5\), having anti-inflammatory\(^6\) and antiplatelet aggregation activities\(^7\). Additionally, there is evidence that olive oil phenolic compounds decrease plasma glucose levels in rats\(^8\)-\(^9\). HT can be efficiently recovered from olive by-products, which has prompted the study of the biological and health effects of HT from alperujo\(^8\),\(^10\) or olive leaves.\(^9\),\(^11\)

On the other hand, the food industry demands new lipophilic antioxidants to preserve fats and oils against autoxidation. Two series of hydrophobic derivatives of HT, hydroxytyrosyl esters\(^12\) and hydroxytyrosyl ethers\(^13\), have been synthesized. Hydroxytyrosyl acetate (HT-Ac) merits special interest among hydroxytyrosyl esters, since it is an antioxidant naturally present in virgin olive oil\(^14\) that is transported across the small intestinal epithelial cell barrier more efficiently than HT,\(^15\) showing a higher hepatic bioavailability than HT\(^16\). Moreover, HT-Ac has shown protective effects against oxidative DNA damage in blood cells\(^17\), iron-induced oxidative stress in human cervical cells,\(^18\) and oxidative stress in HepG2 cells\(^19\). Hydroxytyrosyl ethyl ether (HT-Et), with an alkyl chain with the same length as the acyl chain in HT-Ac, was included in this study to assess the influence of the different functional groups (etherification versus esterification) on the biological activity of the two phenolic compounds. HT-Et is absorbed to a higher extent in Caco-2 cells than its precursor HT,\(^20\) with absorption rate similar to that of HT-Ac,\(^15\) being also broadly taken-up by HepG2 cells\(^21\). In addition, HT-Et protects hepatic human HepG2 cells against oxidative stress\(^22\) and inhibits platelet activation after oral
administration in rats\textsuperscript{23}. These antecedents justify using this compound although it is not naturally present in virgin olive oil.

Both HT-Ac and HT-Et maintain the orthodiphenolic group intact and show higher antioxidant capacity than HT\textsuperscript{24,25}, preserving their potential application as antioxidants to stabilize foodstuffs or as functional food ingredients.

With these antecedents, the aim of the present study was to comparatively analyse the ability of HT and its lipophilic derivatives, HT-Ac and HT-Et, counteracting the metabolic deregulation derived from consuming a high-cholesterol diet, focusing on the effects on plasma lipids, glucose levels, hormone response, oxidative stress and pro-inflammatory status. In addition, body weight gain, adipose tissue distribution and secretion of adipokines from visceral fat were evaluated.

2. Experimental

2.1. Materials and Chemicals

Cholesterol, cholic acid, palmitic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxilic acid (Trolox), 1,1,3,3-tetraethoxypropane, fluorescein, 2,2’-azobis(2-amidinopropane) dihydrochloride (AAPH), trizmabase, dithiothreitol, 1,1,3,3-tetraethoxypropane and 2,4-dinitrophenylhydrazine were purchased from Sigma-Aldrich Chemical (Madrid, Spain). Sodium hydroxide, sulphuric acid, sodium hydrogen phosphate and potassium dihydrogen phosphate were from Panreac (Madrid, Spain). The Bradford reagent was from BioRad (BioRad Laboratories S.A., Madrid, Spain). Free fatty acids (FFA) colorimetric kit was purchased from Roche (Roche Applied Science, Madrid Spain). Rat adipocyte (RADPCYT-82K, MilliplexMap Rat Adipocyte Panel) and adipokine (RADPK-81K, MilliplexMap Kit Rat Serum Adipokine Panel) Milliplex kits were acquired from
Millipore (Millipore, Billerica, MA, US, USA). Other reagents were of analytical or chromatographic quality.

HT was isolated with 98% purity from olive oil waste water following a patented procedure and further purified by column chromatography.

HT-Ac was obtained from HT in ethyl acetate after incubation with p-toluenesulfonic acid and purification by column chromatography following a patented procedure.

HT-Et was obtained from HT by chemical synthesis as described elsewhere.

2.2. Diet, animals and experimental design

Forty male Wistar rats (200–225 g body weight) were purchased from an accredited supplier (Charles River Laboratorios España, S.A. Barcelona, Spain) and housed in metabolic cages. They had free access to food and water, and were maintained under a normal light–dark cycle in the Experimental Surgery Service of La Paz University Hospital (registration number: 280790001941). After one week of acclimation, animals were randomly distributed into 5 different experimental groups (8 rats per group). One group received the standard, maintenance rodent diet (A04-SAFE, Augy, France), which is the control (C group), and four groups were fed the standard diet supplemented with 2% cholesterol and 0.4% cholic acid in order to generate the hypercholesterolemic model as described elsewhere. In three of these groups the hypercholesterolemic diet was supplemented with 0.04% of HT (HT group), HT-Ac (HT-Ac group) and HT-Et (HT-Et group), respectively, to assess the potential beneficial effects of these phenolic compounds in the hypercholesterolemic rat model. Nutritional and energetic content of each diet is summarized in Table 1. During the whole intervention, animal health status and dietary tolerance were monitored by veterinary individual observation in addition to weekly control.
of animals’ weight (Table 2). After 8 weeks animals were subjected to complete
exsanguination under general anesthesia (isoflurane 2%). Plasma and serum fractions were
separated by centrifugation (10 minutes at 2500 g) using pretreated EDTA or Silica Act Cot
Activator blood collection tubes (BD Vacutainer, Plymouth, UK) respectively, and stored at -
20°C until further analysis. Liver, kidney, heart and adipose tissue from retroperitoneal and
epididymal areas were collected, weighed and washed in ice-cold phosphate buffered saline.
Samples were immediately frozen in liquid nitrogen and stored at -80°C until analysis. The
study protocol was approved by the Institutional Animal Ethics Committee of La Paz
University Hospital (Madrid, Spain) and procedures were performed in accordance with
Spanish law for the protection of experimental animals and other research purposes (RD
53/2013, BOE nº 34 Sec I pg 11370 8th February 2013).

2.3. Blood biochemical analyses

Triglycerides, total cholesterol, LDL-cholesterol, HDL-cholesterol, glucose and
creatine in plasma were determined using an automated analyser (Beckman Coulter-Former
Olympus Diagnostics AU 5420, Nyon, Switzerland). Plasma free fatty acids (FFA)
concentration was determined with a colorimetric commercial assay kit (Free Fatty Acids,
Half Micro Test, Roche Applied Science) using palmitic acid as standard.

2.4. Determination of oxygen radical scavenging capacity (ORAC) in serum.

Serum antioxidant activity was analysed using the hydrophilic oxygen radical
scavenging capacity (ORAC) assay according to the method developed by Huang, Ou,
Hampsch-Woodill, Flanagan& Prior28. The fluorescence at 485 and 528nm excitation and
emission wavelengths, respectively, was determined in a 96 well microplate reader (Bio-Tek,
Winooski, VT, USA). ORAC values were expressed as micromoles of Trolox / mL serum.
2.5. Determination of Malondialdehyde (MDA) in serum and liver.

MDA was determined as its hydrazone by high-performance liquid chromatography using 2,4-dinitrophenylhydrazine for derivatization. Livers (0.5g) were homogenized (1:5 w/v) in ice-cold 0.25M Trizma base buffer pH 7.4 containing 5mM dithiothreitol using an Ultra Turrax (IKA® Works Inc., Wilmington, NC) at 18000 rpm. After centrifugation (11000rpm 30min, 4°C), supernatants were collected for MDA quantification. Serum samples were analysed directly. Standard MDA was prepared by acidic hydrolysis of 1,1,3,3-tetraethoxypropane in 1% sulphuric acid. Concentrations were expressed as nanomoles of MDA per milligram of protein in liver tissue and per millilitre in serum. Protein content in liver homogenates was estimated by the Bradford method using a Bio-Rad protein assay kit.

2.6. Determination of cytokines and hormones in plasma and adipose tissue by immunoassay.

A sample of white adipose tissue from the retroperitoneal area (0.2g) was collected and homogenized (1:3.5 w/v) in PBS (pH 7.4) with Triton X-100 and protease inhibitor by low temperature sonication (17 microns, 3 consecutive pulses of 15 seconds at 30 seconds intervals). After centrifugation (5000rpm, 10min, 4°C), the lipid layer was removed and supernatants were collected for adipokine determination. The concentration of leptin, interleukin-1β (IL-1β), interleukin-6 (IL-6), tumour necrosis factor-α (TNF-α), monocyte chemoattractant protein-1 (MCP-1) and plasminogen activator inhibitor-1 (PAI-1) in the retroperitoneal adipose tissue were determined using a Rat Adipocyte Multiplex Kits (RADPCYT-82K Milliplex Map Kit, Rat Adipocyte Panel, Millipore, Billerica, MA, US). IL-1β, IL-6, TNF-α, insulin and leptin concentrations were determined in plasma samples obtained at the end of the intervention using a Rat Adipokine multiplex kits (RADPK-81K,
Milliplex Map Kit, Rat Serum Adipokine Panel, Millipore, Billerica, MA, US). Multianalyte profiling panels were used according to manufacturer’s instructions and analysed on a LuminexLX200 Analyzer. Data were analysed using the 3.1 xPONENT software (Millipore); high and low concentration quality controls were used with all the biomarkers. Protein content in adipose tissue homogenates was estimated by the Bradford method using a Bio-Rad protein assay kit.

2.7. Statistical analysis.

All data were expressed as the mean ± standard deviation (SD). Non parametric significant test (Kruskal-Wallis one-way analysis of variance and Mann-Whitney $U$ test) was performed to compare values. Differences were considered statistically significant when $p < 0.05$ and in Mann-Whitney $U$ test the significance was adjusted with a Bonferroni test. Data were analysed using the statistical package SPSSv.19.0 software (SPSS, Institute Inc, Cary, NC).

3. Results

3.1. Food intake, body weight gain and tissues weights

All diets provided equivalent amounts of carbohydrates and proteins. However, cholesterol-rich diets led to an excess of lipids resulting in an 8.5 Kcal/100g higher caloric intake. During the 8-week study period, animals in the five experimental groups had similar daily food intakes (approximately 20g/d).

Therefore, the groups consuming the cholesterol-rich diets ($Chol$, $HT$, $HT$-$Ac$ and $HT$-$Et$) presented a daily caloric excess of approximately 2 Kcal/day. However, this did not induce significant differences in the body weight gain compared with the control animals, as can be seen in Table 2.
Considering an average daily food intake of 20g and an average body weight of 300g, the daily phenolic compound intake was 25mg/kg body weight/day. This is a relatively low dose, comparable with doses used in some other studies and within a range proven to elicit no toxic effects in rats. Accordingly, no pathologic alterations or behaviours were observed in the animals during the study. Organs, including kidney or heart among others, presented normal appearance by gross observation. However, livers in animals fed the cholesterol-rich diets showed a whitish appearance compared with the C group, suggestive of fat accumulation, being significantly heavier than those of the C group (Table 2). Adipose tissue depots were obtained from retroperitoneal and epididymal areas, and when their weights were normalized for the final animal body weight, no differences were observed among different treatments. However, the ratio obtained with the adipose tissue from retroperitoneal area/epididymal area showed significant differences, being higher in the Chol and HT groups than in the C group.

3.2. Plasma cholesterol profile

As Figure 1 shows, animals fed the cholesterol-rich diets had a marked hyperlipidaemia, with high LDL-cholesterol and total cholesterol concentrations, although HDL-cholesterol levels were not affected as compared to C group. Although not reaching the values of control animals, supplementation of the cholesterol-rich diet with 0.04% HT-Ac significantly decreased the total cholesterol and LDL-cholesterol levels as compared with the Chol group (p<0.05), which was not attained in the HT and HT-Et groups.

3.3. Biochemical parameters and hormones involved in energy homeostasis

The high-cholesterol diet used in the present study induced a significant increase in glucose levels in the Chol group compared to control animals (p<0.05). All the tested
phenolic compounds counteracted the glucose increase, with a partial effect of HT-Et whereas values in HT-Ac and HT groups returned to control levels (Table 3).

Triglycerides concentrations were similar in all groups, although phenolic compounds supplementation induced a moderate yet non-significant decrease. No differences were observed in plasma concentrations of free fatty acids, whereas creatinine concentrations were statistically higher in Chol and HT groups, compared to C, HT-Ac and HT-Et groups (Table 3).

After consuming the high-cholesterol diet for 8 weeks significant differences in plasma insulin and leptin concentrations were observed in Chol compared to C group. However, rats consuming diets supplemented with HT and HT-Ac had significantly lower plasma insulin levels than Chol, decreasing to control or even lower values as in the HT-Ac group (Table 3). HT-Et also presented lower insulin levels than Chol group although not statistically different (p=0.070), showing intermediate values between Chol and C groups. Leptin levels were lower in HT and HT-Et groups than in the Chol group, although not reaching values of control animals. Again, HT-Ac group had significantly lower plasma leptin levels than Chol group and in the range of the control values. In the retroperitoneal adipose tissue, leptin concentration was slightly lower in all cholesterol treated groups compared to the C animals, although not significantly different.

3.4. Redox status and inflammatory biomarkers in blood and tissue samples

Serum antioxidant activity (Table 4), analysed using the ORAC assay, was lower in Chol group compared to the control group without reaching the level of statistical significance. However, rats fed with HT and HT-Ac diets showed a significantly higher serum antioxidant activity compared with Chol group (p<0.05).
Regarding the biomarker of lipid peroxidation, Chol group showed MDA levels significantly higher than C animals in serum and liver (p<0.05). Supplementation with any of the studied phenolic compounds decreased MDA levels to control values in serum (Table 4). However, although the three phenolic compounds-supplemented groups showed lower hepatic MDA levels than Chol group, differences were statistically significant only in the HT-Et group (p<0.05) (Table 4).

Consuming the cholesterol-rich diet significantly increased the pro-inflammatory cytokine TNFα plasma concentrations compared to C group, effect that was totally counteracted by the three phenolic compounds (Table 4). A similar response was observed with IL-1β, with increased values in the Chol group returning to control levels in the HT, HT-Ac and HT-Et animals, although differences did not reach the level of statistical significance (Table 4). Plasma IL-6 was not affected by the cholesterol-rich diets or the phenolic supplementation.

Cytokine concentrations in the visceral adipose tissue from retroperitoneal area of the rats in the Chol group were similar to those in C group. TNFα and IL-6 concentrations were unchanged in all the experimental groups; however, IL-1β concentrations were lower in HT-Ac and HT-Et groups. Accordingly, MCP-1 showed significantly lower concentrations in HT-Ac and HT-Et groups compared to C group (Table 4). PAI-1 concentrations showed similar tendency, but differences did not reach statistical significance.

4. Discussion

In the present study the effects of HT were studied in comparison with HT-Ac and HT-Et, two lipophilic derivatives of HT with a higher intestinal bioavailability than their precursor. The evaluation of the biological properties of these three compounds was performed focusing on plasma lipids, energy homeostasis and oxidative and inflammatory
status in rats fed a cholesterol-rich diet. This study shows that HT and its hydrophobic
derivatives induce beneficial metabolic effects in hypercholesterolemic rats. Particularly,
HT-Ac showed the highest capacity to counteract the metabolic stress induced by the high-
cholesterol diet.

Supplementation of the diets with a moderate amount of phenolic compounds
(0.04%) resulted in a daily intake of 25mg/kg body weight, which is far beyond any possible
toxic level considering a recently published a study where consumption of up to 500
mg/kg/day was described as a No Observed Adverse Effects Level (NOAEL)\textsuperscript{30}.

As expected, feeding rats with a diet rich in cholesterol resulted in an increase in
total cholesterol and LDL cholesterol levels. In this hypercholesterolemic model, HT and
HT-Et showed modest plasma LDL-cholesterol lowering effects, in contrast to HT-Ac that
significantly reduced total and LDL-cholesterol levels compared to \textit{Chol} group. These results
are in line with previous human and animal studies showing that phenolic compounds present
in virgin olive oil exert beneficial cardiovascular effects, particularly improving lipid profile\textsuperscript{8,}
\textsuperscript{10,} \textsuperscript{31}. In fact, the European Food Safety Authority has recently issued a positive opinion on
olive oil phenolic compounds capacity to protect LDL cholesterol from oxidative damage\textsuperscript{32}.

Several markers show that rats in the present study suffered hepatic stress induced
by the cholesterol-rich diet, which may have led to an incipient insulin resistance\textsuperscript{2}.
Comparing the \textit{Chol} and \textit{C} groups, the high-cholesterol diet induced a significant increase in
glucose, creatinine, insulin and leptin plasma concentrations. In addition, \textit{Chol}
hypercholesterolemic animals presented higher liver weights than \textit{C} animals and the adipose
depots were mainly distributed in the retroperitoneal area. Interestingly, the diets
supplemented with phenolic compounds counteracted the glucose and insulin increase,
particularly HT and HT-Ac. This outcome is in accordance with previous studies showing
that HT and other olive oil phenolic compounds reduce plasma glucose concentration in
alloxan-diabetic rats\textsuperscript{8-9} by means of alleviating oxidative stress and free radicals as well as enhancing enzymatic defences. A recent paper evidenced the capacity of oleuropein aglycone in the prevention of cytotoxic amyloid aggregation of human amylin, a hallmark of Type-II diabetes\textsuperscript{33}. In accordance, the PREDIMED study showed that consuming a virgin olive oil-enriched traditional Mediterranean diet for three months decreased total and LDL cholesterol together with plasma glucose in asymptomatic high cardiovascular-risk patients, although no changes were observed in insulin levels\textsuperscript{34}. In line with these results, a significant decrease of glycaemia related to a long term daily intake of a virgin olive oil rich in phenolic compounds was observed in healthy young subjects\textsuperscript{35}. HT-Ac also maintained plasma leptin at concentrations similar to those in the C group, while this effect was more discrete in the case of HT and HT-Et. Similarly, in rats that consumed the diet supplemented with HT-Ac and HT-Et creatinine concentrations were restored to control levels, but not with HT. The renal protective effect of olive phenols reducing creatinine levels has already been described in diabetic rats\textsuperscript{8}.

The retroperitoneal/epididymal fat ratio provides information about adipose tissue distribution, being directly proportional to the abdominal fat accumulation. Both retroperitoneal and epididymal adipose depots are considered visceral fat associated with higher levels of inflammation and lipolysis than subcutaneous fat, having been related with insulin resistance\textsuperscript{36}. The retroperitoneal adipose depots in rats could be related to abdominal adiposity in humans since they are located inside the peritoneum attached to the dorsal area of the abdomen, whereas epididymal adipose tissue would correspond to gonadal fat, which exists in mice and rats but not in humans. The groups supplemented with the lipophilic compounds (HT-Ac and HT-Et) moderately improved the retroperitoneal/epididymal fat ratio compared to Chol group, whereas the group that consumed HT did not show changes. Our results are in agreement with the reduction in abdominal fat deposition described in a
metabolic syndrome animal model that consumed a diet supplemented with an olive leaf extract rich in HT.\(^{37}\) Metabolic disturbance which includes hyperlipidaemia, hyperglycaemia and hyperinsulinemia often involves chronic inflammation and oxidative stress\(^ {2,8,11} \). Therefore, redox status and inflammatory biomarkers were analyzed in plasma in order to understand the potential mechanisms underlying the effects of the studied phenolic compounds. Although the possible health implications related to MDA changes remain unknown, in this study elevated systemic MDA concentrations were observed in the Chol group, suggesting a marked oxidative stress in these animals that was counteracted by the consumption of the phenolic compounds. Moreover, the serum antioxidant capacity of animals consuming the phenolic compounds was higher than in the Chol group, particularly HT and HT-Ac. However, at the hepatic level phenolic compounds supplementation did not decrease MDA levels to control values although hepatic peroxidation was reduced in all cases, especially in the HT-Et group (Table 4). It is noteworthy that the studied phenolic compounds follow different metabolic pathways when ingested. HT-Ac is extensively hydrolysed into free HT, whereas HT-Et remains unaltered, yielding more lipophilic metabolites than those generated after HT and HT-Ac hepatic metabolism\(^ {15,20} \). This would result in higher HT-Et bioaccumulation in hepatic tissue, conferring higher protection against oxidation than HT and HT-Ac metabolites.

The protective role of olive oil phenolic compounds against oxidative damage is well established and has been reviewed recently\(^ {1} \). The antioxidant and free radical scavenging capacity of olive oil phenols seem to be related to their anti-inflammatory effects, which have been reported in different animal models\(^ {38,39} \). Gong, Geng, Jiang, Cao, Yoshimura\&Zhong\(^ {40} \) showed the capacity of HT to decrease the pro-inflammatory cytokines IL-1β and TNFα but not to increase the anti-inflammatory cytokine IL-10 in carrageenan-
induced acute inflammation and hyperalgesia in rats. Moreover, the role of olive oil phenols reducing postprandial inflammatory response in obese subjects has been recently attributed to the inhibition of NF-κB, which is an important link between oxidation and inflammation in the postprandial state\textsuperscript{41}. In the present study, diets supplemented with HT, HT-Ac and HT-Et had anti-inflammatory effects decreasing plasma TNF\textsubscript{α} in rats fed cholesterol-rich diet. Similar behaviour was observed with IL-1\textbeta although not reaching statistical significance probably due to the high variability that this parameter presented in the Chol group. In adipose tissue, supplementation with HT-Ac and HT-Et decreased MCP-1 and IL-1\textbeta below control levels.

Nutrients’ mechanisms of action are strongly related with the capacity to modulate gene expression. Llorente-Cortes and co-workers\textsuperscript{34} showed that consuming a traditional Mediterranean diet enriched with virgin olive oil for three months improved lipid profile and plasma glucose levels, prevented the increased expression of cyclooxygenase-2 (COX2) and LDL receptor-related protein-1 (LRP-1) genes, and reduced the expression of MCP1 gene compared with a traditional Mediterranean diet enriched with nuts or with a low fat diet. COX2 and MCP1 genes are involved in inflammation whereas LRP1 takes part in foam cell formation. Likewise, Konstantinidou and co-workers\textsuperscript{31} observed lower plasma oxidative and inflammatory status in healthy subjects after consuming for three months a Mediterranean diet supplemented with virgin olive oil rich in phenolic compounds compared to the control group. In the study, pro-atherogenic genes related with inflammation (IFN\textgamma, ARHGAP15 and IL7R) and oxidative stress (ADRB2) were down-regulated in peripheral blood mononuclear cells (PBMCs). Changes in gene expression were associated with decreases in lipid oxidative damage and systemic inflammation markers. In vitro studies have established the capacity of HT to modulate adipocyte lipid content and gene expression partially mediated by the reduction of the transcription factors PPAR\textalpha and C/EBP\textalpha\textsuperscript{42}. In phytochemicals’ capacity to
modify gene expression in different tissues and cell lines, the chemical structure of the phytochemical plays an important role not only in their direct scavenging of free radicals, but also in the target protein and therefore in the molecular mechanism involved in its biological action. Thus, considering the extensive hydrolysis that HT-Ac undergoes during intestinal absorption yielding HT\textsuperscript{15}, both compounds could follow similar mechanisms to regulate the unbalance induced by the high-cholesterol rich diet. Our results suggest changes in the expression of redox and inflammatory related genes, based on studies recently published by other research groups; however, further work is required to elucidate the molecular mechanisms underlying the regulatory metabolic effect of HT and its derivatives.

A limitation of this study is that a control group fed a standard diet supplemented with HT was not included to assess the effect of this compound within a balanced diet in healthy animals.

In summary, HT and its lipophilic derivatives, HT-Ac and HT-Et, were able to reduce the metabolic unbalance induced by a high-cholesterol diet in rats, being HT-Ac, the lipophilic HT derivative naturally present in virgin olive oil, the most effective phenolic compound. Since HT-Ac can be easily obtained from natural HT\textsuperscript{12}, this compound might be proposed as an interesting bioactive ingredient in the production of functional foods, having the added value of contributing to induce beneficial metabolic properties.
List of abbreviations: **C group:** Control group; **Chol group:** cholesterol-rich diet group; **AAPH:** 2,2’-azobis (2-amidinopropane) dihydrochloride; **COX 2:** cyclooxygenase-2; **FFA:** Free fatty acids; **HT:** hydroxytyrosol; **HT-Ac:** hydroxytyrosyl acetate; **HT-Et:** ethyl hydroxytyrosyl ether; **IL-1β:** interleukin-1β; **IL-6:** interleukin-6; **LRP1:** LDL receptor-related protein-1; **MCP-1:** monocyte chemoattractant protein-1; **ORAC:** oxygen radical scavenging capacity; **PAI-1:** plasminogen activator inhibitor-1; **SD:** standard deviation; **TNF-α:** tumour necrosis factor-α; **Trolox:** 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxilic acid

**Acknowledgements**

Study funded by projects RTA2007-00036 (INIA), Excellence Project AGR-5098 (Junta de Andalucia) and Consolider-Ingenio (CSD2007-00063). The authors thank the technical support and expertise in blood analysis provided by CORE lab (University Hospital La Paz). SM-L is a JAE predoctoral fellow funded by CSIC and the European Social Fund.

**Conflict of interest statement**

The authors have declared no conflict of interest.
References


Figure Legends

Figure 1. Plasma lipid profile (total-cholesterol, LDL- and HDL-Chol) of rats fed for 8 weeks with standard diet (C group), cholesterol-rich diet (Chol group) and cholesterol-rich diet supplemented with hydroxytyrosol (HT group), hydroxytyrosyl acetate (HT-Ac) and ethyl hydroxytyrosyl ether (HT-Et). Data represent the means of 8 determination ± SD. Bars without a common letter differ, p< 0.05.
Figure 1.

[Bar charts showing cholesterol levels under a hypercholesterolemic diet]

- Total Cholesterol (mg/dL)
  - Group C: Lower than other groups
  - Chol, HT, HT-Ac, HT-Et: Significantly different from Group C

- LDL-Cholesterol (mg/dL)
  - Group C: Lower than other groups
  - Chol, HT, HT-Ac, HT-Et: Significantly different from Group C

- HDL-Cholesterol (mg/dL)
  - All groups have similar levels, with no significant differences
Table 1. Dietary composition of the experimental diets.

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<tr>
<td>Cholic acid</td>
<td>0.00</td>
<td>0.44</td>
<td>0.44</td>
<td>0.44</td>
<td>0.44</td>
</tr>
<tr>
<td>HT and derivatives</td>
<td>0.00</td>
<td>0.00</td>
<td>0.04</td>
<td>0.04</td>
<td>0.04</td>
</tr>
<tr>
<td>Caloric content (Kcal/100g)</td>
<td>219.2</td>
<td>227.7</td>
<td>227.7</td>
<td>227.7</td>
<td>227.7</td>
</tr>
</tbody>
</table>
Table 2. Body and tissue weights of rats in the control group (C), the group consuming the non-supplemented cholesterol-rich diet (Chol) or the cholesterol-rich diet supplemented with hydroxytyrosol (HT), hydroxytyrosyl acetate (HT-Ac) and ethyl hydroxytyrosyl ether (HT-Et). Data represents the mean of 8 determinations ± SD*.

<table>
<thead>
<tr>
<th></th>
<th>Standard diet</th>
<th>Hypercholesterolemic diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>Chol</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inicial</td>
<td>242 ± 6</td>
<td>244 ± 8</td>
</tr>
<tr>
<td>Final</td>
<td>336 ± 18</td>
<td>326 ± 30</td>
</tr>
<tr>
<td>Body weight gain (g/56d)</td>
<td>94 ± 15</td>
<td>82 ± 32</td>
</tr>
<tr>
<td>Tissue weights (g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epididymal fat/ body weight</td>
<td>0.016 ± 0.003</td>
<td>0.016 ± 0.002</td>
</tr>
<tr>
<td>Retroperitoneal fat/ body weight</td>
<td>0.018 ± 0.004</td>
<td>0.015 ± 0.002</td>
</tr>
<tr>
<td>Ratio Retroperitoneal/epididymal</td>
<td>0.88 ± 0.09 (\text{a})</td>
<td>1.07 ± 0.08 (\text{b})</td>
</tr>
<tr>
<td>Liver / body weight</td>
<td>0.024 ± 0.002 (\text{a})</td>
<td>0.038 ± 0.003 (\text{b})</td>
</tr>
<tr>
<td>Kidney / body weight</td>
<td>0.0053 ± 0.0022</td>
<td>0.0057 ± 0.0005</td>
</tr>
<tr>
<td>Heart / body weight</td>
<td>0.0026 ± 0.0003</td>
<td>0.0028 ± 0.0005</td>
</tr>
</tbody>
</table>

* Mean values in a row with unlike superscripts are significantly different according to Kruskal-Wallis one-way analysis of variance and Mann-Whitney U test \((p< 0.05)\).
Table 3. Metabolic biomarkers in plasma and adipose tissue of rats in the control group (C), the group consuming the un-supplemented cholesterol-rich diet (Chol) or the group fed the cholesterol-rich diet supplemented with hydroxytyrosol (HT), hydroxytyrosyl acetate (HT-Ac) and ethyl hydroxytyrosyl ether (HT-Et). Data represents the means of eight determinations ± SD*.

<table>
<thead>
<tr>
<th>Plasma nutrients</th>
<th>Standard diet</th>
<th>Hypercholesterolemic diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>Chol</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>140.50 ± 15.23a</td>
<td>182.29 ± 18.17b</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>106.71 ± 21.69</td>
<td>100.29 ± 18.33</td>
</tr>
<tr>
<td>Free Fatty Acids (mM equivalents palmitic acid)</td>
<td>0.33 ± 0.07</td>
<td>0.30 ± 0.13</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>0.52 ± 0.02a</td>
<td>0.58 ± 0.05b</td>
</tr>
</tbody>
</table>

Hormones

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Plasma</th>
<th>Adipose tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Insulin (ng/mL)</td>
<td>Leptin (ng/µg pr)</td>
</tr>
<tr>
<td></td>
<td>972 ± 341a</td>
<td>682 ± 189</td>
</tr>
<tr>
<td></td>
<td>1560.2 ± 997.9b</td>
<td>501 ± 178</td>
</tr>
<tr>
<td></td>
<td>934.1 ± 390a</td>
<td>503 ± 137</td>
</tr>
<tr>
<td></td>
<td>608.1 ± 301.6a</td>
<td>503 ± 148</td>
</tr>
<tr>
<td></td>
<td>1121.3 ± 617.1ab</td>
<td>671 ± 283</td>
</tr>
<tr>
<td></td>
<td>Leptin (ng/mL)</td>
<td>Adiponectin (ng/µg pr)</td>
</tr>
<tr>
<td></td>
<td>2786 ± 908a</td>
<td>726415 ± 139506</td>
</tr>
<tr>
<td></td>
<td>4174± 1116a</td>
<td>658737 ± 145887</td>
</tr>
<tr>
<td></td>
<td>3609 ± 1022ab</td>
<td>593934 ± 62860</td>
</tr>
<tr>
<td></td>
<td>2970 ± 1150a</td>
<td>693282 ± 139463</td>
</tr>
<tr>
<td></td>
<td>3468.1 ± 1291ab</td>
<td>653668 ± 66473</td>
</tr>
</tbody>
</table>

* Mean values in a row with unlike superscripts are significantly different according to Kruskal-Wallis one-way analysis of variance and Mann-Whitney U test (p< 0.05).
Table 4. Redox status and inflammatory biomarkers in plasma and adipose tissue of rats fed the control diet (C), the un-supplemented cholesterol-rich diet (Chol) and the cholesterol-rich diet supplemented with hydroxytyrosol (HT), hydroxytyrosyl acetate (HT-Ac) and ethyl hydroxytyrosyl ether (HT-Et). Data represents the mean of 8 determinations ± SD*.

<table>
<thead>
<tr>
<th></th>
<th>Standard diet</th>
<th>Hypercholesterolemic diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>Chol</td>
</tr>
<tr>
<td>Antioxidant capacity</td>
<td>Serum ORAC (mM T.eq)</td>
<td>27.4 ± 1.8&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Lipid oxidation</td>
<td>Serum (nmol/mL)</td>
</tr>
<tr>
<td></td>
<td>Liver (nmol/mg pr)</td>
<td>0.98 ± 0.33&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Inflammatory mediators</td>
<td>Plasma</td>
<td>TNFα (ng/mL)</td>
</tr>
<tr>
<td></td>
<td>IL-1β (ng/mL)</td>
<td>11.6 ± 6.3</td>
</tr>
<tr>
<td></td>
<td>IL-6 (ng/mL)</td>
<td>4.9 ± 4.4</td>
</tr>
<tr>
<td>Adipose tissue</td>
<td></td>
<td>TNFα (ng/µg pr)</td>
</tr>
<tr>
<td></td>
<td>IL-1β (ng/µg pr)</td>
<td>3.9 ± 0.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>IL-6 (ng/µg pr)</td>
<td>24.6 ± 7.0</td>
</tr>
<tr>
<td></td>
<td>MCP-1 (ng/µg pr)</td>
<td>32.3 ± 7.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>PAI-1(ng/µg pr)</td>
<td>87.6 ± 22.9</td>
</tr>
</tbody>
</table>

* Mean values in a row with unlike superscripts are significantly different according to Kruskal-Wallis one-way analysis of variance and Mann-Whitney U test (p< 0.05).
HTy-Ac

HTy

HTy-Ether

Cholesterol

Metabolism

Redox

Adipokines

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