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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

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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 its lipophilic derivatives, hydroxytyrosyl acetate (HT-Ac) and ethyl hydroxytyrosyl ether (HT-Et), in high-cholesterol fed animals. Male Wistar rats ($n = 8$) were fed a standard diet (*C* group), a cholesterol-rich diet (*Chol* group) or a cholesterol-rich diet supplemented with phenolic compounds (*HT* group, *HT-Ac* group and *HT-Et* group) for 8 weeks. Body and tissue weights, the lipid profile, redox status, and biochemical, hormonal, and inflammatory biomarkers were evaluated. Plasma levels of total cholesterol, 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 had improved glucose, insulin, leptin and MDA levels and antioxidant capacity status, with HT-Ac being 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 hydrophobic derivatives, particularly HT-Ac.

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 is transiently associated 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 properties.[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 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 of the same length as the acyl chain in HT-Ac, was included in this study to assess the influence of 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 an 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.[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,[24,25]

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preserving their potential application as antioxidants to stabilize foodstuffs or as functional food ingredients.

Keeping these in mind, the aim of the present study is 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), trizma base, dithiothreitol, 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, Milliplex Map Rat Adipocyte Panel) and adipokine (RADPK-81K, Milliplex Map 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[26] 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.[12]

HT-Et was obtained from HT by chemical synthesis as described elsewhere.[13]

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.[27] 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 on 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 with 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 the Spanish law for the protection of experimental animals (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 creatinine in plasma were determined using an automated analyser (Beckman Coulter-Former Olympus Diagnostics AU 5420, Nyon, Switzerland). Plasma free fatty acid (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 & Prior.[28] The fluorescence at 485 and 528 nm 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^{-1} serum.

Table 1 Dietary composition of experimental diets

Component (g per 100 g dry weight)	*C*	*Chol*	*HT*	*HT-Ac*	*HT-Et*
Carbohydrates	65.93	64.20	64.17	64.17	64.17
Protein	17.58	17.12	17.11	17.11	17.11
Lipids (other than cholesterol)	3.30	3.21	3.21	3.21	3.21
Cholesterol	0.00	2.19	2.19	2.19	2.19
Cholic acid	0.00	0.44	0.44	0.44	0.44
HT and derivatives	0.00	0.00	0.04	0.04	0.04
Caloric content (kcal per 100 g)	219.2	227.7	227.7	227.7	227.7

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 represent the mean of 8 determinations ± SD*

	Standard diet	Hypercholesterolemic diet			
	C	*Chol*	*HT*	*HT-Ac*	*HT-Et*
Body weight (g)					
Initial	242 + 6	244 + 8	244 + 7	239 + 11	243 + 4
Final	336 + 18	326 + 30	331 + 16	317 + 10	329 + 14
Body weight gain (g per 56 day)	94 + 15	82 + 32	87 + 12	78 + 11	86 + 12
Tissue weights (g)					
Epididymal fat/body weight	0.016 + 0.003	0.016 + 0.002	0.015 + 0.001	0.017 + 0.003	0.015 + 0.004
Retroperitoneal fat/body weight	0.018 + 0.004	0.015 + 0.002	0.014 + 0.002	0.017 + 0.001	0.016 + 0.005
Ratio retroperitoneal/epididymal	$0.88 + 0.09^{a}$	$1.07 + 0.08^{b}$	$1.07 + 0.08^{b}$	$1.00 + 0.05^{ab}$	$0.93 + 0.07^{ab}$
Liver/body weight	$0.024 + 0.002^{a}$	$0.038 + 0.003^{b}$	$0.029 + 0.018^{ab}$	$0.036 + 0.002^{b}$	$0.037 + 0.002^{b}$
Kidney/body weight	0.0053 + 0.0022	0.0057 + 0.0005	0.0058 + 0.0008	0.0061 + 0.0005	0.0059 + 0.0006
Heart/body weight	0.0026 + 0.0003	0.0028 + 0.0005	0.0025 + 0.0001	0.0029 + 0.0005	0.0028 + 0.0003

* 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 ($p < 0.05$).

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.[29] Livers (0.5 g) were homogenized (1 : 5 w/v) in ice-cold 0.25 M Trizma base buffer pH 7.4 containing 5 mM dithiothreitol using an Ultra Turrax (IKA® Works Inc., WilmingtonNC) at 18 000 rpm. After centrifugation (11 000 rpm 30 min, 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. The 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 (0.2 g) 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 interval). After centrifugation (5000 rpm, 10 min, 4 °C), the lipid layer was removed and supernatants were collected for adipokine determination. The concentrations 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 Kit (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 kit (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 3.1 xPONENT software (Millipore); high and low concentration quality controls were used with all the biomarkers. The 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 mean ± standard deviation (SD). Non-parametric significant tests (Kruskal–Wallis one-way analysis of variance and Mann–Whitney *U* test) were performed to compare values. Differences were considered statistically significant when $p < 0.05$, and in the Mann–Whitney *U* test the significance was adjusted with a Bonferroni test. Data were analysed using statistical package SPSSv.19.0 software (SPSS, Institute Inc, Cary, NC).

3. Results

3.1. Food intake, body weight gain and tissue weights

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

Therefore, the groups consuming the cholesterol-rich diets (*Chol*, *HT*, *HT-Ac* and *HT-Et*) presented a daily caloric excess of approximately 2 kcal per 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 20 g and an average body weight of 300 g, the daily phenolic compound intake was 25 mg kg^{-1} body weight per day. This is a relatively low dose, comparable with doses used in other studies[8–11] 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 diet 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 the 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 shown in Fig. 1, animals fed the cholesterol-rich diet had a marked hyperlipidaemia, with high LDL cholesterol and total cholesterol concentrations, although HDL cholesterol levels were not affected as compared to the *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).

Triglyceride concentrations were similar in all groups, although phenolic compound 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 a high-cholesterol diet for 8 weeks, significant differences in plasma insulin and leptin concentrations were observed in *Chol* compared to the *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 the *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 the level found in control animals. Again, the *HT-Ac* group had significantly lower plasma leptin levels than the *Chol* group and in the range of control values. In the retroperitoneal adipose tissue, the leptin concentration was slightly lower in all cholesterol-treated groups compared to the *C* animals, although not significantly different.

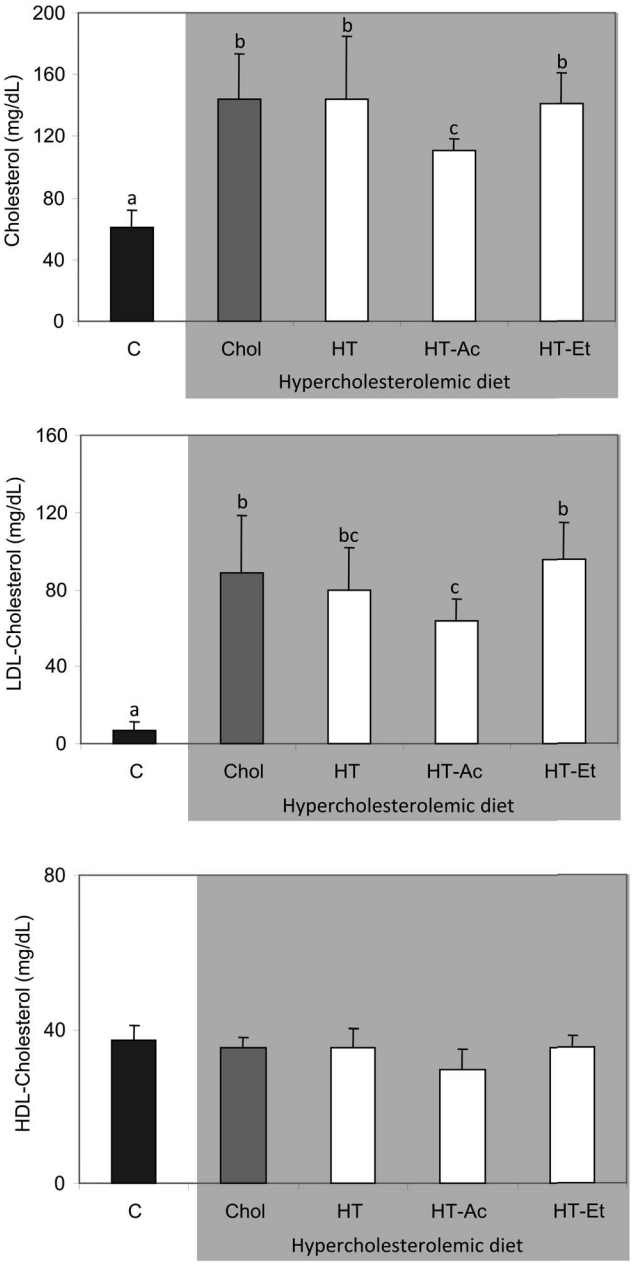

Fig. 1 Plasma lipid profile (total cholesterol, LDL- and HDL-Chol) of rats fed for 8 weeks with standard diet (*C* group), cholesterol-rich diet (*Chol* group) and cholesterol-rich diet supplemented with hydroxytyrosol (*HT* group), hydroxytyrosyl acetate (*HT-Ac*) and ethyl hydroxytyrosyl ether (*HT-Et*).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

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 the *Chol* group compared to the control

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 represent the mean of 8 determinations ± SD*

		Standard diet	Hypercholesterolemic diet			
		C	*Chol*	*HT*	*HT-Ac*	*HT-Et*
Plasma biomarkers						
Glucose (mg dL^{-1})		140.50 ± 15.23^{a}	182.29 ± 18.17^{b}	143.25 ± 19.83^{a}	154.75 ± 23.36^{a}	160.00 ± 22.53^{ab}
Triglycerides (mg dL^{-1})		106.71 ± 21.69	100.29 ± 18.33	83.00 ± 29.50	90.50 ± 19.49	84.57 ± 13.90
Free fatty acids (mM equivalents palmitic acid)		0.33 ± 0.07	0.30 ± 0.13	0.34 ± 0.07	0.31 ± 0.08	0.36 ± 0.07
Creatinine (mg dL^{-1})		0.52 ± 0.02^{a}	0.58 ± 0.05^{b}	0.55 ± 0.02^{b}	0.53 ± 0.03^{a}	0.53 ± 0.03^{a}
Hormones						
Plasma	Insulin (ng mL^{-1})	972 ± 341^{a}	1560.2 ± 997.9^{b}	934.1 ± 390^{a}	608.1 ± 301.6^{a}	1121.3 ± 617.1^{ab}
	Leptin (ng mL^{-1})	2786 ± 908^{a}	4174 ± 1116^{b}	3609 ± 1022^{ab}	2970 ± 1150^{a}	3468.1 ± 1291^{ab}
Adipose tissue	Leptin (ng μg^{-1} pr)	682 ± 189	501 ± 178	503 ± 137	503 ± 148	671 ± 283

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

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 represent the mean of 8 determinations ± SD*

			Standard diet	Hypercholesterolemic diet			
			C	*Chol*	*HT*	*HT-Ac*	*HT-Et*
Antioxidant capacity	Serum ORAC (mM T. eq.)		27.4 ± 1.8^{ab}	25.6 ± 2.3^{a}	28.4 ± 1.6^{b}	28.0 ± 1.2^{b}	26.2 ± 1.1^{a}
Lipid oxidation	Serum (nmol mL^{-1})		1.97 ± 0.75^{a}	3.54 ± 0.24^{b}	2.36 ± 0.23^{a}	2.19 ± 0.39^{a}	2.36 ± 0.30^{a}
	Liver (nmol mg^{-1} pr)		0.98 ± 0.33^{a}	3.33 ± 1.14^{b}	2.52 ± 0.92^{bc}	2.35 ± 0.81^{bc}	2.11 ± 0.53^{c}
Inflammatory mediators	Plasma	TNFα (ng mL^{-1})	3.0 ± 0.7^{a}	4.2 ± 0.7^{b}	3.7 ± 0.8^{a}	2.7 ± 0.6^{a}	3.4 ± 0.7^{ab}
		IL-1β (ng mL^{-1})	11.6 ± 6.3	26.50 ± 21.5	13.8 ± 14.6	11.5 ± 7.1	8.0 ± 4.9
		IL-6 (ng mL^{-1})	4.9 ± 4.4	3.66 ± 1.63	6.0 ± 3.3	5.7 ± 3.7	5.9 ± 2.4
	Adipose tissue	TNFα (ng μg^{-1} pr)	2.5 ± 0.6	2.2 ± 0.5	2.2 ± 0.5	2.0 ± 0.4	1.9 ± 0.3
		IL-1β (ng μg^{-1} pr)	3.9 ± 0.9^{a}	3.7 ± 0.8^{a}	3.8 ± 0.6^{a}	2.9 ± 0.4^{b}	3.1 ± 0.2^{b}
		IL-6 (ng μg^{-1} pr)	24.6 ± 7.0	23.7 ± 6.1	25.7 ± 7.4	20 ± 8	23.4 ± 6.5
		MCP-1 (ng μg^{-1} pr)	32.3 ± 7.4^{a}	27.6 ± 3.9^{a}	28.5 ± 3.9^{a}	26 ± 4^{b}	25.6 ± 3.4^{b}
		PAI-1 (ng μg^{-1} pr)	87.6 ± 22.9	98.9 ± 32.9	92.4 ± 15.4	73.3 ± 18.0	81.5 ± 12.4

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

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 the *Chol* group ($p < 0.05$).

Regarding the biomarker of lipid peroxidation, the *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 compound-supplemented groups showed lower hepatic MDA levels than the *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 the *C* group, an 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 diet or the phenolic supplementation.

Cytokine concentrations in the visceral adipose tissue from the retroperitoneal area of rats in the *Chol* group were similar to those in the *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 the *C* group (Table 4). PAI-1 concentrations showed a 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.[15,20] 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 diets with a moderate amount of phenolic compounds (0.04%) resulted in a daily intake of 25 mg kg^{-1} body weight, which is far beyond any possible toxic level considering a recently published study where consumption of up to 500 mg kg^{-1} per day was described as a No Observed Adverse Effects Level (NOAEL).[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 showed significantly reduced total and LDL cholesterol levels compared to the *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 the lipid profile.[8,10,31] In fact, the European Food Safety Authority has recently issued a positive opinion on the capacity of olive oil phenolic compounds to protect LDL cholesterol from oxidative damage.[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.[2] Comparing the *Chol* and *C* groups, the high-cholesterol diet induced a significant increase in glucose, creatinine, insulin and leptin plasma concentrations. In addition, *Chol* hypercholesterolemic animals presented higher liver weights than *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 the plasma glucose concentration in alloxan-diabetic rats[8,9] by means of alleviating oxidative stress and free radicals as well as enhancing enzymatic defence. A recent paper evidenced the role of oleuropein aglycone in the prevention of cytotoxic amyloid aggregation of human amylin, a hallmark of Type-II diabetes.[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.[34] In line with these results, a significant decrease of glycaemia related to long term daily intake of a virgin olive oil rich in phenolic compounds was observed in healthy young subjects.[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 in rats that consumed a diet supplemented with HT. The renal protective effect of olive phenols reducing creatinine levels has already been described in diabetic rats.[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, being related to insulin resistance.[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 lipophilic compounds (HT-Ac and HT-Et) moderately improved the retroperitoneal/epididymal fat ratio compared to the *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 in reducing the 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.[41] In the present study, diets supplemented with HT, HT-Ac and HT-Et had anti-inflammatory effects, decreasing plasma TNFα in rats fed a cholesterol-rich diet. Similar behaviour was observed with IL-1β 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-1β below control levels.

Nutrients' mechanisms of action are strongly related to the capacity to modulate gene expression. Llorente-Cortes and co-workers[34] showed that consuming a traditional Mediterranean diet enriched with virgin olive oil for three months improved the lipid profile and plasma glucose levels, prevented the increased expression of cyclooxigenase-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[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 to inflammation (IFNγ, 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 PPARα and C/EBPα.[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,[15] both compounds could follow similar mechanisms to regulate the imbalance 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 imbalance induced by a high-cholesterol diet in rats, with HT-Ac, the lipophilic HT derivative naturally present in virgin olive oil, being the most effective phenolic compound. Since HT-Ac can be easily obtained from natural HT,[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.

Conflict of interest statement

The authors have declared no conflict of interest.

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

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