One-month administration of hydroxytyrosol, a phenolic antioxidant present in olive oil, to hyperlipemic rabbits improves blood lipid profile, antioxidant status and reduces atherosclerosis development

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Abstract

The present study analysed the effects of hydroxytyrosol (HT) on blood lipids, antioxidant status and the progression of aortic lesions in hyperlipemic rabbits. Sixty-four rabbits were distributed into eight groups of animals (n = 8). Animal groups C, A and H were fed for 1-month with a control diet containing sunflower oil (C), an atherogenic diet (A) high in saturated fat and cholesterol or the A diet together with HT, respectively. The other five groups were fed for 2-months with diets C or A (groups CC or AA, respectively), or for 1-month with the A-diet followed by a further month with diet C, extra virgin olive oil diet (O) or diet C with HT (groups AC, AO and AH, respectively). Four milligram of HT/kg body weight were used in the study. Fifty and 42% decrease in total cholesterol and triacylglycerols, respectively, and a 2.3-fold increase in HDL-cholesterol were observed in the AH group but not in the H group. The HT-supplemented groups improved their antioxidant status and reduced the size of atherosclerotic lesions measured as intimal layer areas of the aortic arch when compared with control animals. We conclude that HT supplementation may have cardioprotective effects in vivo.
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Keywords: Hydroxytyrosol; Atherosclerosis; Lipids; Antioxidant; Aorta; Olive oil; Rabbits

1. Introduction

Atherosclerosis, is characterised by accumulation of lipids and fibrous elements in the large arteries. This disease is the main cause of cardiovascular disease (CVD) in the Western world. A number of risk factors have been associated with the occurrence of CVD including high blood concentrations of total cholesterol (TC), triglycerides (TG) and homocysteine, low HDL-cholesterol (HDL-C), hypertension, obesity and diabetes [1].

There is strong evidence for an association between the Mediterranean dietary pattern and CVD protection where olive oil, high in monounsaturated fatty acids (MUFA), constitutes the main source of fat [2]. As a result, international nutritional guidelines recommend replacing saturated fat by monounsaturated fat to obtain cardiovascular health benefits. [3]. In addition to oleic acid, virgin olive oil contains a wide range of “minor constituents”, like polyphenols, that contribute to the stability of the oil and exhibit potent antioxidant properties. Hydroxytyrosol (3,4-dihydroxyphenyl-ethanol, HT) is an orto-diphenol that has demonstrated the strongest radical-scavenging properties in vitro among all the olive oil polyphenols [4]. Reported in vitro and in vivo studies
indicate that HT and olive oil phenols can reduce LDL oxidation mediated by copper ions [5], reduce endothelial activation [6] and inhibit platelet aggregation [7]. These properties suggest that HT and olive oil phenols may influence atherosclerosis development.

Most in vivo studies describing cardiovascular effects of HT have been carried out when it was administered together with olive oil [8–11] but less attention has been paid to its effects when administered in aqueous solutions or other food matrices [12,13].

The experimental model of diet-induced atherosclerosis in rabbits has been extensively used to evaluate the effects of fats and/or bioactives in the development of atherosclerotic lesions and biomarkers of the disease [8–11].

In this study, we report the effects produced by the administration of HT on blood lipids, antioxidant status and the progression of aortic lesions using an established model of diet-induced atherosclerosis in rabbits [11]. In this model, the atherosclerosis was induced for 1-month period with a diet supplemented with high amounts of saturated fat and cholesterol.

2. Materials and methods

2.1. Animals and diets

Sixty-four male New Zealand rabbits (weight 2.5–3 kg) were obtained from Harlan Interfauna Iberica SA (Barcelona, Spain). The animals were randomly distributed into eight experimental groups and individually housed under standard conditions of lighting (day/night cycles of 12 h), temperature (18 ± 1 °C) and humidity (65%). Drinking water was available ad libitum throughout the study and food intake for each animal was standardised to 150 g/day. The diets used in the study were as follows: 97% standard chow diet, 3% sunflower oil (diet C, control); 96% standard chow diet, 1.3% of cholesterol and 3% of lard to induce atherosclerosis (diet A, atherogenic) [11]; 97% standard chow diet, 3% virgin olive oil containing 5.6 mg/kg of HT or 234 mg/kg of phenols (diet O, virgin olive oil). The three diets used contained 6% of total fat, excluding cholesterol. The composition of the standard chow diet was 13.5% protein, 3% fat, 50% carbohydrates, 15.5% fibre, 7% minerals and 11.0% water. The fatty acid composition of the diets used in the study is shown in Table 1. To avoid fat oxidation, all diets were kept at 4 °C in the dark until use. The diets were prepared by Panlab SA (Barcelona, Spain). HT was synthesised by reducing 3,4-dihydroxyphenyllactic acid with LiAlH4 in tetrahydrofuran under refluxing for 4 h. The reaction product was purified by chromatography on silica gel with CHCl3:MeOH (7:1) as eluting solvent. The HT obtained was 98% pure (Fig. 1). The purified HT was dissolved in sterile saline and stored at −20 °C until use. Phenolic compounds of virgin olive oil were extracted with methanol:water (60:40, v/v) and the concentration was measured by the Folin-Ciocalteau method using caffeic acid as standard [14].

| Table 1 Fatty acid composition of the diets used in the study (expressed as percentage of total fatty acids) |
|-------------------------------------------------|-----------------|-----------------|-----------------|
| Fatty acid | Diet C | Diet A | Diet O |
| C16:0      | 10.16  | 24.54 | 13.5   |
| C16:1      | 0.32   | 1.33  | 0.69   |
| C18:0      | 3.63   | 11.25 | 2.46   |
| C18:1(n-9) | 26.3   | 29.34 | 55.24  |
| C18:2(n-6) | 54.82  | 24.08 | 21.33  |
| C18:3(n-3) | 2.16   | 3.35  | 3.43   |
| SFA (%)    | 15.15  | 38.33 | 16.97  |
| MUFA (%)   | 27.56  | 33.64 | 58.08  |
| PUFA (%)   | 57.28  | 28.03 | 24.95  |
| n6/n3      | 23.29  | 6.73  | 5.95   |

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

The concentrations of HT in the O-diet and in the aqueous solutions used in the study were measured by high performance liquid chromatography (HPLC) as described in [15].

The care and experimental use of all animals of this study was in strict accordance with the animal welfare guidelines of the NIH Guide for care and use of laboratory animals.

2.2. Experimental design

The experimental design is shown in Fig. 2. Animal groups C, A and H were fed for 1-month and animal groups CC, AA, AC, AO and AH were fed for 2-months. Animal groups C, A and H were fed for 1-month with the C-diet, the A-diet or the A diet together with HT, respectively. The other groups were fed for 2-months with diets C or A (groups CC or AA, respectively), or for 1-month with the A-diet followed by a further month with diet C, extra virgin olive oil (O), or diet C with HT (groups AC, AO and AH, respectively). Four

![Fig. 1. HPLC chromatogram of the synthesized HT used in the study.](image-url)
Fig. 2. Experimental design. Sixty-four New Zealand rabbits were randomly distributed into eight groups of animals \( (n=8) \). Animal groups C, A and H were fed for 1-month with control diet (C), atherogenic diet (A), or the A-diet together with HT, respectively. The other groups were fed for 2-months with diets C or A (groups CC or AA, respectively), or for 1-month with the A-diet followed by a further month with diet C, diet O or diet C with HT (groups AC, AO and AH, respectively). Four milligram per kg BW of HT were administered to animal groups H and AH.

milligram of HT/kg of body weight (BW) were administered by oral gavages to animal groups H and AH. Overnight fasted animals were anesthesized intravenously with sodium pento-barbital (4 mg/kg BW). Blood was collected in tubes added with heparin and plasma was separated by centrifugation at 1750 \( g \) for 10 min. Aortas were quickly dissected out and 2–2.5 cm sections of the aortic arch were selected.

2.3. Lipids, total antioxidant capacity (TAC) and malondialdehyde (MDA) in plasma

The plasma concentrations of TC, HDL-C and TG were determined by colorimetry using commercial kits purchased from Biosystems (Barcelona, Spain) according to the manufacturer’s instructions. The plasma fatty acid profile was measured by gas–liquid chromatography as described in [16].

The TAC of plasma was measured as described in [17] using Trolox as standard. Briefly, fresh plasma was 1/10 diluted in PBS and 10 \( \mu \)L were incubated in the dark with 1 mL of ABTS* [2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)] radical cation for 10 min. Absorbance was read at 734 nm. ABTS* cation was prepared by addition of 88 mL of 140 mM potassium persulphate to 5 mL of a 7 mM solution of ABTS (Sigma A-1808) in water and incubation for 12–14 h. The working solution was obtained by dilution of the former with PBS until the absorbance at 734 nm was 0.7 ± 0.02 as described in [17].

The plasma malondialdehyde concentration was measured using an HPLC method as described in [18]. The method is based on the thiobarbituric acid reaction and reversed-phase HPLC separation with fluorescence detection.

2.4. Histological analysis of aortic atherosclerotic lesions

Selected fragments of the aortic arch were quickly washed for 10 min in Krebs solution (118 mM NaCl, 4.75 mM KCl, 25 mM NaHCO\(_3\), 1.2 mM MgSO\(_4\), 1.2 mM KH\(_2\)PO\(_4\), 11 mM glucose). Then, the specimens were fixed with 100 mL of 10% formaldehyde solution made in 0.1 M phosphate buffer saline (PBS), pH 7.33, at 4 °C. All samples were dehydrated and paraffinized. Serial coronal sections of 5 \( \mu \)m were cut with a microtome and further stained with haematoxylin and eosin, Masson trichromic, azan trichromic, orcein and Van Gieson’s elastin stains. The samples were examined blindly at the Department of Ophthalmology and Otorhinolaringology of Complutense University of Madrid (Spain) using a photomicroscope (Leica, LEITZ DMRB) connected to a video camera (SONY, SSC-C357P). In each one of the sections, the total and the intimal layer areas were measured using an image processing program (LEICA QWIN, Image processing and Analysis Software; Cambridge, UK).

2.5. Statistical analysis

The data are expressed as means ± standard error of the means (S.E.M.). Because the data was not normally distributed, Kruskal–Wallis comparisons followed by Mann–Whitney U-test were performed to assess statistical differences between the groups of the study. Differences were considered significant when \( P \)-values were <0.05. The data was analysed using SPSS software (Version 12.0, Chicago, IL).

3. Results

3.1. Animals

The animal weight gain upon consumption of the diets did not vary significantly among the experimental groups at 1 or 2 months. The body weight of the animals increased from 2.67 ± 0.02 to 3.33 ± 0.08 kg at the end of the first month and to 3.52 ± 0.05 kg at the end of the second month. The diets were well tolerated and all of the animals completed the study.

3.2. Plasma fatty acid profile

The average plasma fatty acid profile of the animals fed with the different diets is shown in Table 2. Rabbits that consumed the diet C only (groups C and CC) had the highest percentage of 18:2(\( n \)-6), polyunsaturated fatty acids (PUFA) and saturated fatty acids (SFA) in plasma. The animal groups that consumed A-diet (groups A and AA) showed a higher plasma concentration of 18:1(\( n \)-9) and MUFA compared with the groups fed with the C-diet. The animal group AC,
consuming the C-diet instead of the A-diet during the second month, showed higher plasma percentages of 18:2(n-6), total PUFA and ratio n6/n3 and lower percentage of C18:0 and 18:3(n-3) (groups AC versus AA), whereas consumption of diet O produced higher plasma amounts of oleic acid and PUFA and ratio n6/n3 (groups AC versus AA). The consumption of the O diet instead of the C diet during the second month (groups AO versus AC) increased the concentration of oleic acid and MUFA and reduced 18:2(n-6), PUFA and the ratio n6/n3 in plasma.

The administration of HT during 1 month together with diet A (group H) did not produce any change in the fatty acid profile compared with the consumption of the diet A alone (group H versus A). However, HT administration with diet C during 1 month after the A-diet (group AH), significantly modified the fatty acid profile (group AH versus AC): the AH group showed higher plasma concentrations of C16:1 and an increasing trend in C18:1(n-9) (P = 0.064), whereas reductions were observed in plasma percentages of C18:0, C18:2(n-6) and total PUFA.

3.3. Plasma lipids

The consumption of the A-diet during the first month induction period produced hyperlipemic rabbits as shown by the 7-fold increase in TG and 25-fold increase in TC values (group A versus group C, Table 3). The administration of HT with the A-diet during this period (group H) did not produce any effect on blood lipids. Substitution of the A diet for the C diet at the end of the induction period for a further month (group AC) reduced the plasma concentration of TG and TC but did not modify HDL-C. Administration of HT together with diet C during this period (group AH) produced a ca. 50% decrease in plasma TG (P = 0.002 versus AC) to reach values within the range obtained in the control group (CC). In addition, a 42% decrease in TC (P = 0.015 versus AC) and a 2.3-fold increase in HDL-C (P = 0.001 versus AC) were observed. Although the AO group reduced the average plasma concentration of TG and TC compared with the AC group, these effects were not statistically significant. In addition, the TC/HDL-C ratio of the AH group was the lowest among all the atherosclerotic groups.

### Table 2

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Group/diet</th>
<th>C</th>
<th>A</th>
<th>H</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>±</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>MUFA</td>
<td></td>
<td>20.25 ± 0.99</td>
<td>20.19 ± 0.97</td>
<td>20.83 ± 0.95</td>
</tr>
<tr>
<td>PUFA</td>
<td></td>
<td>1.90 ± 0.9</td>
<td>1.86 ± 0.95</td>
<td>1.94 ± 0.97</td>
</tr>
<tr>
<td>n6/n3</td>
<td></td>
<td>18.09 ± 0.96</td>
<td>17.88 ± 0.93</td>
<td>18.25 ± 0.97</td>
</tr>
</tbody>
</table>

(A), animal groups fed with the diets for 1-month; (B), animal groups fed for 2-months. Groups H and AH were administered with HT (4 mg/kg BW). Results are expressed as means ± S.E.M.

* P < 0.05 vs. group C.
† P < 0.05 vs. group CC.
‡ P < 0.05 vs. group AA.
§ P < 0.05 vs. group AC.
- P < 0.05 vs. group AO.

Plasma fatty acid profile of the different experimental groups (expressed as percentage of total fatty acids)
Table 3
Plasma concentrations of TG, TC, HDL-C, ratio CT/HDL-C, plasma MDA and TAC of the different animal groups at the end of the experimental periods

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group/diet</th>
<th>C</th>
<th>A</th>
<th>H</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TG</strong></td>
<td></td>
<td>0.61 ± 0.07</td>
<td>4.45 ± 0.50*</td>
<td>5.15 ± 0.37*</td>
</tr>
<tr>
<td><strong>TC</strong></td>
<td></td>
<td>2.07 ± 0.22</td>
<td>52.99 ± 8.54*</td>
<td>57.72 ± 2.66*</td>
</tr>
<tr>
<td><strong>HDL-C</strong></td>
<td></td>
<td>0.60 ± 0.04</td>
<td>0.52 ± 0.05</td>
<td>0.54 ± 0.07</td>
</tr>
<tr>
<td><strong>CT/HDL-C</strong></td>
<td></td>
<td>3.44 ± 0.33</td>
<td>104.3 ± 18.3*</td>
<td>125.0 ± 16.6*</td>
</tr>
<tr>
<td><strong>MDA</strong></td>
<td></td>
<td>1.42 ± 0.11</td>
<td>10.42 ± 0.70*</td>
<td>9.75 ± 0.89*</td>
</tr>
<tr>
<td><strong>TAC</strong></td>
<td></td>
<td>116.8 ± 3.8</td>
<td>19.0 ± 8.8</td>
<td>67.4 ± 8.0*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group/diet</th>
<th>CC</th>
<th>AA</th>
<th>AC</th>
<th>AO</th>
<th>AH</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TG</strong></td>
<td></td>
<td>0.75 ± 0.12</td>
<td>3.14 ± 0.39*</td>
<td>1.34 ± 0.09*</td>
<td>1.16 ± 0.43*</td>
<td>0.69 ± 0.10*</td>
</tr>
<tr>
<td><strong>TC</strong></td>
<td></td>
<td>0.90 ± 0.10</td>
<td>62.86 ± 6.29*</td>
<td>34.63 ± 3.41*</td>
<td>28.65 ± 6.87*</td>
<td>20.19 ± 2.34*</td>
</tr>
<tr>
<td><strong>HDL-C</strong></td>
<td></td>
<td>0.33 ± 0.04</td>
<td>0.34 ± 0.03</td>
<td>0.37 ± 0.02</td>
<td>0.45 ± 0.05</td>
<td>0.87 ± 0.03*</td>
</tr>
<tr>
<td><strong>CT/HDL-C</strong></td>
<td></td>
<td>2.8 ± 0.1</td>
<td>204.9 ± 42.4*</td>
<td>95.7 ± 9.7*</td>
<td>72.2 ± 21.6*</td>
<td>23.4 ± 2.7*</td>
</tr>
<tr>
<td><strong>MDA</strong></td>
<td></td>
<td>0.87 ± 0.06</td>
<td>4.84 ± 0.36*</td>
<td>4.65 ± 0.26*</td>
<td>3.80 ± 0.46*</td>
<td>3.41 ± 0.33*</td>
</tr>
<tr>
<td><strong>TAC</strong></td>
<td></td>
<td>111.8 ± 5.3</td>
<td>77.6 ± 8.8*</td>
<td>113.9 ± 3.9*</td>
<td>113.9 ± 2.9*</td>
<td>147.6 ± 1.3*</td>
</tr>
</tbody>
</table>

(A), animal groups fed with the diets for 1-month; (B), animal groups fed for 2-months. Groups H and AH were administered with HT (4 mg/kg BW). Results are expressed as means ± S.E.M.

- *P* < 0.05 vs. group C.
- #P* < 0.05 vs. group CC.
- †P* < 0.05 vs. group AA.
- ‡P* < 0.05 vs. group AC.
- ¶P* < 0.05 vs. group AO.
- §P* < 0.05 vs. group A.

Fig. 3. Total and intimal layer areas measured in cross sections of the aortic arch of the different experimental groups. Panel A, animal groups fed with the diets for 1-month; panel B, animal groups fed for 2-months. Groups H and AH were administered with HT (4 mg/kg BW). Results are expressed as media ± S.E.M.

- *P* < 0.05 vs. group C; ¥P* < 0.05 vs. group A; #P* < 0.05 vs. group CC; †P* < 0.05 vs. group AA; ‡P* < 0.05 vs. group AC; ¶P* < 0.05 vs. group AO.
3.4. Plasma oxidation parameters

The atherogenic diet produced a clear increase in plasma oxidation parameters which was more prominent at the end of the first month (Table 3). The animal groups that received HT showed a reduction in plasma oxidation markers compared with their controls. The H group of rabbits increased their TAC by 3.5-fold \((P = 0.007 \text{ versus } A)\) but did not reduce MDA when compared with the A group. The AH group showed a 25% reduction in plasma MDA and an increase in TAC \((P = 0.021 \text{ and } P = 0.001 \text{ versus } AC, \text{ respectively})\), also significant when compared with the control values \((P = 0.001 \text{ versus } CC)\). No differences in plasma oxidation markers were found between groups AC and AO.

3.5. Histological results

Morphometric analyses of cross-sections of the aortic arch showed that the A-diet produced a typical thickening of the aortic walls, measured as total area and intimal layer area (Figs. 3 and 4). The rabbits from the AA group showed more developed lesions and a more enlarged intimal and total areas than those from the A-group. Orcein stains showed the presence of elastic fibres next to the luminal side only in aortas of animal groups AA and AC (see arrows in Fig. 4), indicating that the lesions were more developed in these groups, whereas accumulations of lipids appear in aortic sections of all atherosclerotic rabbits. The 1-month administration of HT to animal groups H and AH produced a 45% \((0.50 \pm 0.10 \text{ group } H \text{ versus } 0.88 \pm 0.06 \text{ group } C, \text{ } P = 0.043)\) and three-fold reduction \((2.1 \pm 0.2 \text{ group } AH \text{ versus } 6.5 \pm 0.9 \text{ group } AC, \text{ } P = 0.000)\) in the cross-sectional intimal area compared with groups A and AC, respectively (Fig. 3). The total area was also reduced by 50% in the AH group \((6.8 \pm 0.4 \text{ group } AH \text{ versus } 14.3 \pm 1.3 \text{ group } AC, \text{ } P = 0.000)\). Lesser but non-significant reductions were observed in the AO group compared with the AC or AA groups.

4. Discussion

The main effects observed after 1-month administration of hydroxytyrosol to hyperlipemic rabbits were improvements in the blood lipid profile, antioxidant status and a reduction in size of the atherosclerotic lesions. Minor effects on the plasma fatty acid profile were also obtained.

In our study, atherosclerosis was induced for 1-month with a high-fat diet containing 2.5 times more saturated fat than a standard diet and 1.3% cholesterol. The atherogenic diet produced a combined hyperlipemia and the development of typical atherosclerotic lesions in the aortic arch that resembles the human fatty streak [11]. The hyperlipemia was then ameliorated during the second month of the study with diets not supplemented with cholesterol or saturated fat and the effects of 4 mg of HT/kg BW or a virgin olive oil diet, supplementing 10 \(\mu\)g of HT/kg BW per day, were assessed and compared with controls (diets C and A).

The increase in plasma SFA produced by the C-diet (high in PUFA) and the increase in plasma MUFA produced by the A-diet (high in SFA) have been previously described [10] and aortic tissue [9] using the same animal model. To explain these effects, a compensatory mechanism to control membrane fluidity has been suggested by which a high PUFA diet would induce de novo synthesis of fatty acids [19] and/or
The effects of the diets and HT were studied in sections of the aortic arch as atherosclerotic lesions are preferentially developed in these areas. While a non-significant trend was observed for the O diet at this level, HT produced a clear reduction of the intimal and total areas of the aortic arch when administered with the A- or the C-diets. Previous studies using phenolic-rich olive oil versus phenolic-poor olive oil have shown reductions in atherosclerotic lesions using the same animal model [9,11,30] but again in these cases no distinction was made between the effects derived from the fatty acid composition of olive oil and those produced by the polyphenols. In our study, HT was administered with the diets A or C that contained reduced amounts of oleic acid and differed in their SFA and PUFA content. The decreases in the atherosclerotic lesions obtained indicate a beneficial effect of HT independent from the oleic acid content of the olive oil. To explain the benefits of olive oil phenols in atherosclerosis development several mechanisms have been suggested, including antioxidant protection of LDL and lipids either directly or by interaction with endogenous antioxidants like Vitamin E [31,32], improvement of antioxidant status ([5] and references therein), anti-inflammatory activity [33,34], reduction of thromboxane B2 [7], reduction of macrophage uptake of oxidised LDL [14] or increase the size of LDL particles that become less pro-atherogenic [30]. In our study, we measured markers of plasma oxidizability but not of LDL as the latter seems to be a better indicator of atherogenicity in hypercholesterolemic rabbits [35]. In addition, we observed that LDL particles completely lost HT during dialysis when pre-incubated with HT (unpublished) so any direct antioxidant effect on LDL would be missed when using lag-time as a marker of antioxidant protection. Antioxidant markers clearly improved upon administration of HT with diets A or C. Interestingly, the group receiving HT with the A diet did not have a reduction in blood lipids but showed a reduction of lesion size, suggesting further benefits on atherosclerosis independent from the blood lipid-lowering perhaps by an antioxidant mechanism, as described by others [36,37].

Current strategies for cardiovascular disease prevention mainly focus on improving blood lipid profiles. As oxidative modification of LDL seems to be the trigger of the atherosclerotic process [1], prevention of lipoprotein oxidation by antioxidants can be important in terms of the onset and progression of the disease. However, the few controlled clinical trials using dietary antioxidants have not shown conclusive evidence of beneficial effects on risk factors or clinical outcomes [38]. In this work, the administration of HT improved blood lipid profile, antioxidant status and development of atherosclerotic lesions in an animal model of diet-induced atherosclerosis. More research is needed using different doses of HT over different periods of time to demonstrate its beneficial effects and mechanism(s) of action.

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