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Antioxidant activity of hydroxytyrosol in the peroxidation of rat liver mitochondria: Evaluation

María B Ventura, Javier L Barberon, Patricio J Leaden, Pedro A Zeinsteger, Alejandro Palacios*
Department of Biochemistry, Faculty of Veterinary Sciences, National University of La Plata, La Plata, Buenos Aires, Argentina

Abstract
Hydroxytyrosol (HT), also called 3,4-dihydroxyphenylethanol, is a phenolic compound present in the olive plant (Olea europaea) and has a powerful antioxidant effect, which is why it has been proposed for use as an additive to improve diet. HT can be obtained from three sources, either by extraction of olive residues, by chemical synthesis or by biotechnology. This last technique allowed to obtain high purity HT, without variations between extractions and without contaminants. A high purity HT of biological origin (POLYPHENOL - HT 1®, Nova Mentis Ltd, Ireland) has recently been developed. The objective of this work was to investigate the antioxidant effect of POLYPHENOL - HT 1® on the peroxidation of rat hepatic mitochondria membranes. Mitochondria membranes, adjusted to 1 mg of protein, were incubated in an ascorbate-Fe^{2+} dependent pro-oxidant system at 37 °C to determine its oxidative effect and to be able to evaluate the protection against peroxidation generated with 50, 100 and 200 µM HT. Mitochondrial peroxidation was quantified in a Packard 1900 TR liquid scintillation counter by chemiluminescence in cpm (counts per minute). A completely randomized experimental design was used with the following groups: Control group (mitochondria only), Ascorbate-Fe^{2+} group (mitochondria + pro-oxidant substance) and HT groups (mitochondria + pro-oxidant substance + HT in mentioned concentrations). The results of six independent determinations per group show the mean and its standard deviation of the means of the cpm: 15605±5083 (Control); 36151±1177 (Ascorbate-Fe^{2+}); 24907±8113 (HT 50 µM); 18473±6017 (HT 100 µM) and 16190±5274 (HT 200 µM). Data were statistically evaluated using one-way analysis of variance (ANOVA) and Tukey's test. It was observed that the chemiluminescence value was statistically higher in the Ascorbate-Fe^{2+} group than in the Control, while the HT groups showed an increasing reduction in concentration-dependent chemiluminescence that managed to equate the groups with 100 and 200 mM of HT with Control group. These results indicated that HT may act as antioxidant, protecting rat liver mitochondria membranes from peroxidative damage.

Keywords: HT, mitochondria, antioxidants, peroxidation, chemiluminescence

Introduction

HT is a polyphenol of interest to the food, feed, supplements and pharmaceutical sectors (Echeverría *et al*., 2017) [1]. It is well known for reducing inflammation (Elmaksoud *et al*., 2021) [2], cancer (Ramírez-Expósito *et al*., 2021) [3], coronary and neurodegenerative diseases (Achmon and Fishman, 2014) [4] and for its nephroprotective effect (Rodríguez-Pérez *et al*., 2021) [5]. In nature, HT is a polyphenolic compound of olive oil, derived from hydrolysis of oleuropein during maturation of olives. HT is one of the strongest known natural antioxidants and it is known to be one of the compounds responsible for the health benefits of the Mediterranean diet (Noguera-Navarro *et al*., 2023) [6]. European Food Safety Authority (EFSA) recommends 5 mg of HT daily (EFSA, 2010) [7]. Different doses have been used to obtain biological effects, and it is evident that HT is very safe. In rodents, it is known that doses of 20 mg/kg/day orally exert an antioxidant effect (Cao *et al*., 2014) [8], but that doses of up to 300 mg/kg/day of HT are safe (Heilman *et al*., 2015) [9]. Auñon-Calles *et al*. (2013) [10] propose a No Observed Adverse Effects Level (NOAEL) of 500 mg/kg/d. However, its source, its purity and cost can condition its use. There are three main sources of HT. The first option was to obtain it from natural sources, such as olive mill wastewater processing. Unfortunately this system is not very efficient and requires organic solvents (Karković Marković *et al*., 2019) [11]. In addition, the HT obtained from natural sources is impure and suffer from batch to batch variation and seasonality (De Leonardis *et al*., 2008) [12]. Another option is chemical synthesis, but these methods still result in minimal chemical contaminants, low production efficiency, or are expensive (Achmon and Fishman, 2014) [4]. Finally, biotechnological approaches can produce highly pure HT-like chemical catalysis with the additional advantage of not using any heavy-metal catalysts and harsh conditions (Britton *et al*., 2019) [13]. Recently, commercial high purity HT, based on tyrosinase variants of *Ralstonia solanacearum* on *E. coli* BL21 is available (Molloy *et al*., 2013, Nova Mentis GRAS Notice No. 876) [14]. Different doses and cell models have been evaluated HT *in vitro*. Supplementation with HT (1300µM) might be helpful to maintain the human spermatozoon after centrifugation. This can improve sperm quality, in particular viability, and decrease sperm DNA oxidation by reducing ROS levels (Kedechi *et al*., 2016) [16]. Other studies found that the concentrations in the optimal range for HT (25–50 µM) maintained total motility and progressive motility of cryopreserved bull semen with low cryotolerance (Mohsen Sharafi *et al*., 2022) [17]. Studies *in vitro* inducing hemolysis in human erythrocytes (200µM H_20_2) show HT concentration as little as 50 µM had a significant effect, while 100µM completely prevents the oxidative damage (Manna *et al*., 1999) [18]. Other results indicate that HT treatment (10–50 µM) prevents the increase in hemolysis and Reactive Oxygen Species (ROS)

generation induced by exposure of cells to micromolar $HgCl_2$ concentrations as well as the decrease in GSH intracellular levels (Tagliafierro *et al*., 2015) [19]. Mitochondria, and especially their membranes, are targets of oxidative attack (Ott *et al*., 2007) [20]. Lipid peroxidation of cell membranes is a classical model to study the effects of antioxidants (Wadhwa *et al*., 2012) [21]. The objective of this work was to evaluate the antioxidant capacity of increasing doses of a novel HT (>99 % pure) obtain by biotechnology on rat liver mitochondria membranes.

Materials and Methods

Experimental

Female Wistar AH/HOK rats, 8 weeks old, weighing 120-137 g were used. All rats were fed commercial rat chow and water ad libitum. Female Wistar AH/HOK rats were obtained from Laboratory Animal Facility, Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata. BSA (fraction V) was obtained from Wako Pure Chemical Industries Ltd, Japan. L (+) ascorbic acid, dimethyl sulfoxide and methanol were from Merck Laboratories and POLYPHENOL - HT 1® was obtained from Nova Mentis Ltd, Ireland. All other reagents and chemicals were of analytical grade from Sigma-Aldrich.

Preparation of animals and mitochondria

Rats were euthanized by cervical dislocation and the liver was rapidly removed, cut into small pieces and extensively washed with 0.15 M NaCl. A 30 % (w/v) homogenate was prepared in a 0.25 M sucrose solution, 10 mM Tris-HCl pH 7.4 using a Potter-Elvehjem homogenizer. The homogenate was spun at 10,000 x g for 10 min. The supernatant (30 ml) was applied to a Sepharose 4B column (1.6 x 12 cm) equilibrated and eluted with 10 mM Tris-HCl pH 7.4, 0.01 % NaN_3. Mitochondria were obtained by method described by Schneider and Hogeboom, 1958 [22].

Mitochondria peroxidation

Rat liver mitochondria were incubated with different concentrations of HT (50, 100, 200 µM) in an in *vitro* non-enzymatic ascorbic acid-Fe^{+2} system in order to determine the oxidative effect on membranes and quantify peroxidation level in standarized conditions. Peroxidation was quantified in a liquid scintillation counter Packard 1900 TR by chemiluminescence in cpm (counts per minute). Mitochondria without HT were used as a control. Chemiluminescence and peroxidation were initiated by adding ascorbate to mitochondria (Witing *et al*. 1964) [23]. The mitochondria (0.5 mg mitochondria protein) with addition of HT (50, 100, 200 µM) were incubated at 37 °C with 0.01 M phosphate buffer pH 7.4, 0.4 mM ascorbate, obtaining a final volume of 1 ml. The phosphate buffer provides ferrous or ferric iron (final concentration in the incubation mixture was 2.15 µM) for peroxidation (Wright *et al*. 1979) [24]. Mitochondria preparations, which lacked ascorbate, were carried out simultaneously. Membrane light emission was determined over a 180 min period, chemiluminescence was recorded as count per minute (cpm) every 10 min and the sum of the total chemiluminescence was used to calculate cpm/mg protein. Chemiluminescence was measured as counts per min in liquid scintillation analyzer Packard 1900 TR equipment (Japan) with a program for chemiluminescence.

Protein determination

Proteins were determined by the method of Lowry *et al*. 1951 [25] using BSA as standard.

Statistical analysis

Results are expressed as means ± S.D. of five independent determinations. Data were statistically evaluated by one-way analysis of variance (ANOVA) and Tukey's test. The statistical criterion for significance was selected at different *p*-values, which was indicated in each case.

Results

The incubation of rat liver mitochondria in the presence of ascorbate-Fe^{2+} resulted in the peroxidation of membranes as evidenced by emission of light (chemiluminescence). After incubation of mitochondria in an ascorbate-Fe^{2+} system at 37 °C for 180 minutes (cpm every 10 min) the cpm originated from light emission was lower in the HT group than in the control group in a concentration dependent manner (Figure 1).

Figure 1

The Figure 2 shows the total light emission obtained from HT group and control group.

When comparing the control group with the groups in the presence of HT, it was observed that at different concentrations of HT there was greater protection of the mitochondria. Count per minute (mean ± SDM) was in control 15605±5083; in control + ascorbate 36151±11776 and in different concentrations of HT, 50µM: 24907±8113, 100µM: 18473±6017 y 200µM:16190±5274.

Figure 2

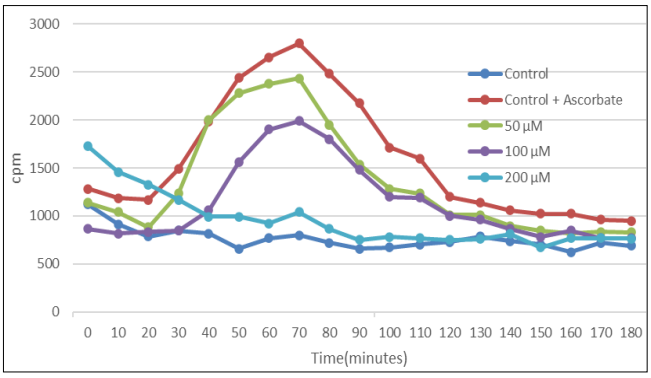

Fig 1: Rat liver mitochondria with different concentrations of HT: peroxidation.

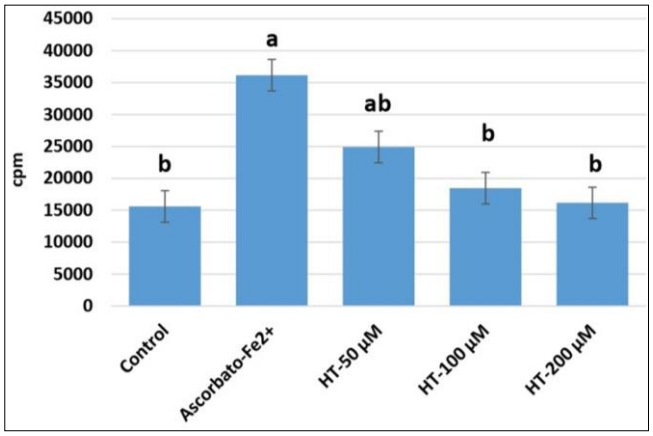

Fig 2: Total chemiluminescence of rat liver mitochondria induced by ascorbate Fe^{2+} system.

Results are expressed as mean ± SD of six independent experiments. The values of light emission showed statistically differences ($p<0.05$), when the control (without ascorbate) and ascorbate-Fe^{2+} (with ascorbate-Fe^{++}) rat liver mitochondria groups were compared. Similarly, differences ($p<0.05$) were found when was compared ascorbate-Fe^{2+} with ascorbate and HT antioxidant addition (50, 100, 200μM) rat liver mitochondria groups.

Discussion

HT has been studied in cellular, animal and human models that demonstrate the safety of the polyphenol; nevertheless, more studies in humans are needed to determine the therapeutic doses (Echeverría *et al*., 2017) [1].

Despite the importance of mitochondrial integrity in diseases that generate oxidative damage, there are few studies evaluating mitochondrial membranes challenged with non-enzymatic peroxidation. In our work, the addition of 50 μM HT partially protected the mitochondrial membranes, and a concentration of at least 100 μM was necessary to fully protect the membranes. Using rat liver microsomes and challenged with Fe^{+2} +ascorbate, complete protection against lipoperoxidation was demonstrated with 50 μM (Gutierrez *et al*., 2001) [26]. These authors also demonstrated that the molecules that generate HT in nature (oleuropein) or in the industrial generation system (tyrosol) do not achieve more important protections such as HT. With other models of non-enzymatic lipid peroxidation, using H_2O_2 as a pro-oxidant, there are some precedents. In this sense, Manna *et al*. (1999) [18], using the human RBC model challenged with H_2O_2 (300 μM), achieved partial protection with 50 μM, which increased with 100 μM HT, but it was also incomplete and did not reduce lipid peroxidation. For control level with milder challenges (100 μM H_2O_2), HT demonstrated partial protection against oxidative damage with concentrations as low as 1 μM (Deiana *et al*., 2008) [27]. This demonstrates that peroxidative damage depends on the concentration and type of prooxidant substance.

Oxidative damage to mitochondrial membranes is central to mitochondrial dysfunction that occurs in cardiovascular (Chistiakov *et al*., 2018) [28], neurological (Johnson *et al*., 2020) and different types of cancer (Sun *et al*., 2014; Luo *et al*., 2020) [30, 31]. Although its antioxidant capacity by inhibiting lipid peroxidation is recognized, HT has several beneficial effects on mitochondrial function (Cao *et al*., 2014) [8]. In an in *vitro* model of endothelial inflammation, HT (1-30 μmol/L) reduced mitochondrial superoxide production and also increased superoxide dismutase activity (Calabriso *et al*., 2018) [32]. In addition, the activation of mitophagy by HT through the AMPK/PINK1 pathway (Dong *et al*., 2022) [33] and the activation of mitochondrial biogenesis (Zhu *et al*., 2010) [34], also resulting in increased ATP synthesis (Visioli *et al*., 2022) [35].

Conclusions

It is concluded that HT succeeded in protecting rat liver mitochondrial membranes from peroxidation, but with concentrations of 100 and 200 μM, while an intermediate effect was achieved with a concentration of 50 μM**.**

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Referencias

1. Echeverría F, Ortiz M, Valenzuela R, Videla L. Hydroxytyrosol and Cytoprotection: A Projection for Clinical Interventions. International Journal of Molecular Sciences,2017:18(5):930. doi:10.3390/ijms18050930
2. Elmaksoud HAA, Motawea MH, Desoky AA, Elharrif MG, Ibrahimi A. Hydroxytyrosol alleviate intestinal inflammation, oxidative stress and apoptosis resulted in ulcerative colitis. Biomedicine & Pharmacotherapy,2021:142:112073. doi:10.1016/j.biopha.2021.112073
3. Ramírez-Expósito MJ, Carrera-González MP, Mayas MD, Martínez-Martos JM. Gender differences in the antioxidant response of oral administration of hydroxytyrosol and oleuropein against N-ethyl-N-nitrosourea (ENU)-induced glioma. Food Research International,2021:140:110023. doi:10.1016/j.foodres.2020.110023
4. Achmon Y, Fishman A. The antioxidant hydroxytyrosol: biotechnological production challenges and opportunities. Applied Microbiology and Biotechnology,2014:99(3):1119–1130. doi:10.1007/s00253-014-6310-6.
5. Rodríguez-Pérez MD, López-Villodres JA, Arrebola MM, Martín-Aurioles E, Fernández-Prior Á, Bermúdez-Oria A, Ríos MC, *et al*. Nephroprotective Effect of the Virgin Olive Oil Polyphenol Hydroxytyrosol in Type 1-like Experimental Diabetes Mellitus: Relationships with Its Antioxidant Effect. Antioxidants (Basel),2021:10(11):1783.
6. Noguera-Navarro C, Montoro-García S, Orenes-Piñero E. Hydroxytyrosol: Its role in the prevention of cardiovascular diseases. Heliyon. Volume 9, Issue 1, Januar, 2023, e1296. doi.org/10.1016/j.heliyon.2023.e12963
7. EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA). Scientific Opinion on the substantiation of health claims related to vitamin E and protection of DNA, proteins and lipids from oxidative damage. EFSA J,2010:8:1816.
8. Cao K, Xu J, Zou X, Li Y, Chen C, Zheng A, Feng Z. Hydroxytyrosol prevents diet-induced metabolic syndrome and attenuates mitochondrial abnormalities in obese mice. Free Radical Biology and Medicine,2014:67:396–407.
9. Heilman J, Anyangwe N, Tran N, Edwards J, Beilstein P, López J. Toxicological evaluation of an olive extract, H35: Subchronic toxicity in the rat. Food and Chemical Toxicology,2015:84:18–28. doi:10.1016/j.fct.2015.07.007
10. Auñon-Calles D, Canut L, Visioli F. Toxicological evaluation of pure hydroxytyrosol. Food and Chemical Toxicology,2013:55:498–504. doi:10.1016/j.fct.2013.01.030.
11. Karković Marković A, Torić J, Barbarić M, Jakobušić Brala C. Hydroxytyrosol, Tyrosol and Derivatives and Their Potential Effects on Human Health. Molecules,2019:24(10):2001.
12. De Leonardis A, Aretini A, Alfano G, Macciola V, Ranalli G. Isolation of a hydroxytyrosol-rich extract from olive leaves (Olea Europaea L.) and evaluation of its antioxidant properties and bioactivity. Eur Food Res Technol,2008:226:653–659

13. Britton J, Davis R, O'Connor K. Chemical, physical and biotechnological approaches to the production of the potent antioxidant hydroxytyrosol. Applied Microbiology and Biotechnology,2019:103:5957–5974. https://doi.org/10.1007/s00253-019-09914-9
14. Molloy S, Nikodinovic-Runic J, Martin LB, Hartmann H, Solano F, Decker H, O'Connor KE. Engineering of a bacterial tyrosinase for improved catalytic efficiency towards D-tyrosine using random and site directed mutagenesis approaches. Biotechnol Bioeng,2013:110(7):1849-57.
15. Nova Mentis GRAS Notice (GRN) No. 876. Office of Food Additive Safety. (accessed on 7 april 2023), 2019. Available online: https://www.fda.gov/media/134474/download.
16. Kedechi S, Zribi N, Louati N, Menif H, Sellami A, Lassoued S, *et al*. Antioxidant effect of hydroxytyrosol on human sperm quality duringin *vitro*incubation. Andrologia,2016:49(1):e12595. doi:10.1111/and.12595
17. Mohsen Sharafi, Patrick Blondin, Patrick Vincent, Muhammad Anzar, James D Benson. Hydroxytyrosol and resveratrol improves kinetic and flow cytometric parameters of cryopreserved bull semen with low cryotolerance. Anim Reprod Sci,2022:245:107065. doi: 10.1016/j.anireprosci.2022.107065.
18. Manna C, Della Ragione F, Cucciolla V, Borriello A, D'Angelo S, Galletti P, *et al*. Biological Effects of Hydroxytyrosol, a Polyphenol from Olive Oil Endowed with Antioxidant Activity. Advances in Experimental Medicine and Biology, 1999, 115–130. doi:10.1007/978-1-4757-3230-6_11
19. Tagliafierro L, Officioso A, Sorbo S, Basile A, Manna C. The protective role of olive oil hydroxytyrosol against oxidative alterations induced by mercury in human erythrocytes. Food and Chemical Toxicology,2015:82:59–63. doi:10.1016/j.fct.2015.04.029
20. Ott M, Gogvadze V, Orrenius S, Zhivotovsky B. Mitochondria, oxidative stress and cell death. Apoptosis,2007:12(5):913-22.
21. Wadhwa N, Mathew BB, Jatawa S, Tiwari A. Lipid peroxidation: mechanism, models and significance. Int J Curr Sci,2012:3:29-38.
22. Schneider WC, Hogeboom GH. Cytochemical studies of mammalian tissues; the isolation of cell components by differential centrifugation: a review. Cancer Res,1951:11(1):1-22.
23. Witing LA, Hornitt MK. J Nutr Efect of degree of fatty acid unsaturation in tocopherol deficiency-induced creatinuria. The Journal of Nutrition,1964:82:19-25.
24. Wright JR, Rumbaugh RC, Colby HD. The relationship between Chemiluminescence and lipid peroxidation in rat hepatic microsomes. Arch. Biochem. Biophys,1979:192:344-351.
25. Lowry OH, Rosebrough NJ, Farr NJ. Protein measurement with the folin phenol reagent. Biol. Chem,1951:193:265-275.
26. Gutierrez VR, de la Puerta R, Catalá A. The effect of tyrosol, hydroxytyrosol and oleuropein on the non-enzymatic lipid peroxidation of rat liver microsomes. Mol Cell Biochem,2001:217(1-2):35-41. doi: 10.1023/a:1007219931090. PMID: 11269663.
27. Deiana M, Incani A, Rosa A, Corona G, Atzeri A, Loru D, *et al*. Protective effect of hydroxytyrosol and its metabolite homovanillic alcohol on H(2)O(2) induced lipid peroxidation in renal tubular epithelial cells. Food Chem Toxicol,2008:46(9):2984-90.
28. Chistiakov DA, Shkurat TP, Melnichenko AA, Grechko AV, Orekhov AN. The role of mitochondrial dysfunction in cardiovascular disease: a brief review. Ann Med,2018:50(2):121-127. doi: 10.1080/07853890.2017.1417631. Epub 2017 Dec 18. PMID: 29237304.
29. Johnson J, Mercado-Ayon E, Mercado-Ayon Y, Dong YN, Halawani S, Ngaba L, *et al*. Mitochondrial dysfunction in the development and progression of neurodegenerative diseases. Arch Biochem Biophys,2021:702:108698.
30. Sun L, Luo C, Liu J. Hydroxytyrosol induces apoptosis in human colon cancer cells through ROS generation. Food Funct,2014:5(8):1909-14.
31. Luo Y, Ma J, Lu W. The Significance of Mitochondrial Dysfunction in Cancer. Int J Mol Sci,2020:21(16):5598. doi: 10.3390/ijms21165598. PMID: 32764295; PMCID: PMC7460667.
32. Calabriso N, Gnoni A, Stanca E, Cavallo A, Damiano F, Siculella L, *et al*. Hydroxytyrosol Ameliorates Endothelial Function under Inflammatory Conditions by Preventing Mitochondrial Dysfunction. Oxid Med Cell Longev,2018:18:2018:9086947.
33. Dong Y, Yu M, Wu Y, Xia T, Wang L, Song K, *et al*. Hydroxytyrosol Promotes the Mitochondrial Function through Activating Mitophagy. Antioxidants (Basel),2022:11(5):893. doi: 10.3390/antiox11050893. PMID: 35624756; PMCID: PMC9138034.
34. Zhu L, Liu Z, Feng Z, Hao J, Shen W, Li X, *et al.* Hydroxytyrosol protects against oxidative damage by simultaneous activation of mitochondrial biogenesis and phase II detoxifying enzyme systems in retinal pigment epithelial cells. J Nutr Biochem,2010:21(11):1089-98.
35. Visioli F, Rodríguez-Pérez M, Gómez-Torres Ó, Pintado-Losa C, Burgos-Ramos E. Hydroxytyrosol improves mitochondrial energetics of a cellular model of Alzheimer's disease. Nutr Neurosci,2022:25(5):990-1000.