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Hydroxytyrosol-Rich Olive Mill Wastewater Extract Protects Brain Cells in Vitro and ex Vivo

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Elevated oxidative and nitrosative stress both impair the integrity and functioning of brain tissue, especially in aging. As long-term intake of plant foods rich in antioxidant phenolics, such as extra virgin olive oil, positively modulates surrogate markers of many human pathological alterations, the interest in cheap and abundant sources of such phenolics is rapidly growing. Olive mill wastewater is particularly rich in hydroxytyrosol, an *o*-diphenol with powerful antioxidant, anti-inflammatory, and antithrombotic activities. Due to the deleterious effect of oxidative stress on brain cell survival, the efficacy of a hydroxytyrosol-rich extract to attenuate Fe^{2+}- and nitric oxide (NO)-induced cytotoxicity in murine-dissociated brain cells was investigated. The addition of either Fe^{2+} or SNP (an NO donor) caused both a severe loss of cellular ATP and a markedly depolarized mitochondrial membrane potential. Preincubation with hydroxytyrosol significantly attenuated the cytotoxic effect of both stressors, although with different efficiencies. Mice feeding studies were performed to assess the brain bioactivity of hydroxytyrosol ex vivo. Subchronic, but not acute, administration of 100 mg of hydroxytyrosol per kilogram body weight for 12 days enhanced resistance of dissociated brain cells to oxidative stress, as shown by reduced basal and stress-induced lipid peroxidation. Also, basal mitochondrial membrane potential was moderately hyperpolarized ($P < 0.05$), an effect suggestive of cytoprotection. In synthesis, the ex vivo data provide the first evidence of neuroprotective effects of oral hydroxytyrosol intake.

KEYWORDS: Hydroxytyrosol; olive mill wastewater; dissociated brain cells; oxidative stress; brain; Mediterranean diet

INTRODUCTION

Mediterranean diets are characterized by abundant intake of plant foods, such as fruits, vegetables, nuts, seeds, and wild plants (*1*). These diets are associated with lower mortality from coronary heart disease and cancer (*2*). One of the main features of Mediterranean diets is the use of olive oil as the principal source of fat. Accumulating evidence suggests that, aside from oleic acid, extra virgin olive oil (EVOO) minor constituents, namely, phenolic compounds, might provide health benefits (reviewed in ref *3*). Among EVOO phenolics, hydroxytyrosol (HT) is attracting distinct attention because of its ortho-diphenolic structure. HT is bioavailable, and its metabolism has been elucidated in animals and humans (*4*, *5*). Many in vitro studies indicate that HT is a powerful antioxidant and exerts biological, for example, anti-inflammatory and antithrombotic, activities that are being investigated in vivo. As an example, HT administration enhances plasma antioxidant capacity and lowers the production of pro-inflammatory and prothrombotic mediators in laboratory animals, where it also exerts hypocholesterolemic effects (*6*). In summary, the majority of human studies demonstrate modulation of surrogate markers of coronary heart disease and oxidative DNA damage (reviewed in ref *3*). As a consequence, the interest in cheap and abundant sources of olive phenolics is rapidly growing. One such source is olive mill wastewater, currently discarded although rich in polyphenols—including HT—that can be recovered with ad hoc techniques (*7*−*9*).

Thus far, the near entirety of studies focused on the potential healthful effects of HT on the cardiovascular system (*10*). In contrast, nothing is known about the possible impact of HT and other olive phenols, for example, oleuropein, on the brain. Yet, recent epidemiological and human intervention studies as well as detailed animal experiments are suggestive of neuroprotective effects of long-term intake of fruit- and vegetable-rich diets (*11*−*13*). Whereas a mechanism of action has been recently suggested

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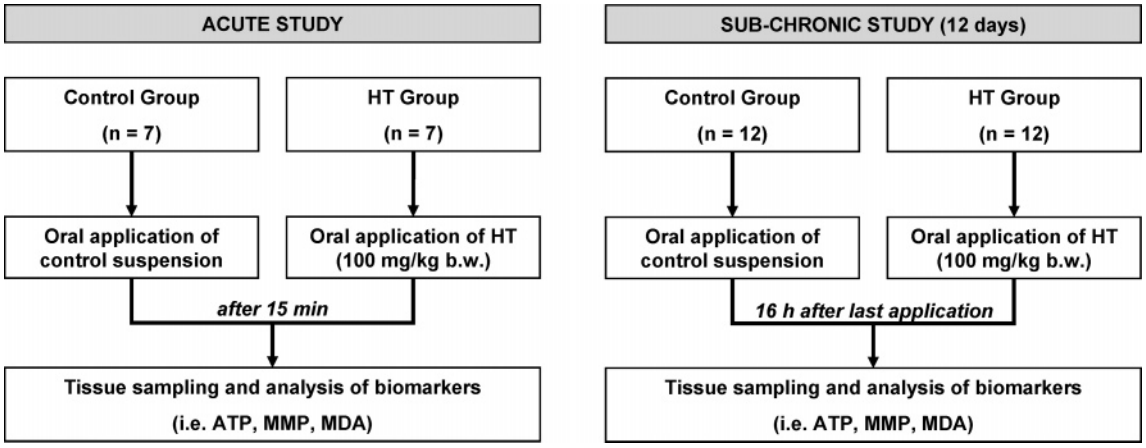

Figure 1. Design of the ex vivo studies.

for phenolics such as catechins from tea (*14*), the extent and precise nature of neuroprotection have yet to be revealed for most secondary plant metabolites.

The aim of the present study was to test the potential of an HT-rich extract (HT-E), which was prepared from olive mill wastewater, to improve the in vitro and ex vivo (**Figure 1**) resistance of dissociated brain cells (DBC) to oxidative stress.

MATERIALS AND METHODS

Chemicals. Hytolive, an HT-rich extract prepared from olive mill wastewater, was provided by Genosa I+D (Malaga, Spain). The extract contains 45.5% HT, 8.07% tyrosol, and 2.32% oleuropein, as assessed by HPLC analysis.

Dulbecco's modified Eagle's medium (DMEM) and rhodamine 123 (R123) were purchased from Invitrogen (Grand Island, NY); horse serum was from PAA (Pasching, Austria); agarose (type XI), bovine serum albumin, hydrogen peroxide (H_2O_2; 30% solution), fetal calf serum, Hank's balanced salt solution (HBSS), and sodium nitroprusside (SNP) were from Sigma (St. Louis, MO), and heparin (25000 IU/5 mL) was from Ratiopharm (Ulm, Germany).

All other reagents were of the highest available purity and purchased from Sigma (Grand Island, NY) or Merck (Darmstadt, Germany). Aqueous solutions were prepared with deionized, filtered water (Millipore, Billerica, MA).

HPLC Analysis. HPLC analyses were performed with solvents of analytical grade (Romil Ltd., Cambridge, U.K.) using an HP 1100 liquid chromatograph equipped with a diode array UV detector. The experimental conditions were similar to those previously reported (*15*). Briefly, as analytical column a 4.0 × 250 mm Lichrospher, 5 μm (Merck), maintained at 30 °C, was used. Elution was performed at a flow rate of 1.0 mL/min, using as mobile phase a mixture of water/acetic acid (97:3, v/v; solvent A) and methanol/acetonitrile (50:50, v/v; solvent B). Chromatograms were acquired at 240, 280, and 335 nm.

Preparation of DBC for in Vitro Studies. Female NMRI mice (3−4 months old) used in this study were purchased from Harlan-Winkelmann GmbH (Borchen, Germany). The protocol for animal feeding studies and tissue collection conducted in our laboratory had been approved by the local authorities for animal welfare, and all experiments were handled according to the European Communities Council Directive 86/606/EEC for animal care; that is, mice were housed in groups of 6−10 animals in plastic cages at room temperature and under a 12-h light/dark cycle, with ad libitum access to drinking water and standard pelleted diet.

Tissues were collected after cervical dislocation, followed by decapitation. After removal of the cerebellum, brains were quickly dissected on ice. A previously published method was used for preparing DBC (*16*). This method offers the advantage to perform studies in vital, differentiated brain cells and has been successfully used for the study of signal transduction mechanisms and changes in calcium and potassium levels as well as drug-induced neuroprotection (*16*−*18*).

After dilution in DMEM supplemented with 10% heat-inactivated fetal calf serum and 5% heat-inactivated horse serum, DBC were seeded in multiwell plates and cultured at 37 °C in a humidified incubator containing 5% CO_2.

DBC were incubated for 1 h with HT-E samples freshly diluted from stock solutions prepared in DMSO (≤0.5% final concentration). Thereafter, DBC were centrifuged for 5 min at 1500 rpm, and the medium was replaced with new DMEM. DBC were incubated with various concentrations of SNP or Fe^{2+} ($FeCl_2 \times 4\ H_2O$) for another 3 h prior to the analysis of oxidative stress markers. Stressors were used at concentrations causing a 40−50% reduction in the signal of the MMP and ATP assay, respectively.

Animals and Extract Administration for ex Vivo Studies. For the acute feeding study, female NMRI mice (4 months old) were treated once by oral gavage (pharyngeal tube) with 0.2% agarose suspension containing 53.6 mg of Hytolive/mL, thus providing 100 mg of HT/kg of body weight (bw). Control animals received pure agarose suspension. Animals were sacrificed 15 min after sample administration, and DBC were prepared as described above.

For the subchronic feeding study (12 days), female NMRI mice (4 months old) were treated once daily (at 4:00 p.m.) by oral gavage (pharyngeal tube) with 0.2% agarose suspension containing 53.6 mg of Hytolive/mL, thus providing 100 mg of HT/kg of bw. Control animals received pure agarose suspension. Animals were sacrificed 16 h after the last feeding, and DBC were prepared as described above.

In both studies, DBC were incubated with various concentrations of SNP or Fe^{2+} ($FeCl_2 \times 4\ H_2O$) prior to the analysis of oxidative stress markers.

Measurement of Mitochondrial Membrane Potential (MMP). DBC were seeded in 48-well plates (Greiner, Frickenhausen, Germany) and added, in triplicate, with the oxidative stressors (2 mM SNP or 2.5 mM Fe^{2+}). MMP was measured with the fluorescence dye R123. In detail, DBC were incubated in the dark for 15 min with 0.4 μM R123 on a horizontal shaker. DBC were twice both centrifuged (1500 rpm, 5 min) and washed with HBSS buffer (supplemented with Mg^{2+}, Ca^{2+}, and HEPES; pH 7.4; 37 °C). After supplementing DBC with new HBSS, MMP was assessed by reading the R123 fluorescence at an excitation wavelength of 490 nm and at an emission wavelength of 535 nm (Victor^2 1420 multilabel counter, Perkin-Elmer, Rodgau-Jügesheim, Germany). The fluorescence in each well was read in five consecutive runs.

Measurement of ATP Levels. DBC were seeded in white, 96-well plates (Cambrex, East Rutherford, NJ) and added, in triplicate, with the oxidative stressors (300 μM SNP or 800 μM Fe^{2+}). The ViaLight Plus bioluminescence kit (Cambrex), which is based on the production of light from ATP and luciferin in the presence of luciferase, was employed for assessing ATP levels. Briefly, the multiwell plate was removed from the incubator and allowed to cool to room temperature for 10 min. Thereafter, DBC were incubated for 10 min with the lysis buffer and for an additional 2 min with the monitoring reagent. The emitted light (bioluminescence), which is linearly related to ATP

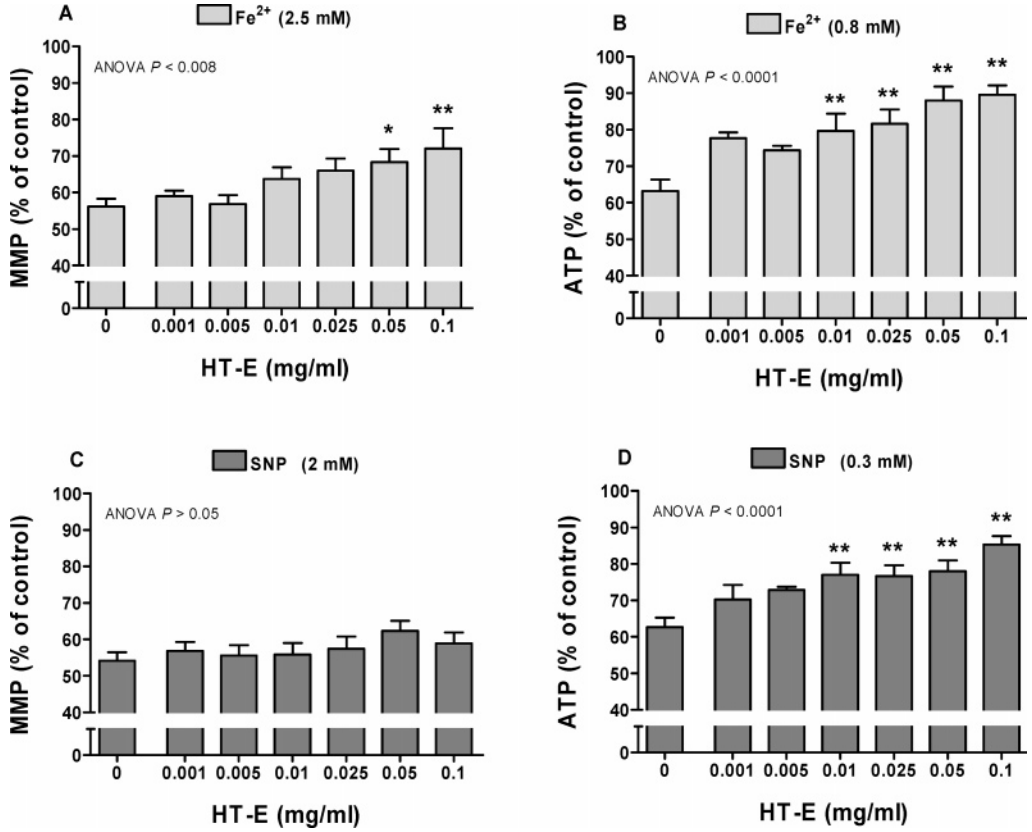

Figure 2. In vitro effect of HT-rich extract on cellular ATP levels and on the mitochondrial membrane potential (MMP) of DBC. Cells were incubated with HT-E (0.001–0.1 mg/mL) for 1 h prior to the addition of (**A**, **B**) oxidative (Fe^{2+}-induced) or (**C**, **D**) nitrosative (SNP-induced) stressor, which were incubated for 3 h. ATP levels were quantified using a luciferase-based bioluminescence assay. MMP was assessed using the fluorescence dye R123. All results are expressed as percent of untreated controls. HT versus stressor only treatment: *, $P < 0.05$; **, $P < 0.01$ ($n = 3$–9).

concentration, was recorded using a luminometer (Victor^2 1420 multilabel counter, Perkin-Elmer, Rodgau-Jügesheim, Germany).

Measurement of Lipid Peroxidation. DBC were seeded in 48-well plates (Greiner, Frickenhausen, Germany) and incubated with the stressor (200 μM Fe^{2+}) for 30 min at 37 °C in a humidified incubator containing 5% CO_2. Lipid peroxidation was assessed as malondialdehyde (MDA), an end product derived from peroxidation of polyunsaturated fatty acids and related esters present in lipid membranes. MDA concentration was measured by the colorimetric reaction of one molecule of malondialdehyde with two molecules of *N*-methyl-2-phenylindole (NMPI) in acetonitrile, leading to the formation of a chromophore with maximal absorbance at 586 nm (*19*). Briefly, after incubation with Fe^{2+}, the 48-well plate was placed on ice, and lipid peroxidation was stopped by the addition of 10 μL of 125 mM butylhydroxytoluene in acetonitrile. Cells were then transferred to Eppendorf tubes, sonicated, and centrifuged for 10 min at 6000 rpm, at 4 °C. For MDA measurement, 200 μL of clear supernatant was incubated with NMPI and 150 μL of 12 N HCl, in covered glass tubes for 60 min at 45 °C. Thereafter, glass tubes were placed on ice for 15 min, and 200 μL of the reaction mixture was transferred, in duplicate, to a 96-well plate (Greiner). Absorbance of the chromophore was measured at 570 nm (ASYS Hitech Digiscan, Eugendorf, Germany). 1,1,3,3-Tetramethoxypropane in Tris-HCl buffer was used as the MDA standard.

Measurement of Antioxidant Capacity (AOC). After decapitation, blood was immediately collected in plastic tubes containing 20 μL of heparin. Samples were centrifuged for 10 min at 3000 rpm and 4 °C. Plasma was transferred to Eppendorf tubes and stored at −80 °C. The AOC of plasma was measured by a validated method based on the reduction of Cu^{2+} to Cu^{+}, using uric acid as the reference compound. Results are expressed as milliequivalents of uric acid (*20*).

Protein Quantification. Protein levels were determined using the Protein Assay Kit from Bio-Rad (Munich, Germany), and bovine serum albumin served as the standard.

Calculations and Statistics. All data were normalized to the amount of protein and are reported as means ± SEM. Statistical analysis was performed by applying one-way ANOVA followed by Dunnett's post test and unpaired Student's *t* test for in vitro and ex vivo studies, respectively (Prism 4.0, GraphPad Software, San Diego, CA). A *P* value of <0.05 was considered to be statistically significant.

RESULTS

HT-Rich Extract Prevents Oxidative Stress-Induced Loss of Cellular ATP Levels and MMP in Vitro. Brain cells are particularly vulnerable to the damaging effects of reactive species (ROS and RNS), when in excess. Oxidative and nitrosative stresses have been suggested to be causally associated with neurodegeneration (*21*). The two stressors we selected in this study, that is, SNP and Fe^{2+}, are known to readily induce cell-damaging compounds. Whereas SNP releases NO, which impairs cell viability either directly or via formation of peroxynitrite ($ONOO^-$), ferrous iron triggers the production of noxious ROS, especially hydroxyl radicals ($OH^•$) (*16*, *22*). The addition of SNP or Fe^{2+} to the culture medium impaired the MMP and ATP levels of DBC by >40% (**Figure 2**). DBC preloaded with HT-E were less susceptible to SNP- and Fe^{2+}-induced damage. In particular, HT-E concentrations as low as 0.01 mg/mL, which correspond to a 24 μM concentration of pure HT, significantly prevented oxidative and nitrosative stress-induced ATP loss. Also, HT-E dose-dependently attenuated the hampering impact of Fe^{2+}, but not of SNP, on MMP. It is noteworthy that a 10-fold concentration (0.1 mg/mL) of HT-E did not induce cellular stress, suggesting that the extract—as well as its main constituent, HT—are nontoxic to DBC, even at high concentrations.

Table 1. Oxidative and Nitrosative Stress Biomarkers in DBC Prepared from Mice Treated Once with 100 mg of HT/kg of bw (Acute Feeding Study)

	MMP[a]		ATP[b]		MDA[c]
	SNP-stimulated	Fe^{2+}-stimulated	SNP-stimulated	Fe^{2+}-stimulated	Fe^{2+}-stimulated
control	8.11 ± 1.14	8.16 ± 1.17	1.36 ± 0.20	1.16 ± 0.17	1.30 ± 0.27
HT	9.70 ± 1.36	9.45 ± 1.47	1.66 ± 0.31	1.33 ± 0.25	1.14 ± 0.28

[a] Mitochondrial membrane potential (FLU/mg of protein [× 10^4]); means ± SEM; $n = 7$. [b] ATP levels (μM/mg of protein); means ± SEM; $n = 7$. [c] MDA (μM/mg of protein); means ± SEM; $n = 6$.

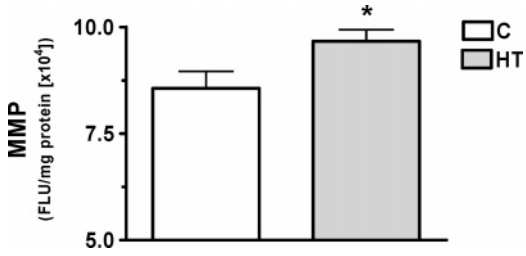

Figure 3. Effect of subchronic HT-E administration on basal MMP of DBC. Mice were orally fed HT-E (HT = 100 mg/kg of bw) once a day for 12 days. MMP was assessed using the fluorescence dye R123. Results are expressed as protein-normalized fluorescence units (FLU). HT group versus control group: *, $P < 0.05$ ($n = 12$).

Tolerance of Feeding Procedure. Treatments with both control and HT-E suspensions were well tolerated during both feeding regimes. No significant differences in live weight occurred between experimental groups at any day of the subchronic study (data not shown).

Acute Treatment with HT-Rich Extract Does Not Improve ex Vivo Resistance to Oxidative Stress. As rapid uptake of HT into the brain has been previously reported (*23*), we assessed whether acute oral HT-E treatment had an impact on basal parameters or was able to improve the resistance of DBC to the noxious effects of ROS and RNS (**Table 1**). Although mice were fed a comparatively high dose of HT-E, providing 100 mg of pure HT/kg of bw, none of the mitochondria-associated parameters, i.e., MMP and ATP levels, differed from those of control animals (**Table 1**). Also, an equivalent increase in iron-induced lipid peroxidation was observed in both groups.

Subchronic Feeding of HT-Rich Extract Ameliorates Basal MMP in DBC. On the basis of data from the acute study, a subchronic feeding experiment was undertaken to test the long-term effects of HT-E intake on biomarkers of brain cell stress after a wash-out period of 16 h. The integrity of mitochondria is, in fact, indispensable to cell function; marked depolarization of MMP triggers brain cell death (*24*). The MMP of unstressed DBC prepared from HT-E-fed mice exhibited a moderate, although statistically significant, hyperpolarization (**Figure 3**), an effect that has been associated with reduced rate of cell death (*25*). Basal ATP levels, however, did not differ between study groups (data not shown).

Subchronic Feeding of HT-Rich Extract Does Not Ameliorate Mitochondria-Associated Stress Parameters in DBC. We next investigated the effects of subchronic HT-E feeding on DBC vulnerability ($n = 12$), by measuring MMP (assessed as FLU/mg of protein [× 10^4]) and ATP levels (assessed as μM/mg of protein) after SNP- and iron-induced oxidative stress. Neither ATP levels (SNP, 1.24 ± 0.065 vs 1.23 ± 0.068; Fe^{2+}, 1.10 ± 0.057 vs 1.13 ± 0.057) nor MMP (SNP, 5.19 ± 0.15 vs 5.30 ± 0.22; Fe^{2+}, 5.68 ± 0.19 vs 5.89 ± 0.20) significantly differed between control and HT-E groups, respectively, indicating that prolonged HT-E intake does not convey mitochondrial protection from severe oxidative and nitrosative stress.

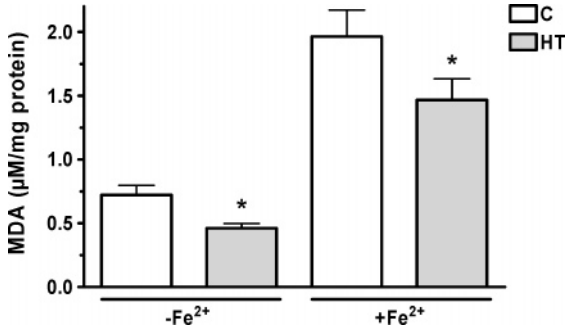

Figure 4. Effect of subchronic HT-E administration on basal ($-Fe^{2+}$) and iron-induced ($+Fe^{2+}$) lipid peroxidation in DBC. Mice were orally fed HT-E (HT = 100 mg/kg of bw) once a day for 12 days. DBC were stressed with Fe^{2+} (200 μM) for 1 h. Extent of lipid peroxidation is expressed as protein-normalized MDA levels. HT group versus control group: *, $P < 0.05$ ($n = 12$).

Subchronic Feeding of HT-Rich Extract Decreases Basal and Iron-Stimulated Lipid Peroxidation in DBC. In contrast to the data from the acute feeding study reported above, subchronic administration of HT-E significantly reduced both basal and iron-stimulated lipid peroxidation (**Figure 4**). These observations are in accordance with previous studies reporting lower lipid peroxidation in the brains of animals fed polyphenol-rich plant extracts (*26*). It is noteworthy that the reduction of basal MDA production in mice fed HT-E cannot entirely explain the observed protection of DBC from iron-induced lipid peroxidation. This suggests that oral HT-E provides DBC with an ability to counteract acute oxidative stress, namely, when it is induced by metal ions.

Plasma Antioxidant Capacity. There was no significant difference in AOC (assessed as μM Cu^{2+} reduced) between the groups ($n = 8-11$) treated with vehicle versus HT at the end of the subchronic study (73.9 ± 6.0 vs 73.2 ± 5.1, respectively).

DISCUSSION

The incidence of neurodegenerative disorders and their sequelae such as impaired cognition and memory is escalating, largely due to increased lifespan. As a consequence, considerable interest exists for feasible and cost-effective means of preventing neurodegeneration, especially in the elderly. In addition to cardioprevention, appropriate diets, such as Mediterranean diets, have been associated with reduced incidence of neurodegenerative disorders (*27*) and are also cost-effective in terms of cost per year of life gained (*28*). In the present study, we assessed the neuroprotective potential of an olive mill wastewater extract rich in hydroxytyrosol (HT-E), both in vitro and ex vivo using DBC. Previously, we showed that >80% of DBC are viable (*18*), and the plethora of data obtained with DBC argue for the suitability of this cellular model for studying brain-relevant nutritional mechanisms (*16*, *29*). In fact, olive oil consumption has been associated with lower cognitive decline, but most of the effects have been—to date—attributed to the intake of monounsaturates (*27*). However, extra virgin olive oil is rich in phenolic compounds (namely, oleuropein and hydroxytyrosol), which are being increasingly held responsible for its putative health effects (*10*, *30*). Nevertheless, whereas the intake of HT, for example, through the consumption of EVOO, has been shown to modulate surrogate markers of cardiovascular

disease (*10*), the effect of HT on brain cell survival has been only rarely investigated in vitro (*31*, *32*) and never ex vivo.

The bioavailability of HT (including that isolated from olive mill wastewater) has been thoroughly studied in numerous rodent and human experiments. In brief, HT is dose-dependently absorbed and is metabolized and mainly excreted in the urine (*33*). In addition, the penetration of HT into brain has been demonstrated in a pharmacokinetic study assessing the metabolic fate of intravenously injected [^{14}C]HT (*23*). On the basis of these notions, we designed two ex vivo studies to assess the neuroprotective potential of HT-E, following acute and subchronic oral administrations. The daily HT dose of 100 mg/kg of bw is high in terms of human consumption, but still safe, as oral intakes of HT and olive mill wastewater extract up to 2 and 500 mg/kg of bw (≈80 mg of polyphenols), respectively, did not show appreciable toxicity in rodents (*8*, *23*).

Although exposure to low concentrations of NO might be neuroprotective, exposure of DBC to sodium nitroprusside (generally used to induce nitrosative stress in vitro) led to severe depolarization of the MMP and a drop in cellular ATP, as previously reported by our group (*16*). Whereas preincubation of DBC with HT-E exerts no effects on MMP after the induction of nitrosative stress, ATP levels are dose-dependently maintained by HT-E. The neuroprotective activity of HT became apparent from HT-E concentrations as low as 0.01 mg/mL (≈24 μM HT) and are in accordance with previous studies reporting neuroprotection of low concentrations of plant extracts, such as *Ginkgo biloba* (*24*). Our results are also in agreement with in vitro data by Deiana et al. (*31*), showing—in neuronal-like cells preloaded with HT—reduced levels of DNA damage and tyrosine nitration after the addition of peroxynitrite, another highly reactive, nitrosative stress-initiating agent (*34*).

In contrast to what we observed after challenge with SNP, the prevention of Fe^{2+}-induced cytotoxicity by HT-E was confirmed by both stress parameters (**Figure 2A**,**B**). HT possesses excellent iron chelating properties due to its catecholic structure (*7*), and it is conceivable that this mechanism is largely responsible for the protection of DBC mitochondrial function from Fe^{2+}-induced oxidative stress in vitro, as already shown for green tea catechins (*14*).

Increased oxidative and nitrosative damage has been repeatedly found in brain and other tissues of subjects with cognitive impairment, and a single administration of isolated polyphenols or polyphenol-rich plant extracts to rodents leads to the amelioration of both behavioral parameters, suggesting that polyphenols are capable of exercising neuronal biological activities and brain biomarkers reflecting antioxidant response (*35*−*38*). Despite the promising results obtained in in vitro experiments, our data from the acute feeding study do not support a prompt protection from nitrosative and oxidative stress of brain tissue (namely, DBC) following acute HT intake. These data support the notion that only long-term intake of diets rich in vitamins, minerals, and secondary plant metabolites are able to afford meaningful brain protection from oxidative stress and neurodegeneration, which is of long latency and multifactorial in nature (*12*). Accordingly, our subchronic study strongly indicates enhanced resistance of DBC to oxidative stress, thus providing experimental evidence for the association between long-term plant food intake and better health.

Mechanistically, enhanced lipid peroxidation is associated with many chronic disorders, even though the extent and precise nature of oxidative processes are yet to be fully determined (*21*, *39*). However, the consensus is that oxidative stress is, to some extent, implicated in neurodegeneration, as oxidative damage of neuronal plasma membranes and their milieu are associated with memory impairment and cognitive decline (*40*). Subchronic HT-E intake significantly reduced basal and Fe^{2+}-induced MDA formation in our ex vivo model, even after a 16 h wash-out period (**Figure 4**). It is noteworthy that the degree of protection recorded under unstressed condition was smaller than that measured after challenge with Fe^{2+}, indicating that HT directly or indirectly exerted antagonistic actions on mechanisms leading to lipid peroxidation. Aside from the well-known iron-scavenging activity of HT, other effects on antioxidant defense lines, such as increased expression of antioxidant enzymes, although yet to be demonstrated, might be responsible for the attenuation of lipid peroxidation in DBC. Indeed, the lack of systemic effects, in terms of antioxidant capacity, that we recorded suggest that the mechanisms of cytoprotection elicited by HT are manifold and not limited to antioxidant activity, as previously suggested for other polyphenols/flavonoids (*41*).

Mitochondrial dysfunction, especially in the from of severe changes in MMP, is believed to be crucial for the onset and progression of neurodegeneration observed in the aged as well as the demented brain (*42*, *43*). In contrast, moderate depolarization, for example, due to activity of uncoupling proteins, and hyperpolarization of MMP have been suggested to lower the release of potentially deleterious metabolites by mitochondria and to improve their stress resistance, respectively (*25*, *44*). We found a moderate MMP hyperpolarization in DBC under basal conditions (**Figure 3**), which might be directly or indirectly responsible for the reduced levels of lipid peroxidation under unstressed conditions. Various mechanisms might have caused the moderately hyperpolarized MMP such as changes in the genomic and proteomic profile, which have been detected in the brains of antioxidant-treated animals, and often affect mitochondrial activity (*45*, *46*).

Despite the above-mentioned moderate MMP hyperpolarization, which has been reported by others to convey cell protection (*25*), the one we recorded was of insufficient magnitude to completely prevent nitrosative and oxidative stress-induced MMP decline and ATP loss ex vivo, even though HT-E effectively preserved (by about 40−50%) MMP and ATP levels in vitro. That degree of damage, however, was too severe to detect cytoprotection following subchronic HT-E treatment. It should be remembered, though, that life-long exposure to macro- and micronutrients differs from that to drugs, generally of short or medium duration. We can speculate that regular intake of HT, for example, through EVOO, contributes to the prevention of neurodegeneration in the Mediterranean basin, to an extent as yet to be fully computed.

In conclusion, this study indicates that HT and EVOO afford neuroprotection and might contribute to the lower incidence of neurodegenerative diseases, as observed in the Mediterranean area. Moreover, we corroborate that olive mill wastewater can be exploited for obtaining HT-rich extracts with promising biological effects (*7*, *47*, *48*). These activities affect several oxidative stress pathways and are apparent not only in vitro but also, partly, ex vivo. Although the first study of its kind, our data provide an informative basis for subsequent in vivo experiments exploring the neuroprotective potential of long-term HT intake.

SAFETY

All experiments have been conducted in accordance with the respective institutional guidelines.

ABBREVIATIONS USED

AOC, total antioxidant capacity; bw, body weight; DBC, dissociated brain cells; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; EVOO, extra virgin olive oil; HBSS, Hank's balanced salt solution; FLU, fluorescence units; HT, hydroxytyrosol; HT-E, hydroxytyrosol-rich extract; LPO, lipid peroxidation; MDA, malondialdehyde; MMP, mitochondrial membrane potential; NMPI, *N*-methyl-2-phenylindole; R123, rhodamine 123; RNS, reactive nitrogen species; ROS, reactive oxygen species; SNP, sodium nitroprusside.

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