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Protective Activity of Hydroxytyrosol Metabolites on Erythrocyte Oxidative-Induced Hemolysis

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ABSTRACT: The capacity of important hydroxytyrosol metabolites (homovanillyl alcohol, hydroxytyrosol acetate, homovanillyl alcohol acetate, hydroxytyrosol 3′ and 4′-*O*-glucuronides, and homovanillyl alcohol 4′-*O*-glucuronide) to protect red blood cells (RBCs) from oxidative injury induced by the radical initiator 2,2′-azo-bis(2-amidinopropane) dihydrochloride (AAPH) or by the natural radical initiator H_2O_2 was evaluated. In the presence of AAPH, all compounds showed to protect RBCs from hemolysis in a dose-dependent manner, exccept for the homovanillyl alcohol glucuronide, with the order of activity being at 20 μM hydroxytyrosol > hydroxytyrosol glucuronides = hydroxytyrosol acetate = homovanillyl alcohol = homovanillyl acetate > homovanillyl alcohol glucuronide. At 10 μM, hydroxytyrosol, hydroxytyrosol acetate, and hydroxytyrosol glucuronides still protected hemoglobine from oxidation and from morphological RBC changes. In the presence of H_2O_2, hydroxytyrosol showed to significantly protect RBCs from oxidative hemolysis in a dose-dependent manner, but the hydroxytyrosol glucuronides showed only a limited protection that was independent of the concentration used.

KEYWORDS: *polyphenols, erythrocytes, olive oil, hydroxytyrosol, homovanillyl alcohol, hydroxytyrosol acetate, glucuronides*

INTRODUCTION

Antioxidants have received particular attention because of their potential to modulate oxidative stress associated with chronic disease. The lower incidence of coronary heart disease and of some cancers in the Mediterranean area led to the hypothesis that a diet rich in fruits, vegetables, and grains has a beneficial effect on health. The major fat component of the so-called "Mediterranean diet" is virgin olive oil (VOO),[1] and several studies have suggested that phenolic compounds, although considered among the minor constituents of VOO, may contribute to the healthy nature of this diet.[2−8]

The classes of phenols present in olives are phenolic alcohols, such as hydroxytyrosol (3,4-dihydroxyphenylethanol, 3,4-DHPEA, or Hy) and tyrosol (4-hydroxyphenylethanol, 4-HPEA, or Ty), secoiridoids, flavonoids, and lignans. Secoiridoids are the most representative class in olives; the glycoside oleuropein (OL), one of the most important secoiridoids, represents up to 14% of the dry weight of olives. During VOO extraction, secoiridoid glycosides such as oleuropein are hydrolyzed. Therefore, the major phenolic compounds found in VOO are the dialdehydic form of elenolic acid linked to Hy (3,4-DHPEA-EDA), the OL aglycone (3,4-DHPEA-EA), and the dialdehydic form of elenolic acid linked to Ty (4-HPEA-EDA). These compounds account for up to 80% of the total phenolic fraction.[9−11]

Although many studies have investigated the in vitro antioxidant properties of the minor VOO phenolics OL and especially of Hy, as well as their protective effects against cell injury, the biological properties of these phenols in vivo will depend on the extent to which they are absorbed and metabolized. Bioavailability studies have demonstrated that Hy is well absorbed in the small intestine[12] and, together with its acetate, glucuronide, and sulfate conjugates and homovanillyl alcohol and its glucuronide and sulfate conjugates, it can be found in urine and plasma after olive oil consumption (Figure 1).[13−15]

Until recently, studies on the bioavailability for secoiridoids, except for oleuropein, were inexistent. However, an important body of evidence has shown that OL aglycones are bioavailable. After the consumption of VOO, the concentration of Hy rises more than expected if only the Hy existent in the oil was absorbed, and it is proportional to the total phenol content and not to the Hy content of the oil.[12] The first study on the bioavailability of 3,4-DHPEA-EDA and 3,4-DHPEA-EA showed that both secoiridoid compounds are well absorbed by Caco-2 cells and by the rat intestine and are not only metabolized to reduced forms and conjugated with glucuronic acid but also hydrolyzed with the production of hydroxytyrosol and its metabolites.[16] Actually, it is believed that olive oil consumption could reduce oxidative damage due to its richness in oleic acid, as well as due to its minor components, particularly the phenolic compounds. However, which components have a major role in this protection is still unknown. Therefore, the potential health benefits of Hy and its derivatives may be attributed to both parental compounds and their phase I and phase II metabolites.

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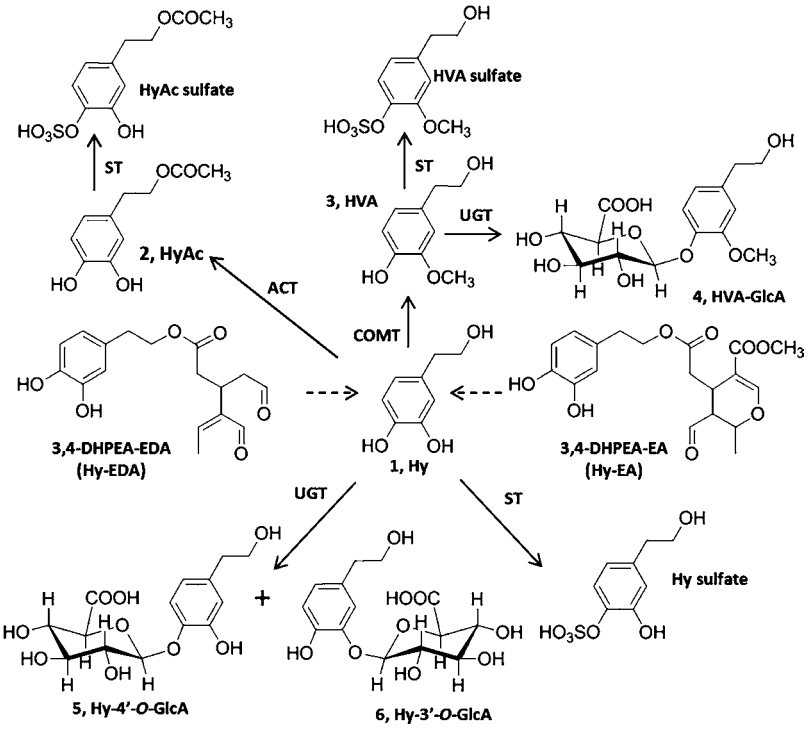

Figure 1. Metabolic pathways of the main polyphenolic compounds found in olive oil. Discontinuous lines indicate that more than a pathway is possible for hydroxytyrosol obtained from secoiridoids. UCT, glucuronosyltransferase; ACT, *O*-acetyltransferase; COMT, catechol methyl transferase; SF, sulfotransferase; Hy, hydroxytyrosol; HyAc, hydroxytyrosol acetate; Hy-4′-*O*-GlcA and Hy-3′-*O*-GlcA, hydroxytyrosol glucuronides; HVA, homovanillyl alcohol; HVAAc, homovanillyl alcohol acetate; HVA-GlcA, homovanillyl alcohol glucuronide.

Human red blood cells (RBC) are particularly useful in the evaluation of the antioxidant properties of several compounds. RBCs, the most abundant blood cells, are particularly susceptible to endogenous oxidative damage because of their specific role as oxygen carriers. In the normal metabolism of RBCs, around 0.3% of the oxygen molecule is shifted from its normal role with the production of superoxide anion.[16] Healthy subjects are equipped with efficient RBC antioxidant endogenous systems. However, if reactive oxygen species (ROS), such as H_2O_2 and O_2^-, are overproduced within the erythrocyte, an "oxidative stress" condition will develop, inducing oxidative damage on erythrocyte constituents, which may lead ultimately to hemolysis.[17] When the capacity of protective hemoglobin-scavenging mechanisms has been saturated, levels of cell-free hemoglobin increase in the plasma.[18] Whenever the hemoglobin is released from erythrocytes, it is potentially dangerous because it can be converted into oxidized forms, powerful promoters of oxidative processes in blood.[18,19] Moreover, the production and modulation of NO blood concentrations[20,21] and ascorbate recycling[22] by RBCs are also very important for the body defense against the development of oxidative stress in the cardiovascular system.

In this work, the capacity of some of the main hydroxytyrosol (Hy) phase I and phase II metabolites, hydroxytyrosol acetate (HyAc), hydroxytyrosol 3′ and 4′ *O*-glucuronides (Hy-GlcA), homovanillyl alcohol (HVA), homovanillyl acetate (HVAAc), and homovanillyl alcohol 4′-*O*-glucuronide (HVA-GlcA), to protect RBCs from oxidative injury induced by the water-soluble radical initiator 2,2-azobis(2-amidinopropane) dihydrochloride (AAPH) was evaluated. At 37 °C, azo-initiator compounds react with molecular oxygen producing peroxyl radicals at a constant rate that mimetizes the attack of free radicals from the plasma to the RBCs. The capacity of hydroxytyrosol 3′ and 4′ *O*-glucuronides was also evaluated against the oxidative injury induced by the physiological oxidizing agent in erythrocytes, the H_2O_2. Our aim was, therefore, to evaluate the antioxidant capacity of these compounds that might be important in protecting cells from oxidative damage in oxidative stress conditions, such as cardiovascular diseases, diabetes, and neoplasic disorders.

MATERIALS AND METHODS

All chemicals were obtained from chemical suppliers and used without further purification, unless otherwise noted. All reactions were monitored by thin layer chromatography (TLC) on precoated Silica-Gel 60 plates F_{254}, and detected by heating with Mostain [500 mL of 10% H_2SO_4, 25 g of $(NH_4)_6Mo_7O_2{\cdot}4H_2O$, 1 g of $Ce(SO_4)_2{\cdot}4H_2O$]. Products were purified by flash chromatography with Silica Gel 60 (200−400 mesh). NMR spectra were recorded on 400 MHz NMR equipment, at room temperature for solutions in $CDCl_3$, D_2O, or CD_3OD. Chemical shifts are referred to the solvent signal and are expressed in ppm. 2D NMR experiments (COSY, TOCSY, ROESY, and HMQC) were carried out when necessary to assign the corresponding signals of the compounds.

Phenolic Compounds. Hydroxytyrosol (**1**) was synthesized from 3,4-dihydroxyphenylacetic acid (Sigma-Aldrich Quimica-S.A. Madrid, Spain) according to the procedure of Baraldi et al.[23] Hydroxytyrosol acetate (**2**) and homovanillyl alcohol acetate were synthesized from hydroxytyrosol and homovanillyl alcohol (**3**), respectively, according to the procedure of Bernini et al.[24]

Synthesis of Hydroxytyrosol Metabolites. The synthesis of glucuronides was performed according to the method described by Lucas et al.[25] with some modifications (Figure 2).

*2-[3′-Hydroxy-4′-(methyl-2,3,4-tri-O-acetyl-β-D-glucopyranosyluronate) phenyl] EtOAc (**8**) and 2-[4′-Hydroxy-3′-(methyl-2,3,4-tri-O-acetyl-β-D-glucopyranosyluronate)phenyl] Ethyl Acetate (**10**).* To a solution of trichloroacetimidate 7 (990 mg, 2 mmol) and

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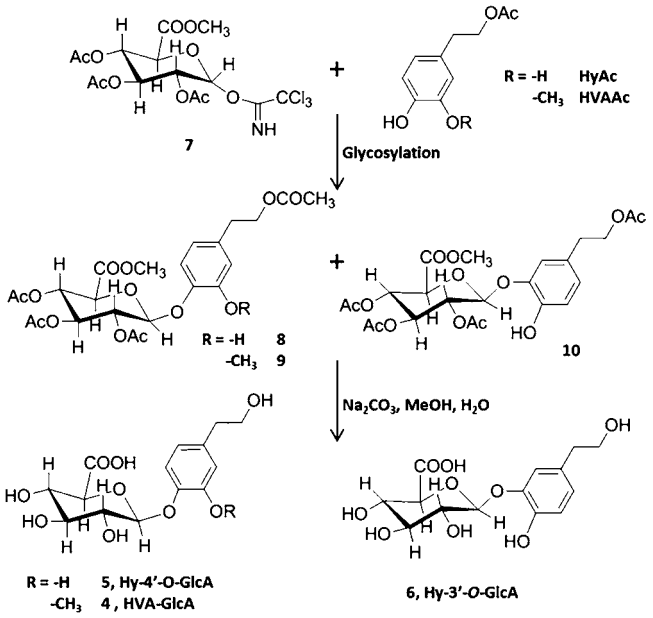

Figure 2. Synthesis of hydroxytyrosol 3′ and 4′-*O*-*β*-D-glucuronides (**5** and **6**) and homovanillyl alcohol-4′-*O*-*β*-D-gucuronide (**4**).

hydroxytyrosol acetate **2** (550 mg, 2.8 mmol) in anhydrous CH_2Cl_2 (6 μL) at −10 °C was added TMSOTf (95 μL, 0.52 mmol) dropwise. After 2 h, TLC (hexane−EtOAc 1:1) showed the formation of a new product and complete consumption of the glycosyl donor. The reaction was neutralized with triethylamine and concentrated in vacuum. The resulting residue was purified by flash column chromatography (hexane−ethyl acetate 1:1) to afford a 1:1 regioisomeric mixture of **9** and **10** (445 mg, 31%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.88 (d, 2H, H_{arom}, J = 8.0 Hz), 6.82 (d, 2H, H_{arom}, J = 1.8 Hz), 6.66 (dd, 2H, H_{arom}), 5.37−5.25 (m, 6H), 5.00 (d, 1H, J = 10.4 Hz), 4.99 (d, 1H, J = 10.4 Hz), 4.23 (t, 2H, J = 7.2 Hz), 4.22 (t, 2H, J = 7.2 Hz), 4.17 (d, 2H, J = 9.2 Hz), 3.73 (s, 6H, CH_3O), 2.84−2.80 (m, 4H, 2 CH_2), 2.11−2.03 (m, 24H, CH_3CO); δ ^{13}C NMR (75 MHz, $CDCl_3$) 171.9, 170.9.0, 170.7, 170.3, 167.7, 148.4, 147.1, 144.8, 143.7, 136.4, 130.9, 126.7, 121.6, 119.3, 119.0, 118.0, 117.5, 102.5, 102.4, 78.3, 73.4, 72.3, 69.9, 65.8, 65.7, 54.1, 35.4, 35.2, 21.9, 21.6, 21.5, 21.4. Spectral data were in accordance with those found in the literature.[26]

*2-[3′-Hydroxy-4′-β-D-glucopyranosyluronic acid)phenyl] Ethanol (**5**) and 2-[4′-Hydroxy-3′-β-D-glucopyranosyluronic acid) phenyl] Ethanol (**6**).* A solution of the regioisomeric mixture of **9** and **10** (200 mg, 0.39 mmol) in methanol (6 mL) was stirred at room temperature under argon with a solution of Na_2CO_3 (70 mg, 0.65 mmol) in H_2O (1.5 mL). After 24 h, glacial acetic acid water was added to adjust the pH to 6. The solvents were then removed, and residue was purified by flash column chromatography [CH_2Cl_2/CH_3OH (1:1)] affording compounds **5** and **6** (116 mg, 90%) as a 1:1 regioisomeric mixture (determined by HPLC and NMR). ^{1}H NMR (400 MHz, D_2O) δ 7.12−6.61 (m, 6H, H_{arom}), 4.71 (d, 1H, J = 7.0 Hz), 4.70 (d, 1H, J = 7.0 Hz), 3.69 (t, 2H, J = 7.2 Hz), 3.68 (t, 2H, J = 7.2 Hz), 3.66−3.49 (m, 8H), 2.70 (t, 2H, J = 7.0 Hz), 2.69 (t, 2H, J = 7.2 Hz); ^{13}C NMR (75 MHz, D_2O) δ 177.0 (C═O), 148.8, 147.3, 146.9, 145.5, 136.8, 132.3, 125.9, 121.7, 120.9, 120.3, 118.1, 117.4, 105.4, 105.2, 77.4, 74.8, 74.7, 73.6, 64.5, 64.4, 39.8, 39.6. Spectral data were in accordance with those found in the literature.[26]

*2-[3′-Metoxy-4′-(methyl-2,3,4-tri-O-acetyl-β-D-glucopyranosyluronate) phenyl]ethyl Acetate (**9**).* To a solution of trichloroacetimidate **7** (990 mg, 2 mmol) and homovanillyl alcohol acetate **8** (590 mg, 2.8 mmol) in anhydrous CH_2Cl_2 (6 mL) at −10 °C was added $BF_3{\cdot}OEt_2$ (70 μL) dropwise (Figure 2). After 6 h, TLC (hexane−ethyl acetate 1:1) showed the formation of a new product and complete consumption of the glycosyl donor. The reaction was neutralized with NEt_3 and concentrated in vacuum. The resulting residue was purified by flash column chromatography (hexane−ethyl acetate 1:3) to afford 2-[3′-metoxy-4′-(methyl-2,3,4-tri-*O*-acetyl-*β*-D-glucopyranosyluronate)phenyl]ethyl acetate, **11** (471 mg, 32%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.06 (d, J = 8.0 Hz, 1H, H_{arom}), 6.74 (d, J = 1.9 Hz, 1H, H_{arom}), 6.72 (dd, 1H, H_{arom}), 5.29 (m, 3H), 4.99 (d, 1H, J = 7.2 Hz), 4.25 (t, 1H, J = 7.1 Hz), 3.80 (s, 3H), 3.74 (s, 3H), 2.88 (t, 2H, J = 7.1 Hz), 2.08 (s, 3H), 2.04 (s, 6H), 2.02 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 171.0, 170.1 (C═O), 169.4, 169.3, 167.0, 150.6, 144.4, 134.8, 121.1, 120.8, 113.4, 100.8, 72.6, 71.9, 71.1, 69.3, 64.8, 56.01, 52.9, 34,8, 21.0, 20.6, 20.5.

*2-[3-Metoxy-4′-β-D-glucopyranosyluronic acid)phenyl] Ethanol (**4**).* A solution of the compound **11** (200 mg, 0.38 mmol) in methanol (6 mL) was stirred at room temperature under argon with a solution of Na_2CO_3 (70 mg, 0.65 mmol) in H_2O (1.5 mL). After 24 h, glacial acetic acid water was added to adjust the pH to 6. The solvents were then removed, and residue was purified by flash column chromatography [CH_2Cl_2/CH_3OH (1:1)] affording compound **4** (87 mg, 67%). ^{1}H NMR (500.13 MHz, CD_3OD) δ 7.10 (d, 1H, J = 8.2 Hz), 6.85 (d, 1H, J = 1.9 Hz), 6.73 (dd, 1H), 4.81 (d, 1H, J = 7.2 Hz), 3.81 (s, 3H), 3.68 (t, 2H, J = 7.1 Hz), 3.64 (s, 3H), 3.48 (m, 3H), 2.73 (t, 2H, J = 7.0 Hz); ^{13}C NMR (100 MHz, CD_3OD) δ 176.2, 150.9, 146.6, 136.0, 122.7, 119.3, 114.7, 103.7, 77.6, 76.5, 74.7, 73.5, 64.3, 56.9, 39.9. MS: m/z = 343.33 $[M - H]^+$, 175.07 $[M - 167]^+$, 167.27 $[M - 175]^+$, 152.27 $[M - H - 175 - CH_3]^+$, 113.02 $[M - H - 175 - CO_2 - H_2O]^+$. Spectral data were in accordance with those found in the literature.[26]

Preparation of Erythrocyte Suspensions. Blood was obtained from healthy, nonsmoker volunteers (two women and two man aged 23−50 years old) by venipuncture, and collected into tubes containing ethylenediaminetetraacetic acid (EDTA), as an anticoagulant. Samples were immediately centrifuged at 400*g* for 10 min; plasma and buffy coat were carefully removed and discarded. RBCs were washed three times with phosphate buffered saline solution (PBS; 125 mM NaCl and 10 mM sodium phosphate buffer, pH 7.4) at 4 °C and, finally, resuspended in PBS, to obtain RBC suspensions at 2% hematocrit. RBC suspensions were used on the day they were prepared.

Induced Hemolysis. RBC suspensions were prepared at 2% hematocrit, and the assays were performed by using AAPH at final concentration of 60 mM or H_2O_2 at 7.5 mM. In all sets of experiments (n = 4, in quadruplicates), a negative control (RBCs in PBS) was used, as well as phenolic compound controls (RBCs in PBS, with each phenolic compound). Controls and sample tests were run in duplicate. Incubations of RBC suspensions were carried out at 37 °C for 4 h, under gentle shaking, in the presence of each individual compound or in the presence of the phenolic compound plus the radical initiator. Phenolic compounds were incubated 15 min with RBCs before the addition of the radical initiator (AAPH or H_2O_2), and they were tested at concentrations of 10, 20, 40, and 80 μM. Hemolysis was determined spectrophotometrically, according to Ko et al.[27] After the incubation period (2 or 4 h), an aliquot of the RBC suspensions was diluted with 20 volumes of saline and centrifuged (1200*g* for 10 min). The absorption (A) of the supernatant was read at 540 nm. The absorption (B), corresponding to a complete hemolysis, was acquired after centrifugation of a RBC suspension that was previously treated with 20 volumes of ice-cold distilled water. The percentage of hemolysis was then calculated ($A/B \times 100$).

Induced Morphological Changes. To study the morphological changes of RBC suspensions by optical microscopy, aliquots (50 μL) were taken from test tubes containing 10 and 80 μM of phenolic compounds, with and without AAPH, and controls at the end of the incubations. The samples were diluted (1:50) and then mounted in a slide with a coverslip. By using the same volume of the RBC suspensions, it was possible to compare in a rough way the number of RBC per microscopic field with the RBC lysis quantified previously by spectrophotometry.

Protective Effect of Phenolic Compounds against AAPH-Induced Hemoglobin Oxidation. To clarify the nature of the hemoglobin linked to RBC membranes and the concentration of oxyhemoglobin in hemolysates,[28] visible absorption spectral scans (450−650 nm) were performed.[29]

Statistical Analysis. The results obtained for the four independent hemolysis experiments (blood obtained each time from a different donor), performed in quadruplicate, are expressed as mean ± SEM. Statistical differences between groups of experiments with different antioxidant compounds were analyzed by two-way analysis of variance with posthoc testing using Tukey's test. A p value lower than 0.05 was accepted as being statistically significant.

RESULTS AND DISCUSSION

Increasing evidence has supported the hypothesis that a number of nutrients or non-nutrient dietary components, labeled as "antioxidants", might have a beneficial role regarding the course of chronic diseases. In particular, it has been claimed that olive oil polyphenols may play a major role on the protective effects of olive oil consumption against oxidative damage. However, little research has been addressed to the study of the antioxidant profile of the most significant olive oil phenolic compounds metabolites in biological systems. In this work, we addressed that issue by studying the protective properties of some of the most important olive oil phenolic compounds metabolites, the hydroxytyrosol metabolites, upon human RBC under AAPH and H_2O_2-induced oxidative stress. This biological model has been extensively studied both as a source of free radicals and as a target for oxidative damage.

After 4 h of incubation, hydroxytyrosol, hydroxytyrosol acetate, hydroxytyrosol glucuronides, homovanillyl alcohol, and homovanillyl alcohol acetate protected RBCs in a significant way from oxidative-AAPH induced hemolysis at the concentration of 80 μM (Figures 3 and 4). At lower concentrations

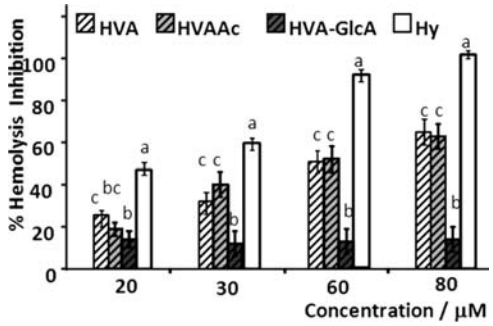

Figure 3. Inhibition of hemolysis of RBCs (mean ± SEM) at 2% hematocrit, incubated for 4 h with with homovanillyl derivative compounds at 10, 20, 40, and 80 μM and with 60 mM AAPH. HVA, homovanillyl alcohol; HVAAc, homovanillyl alcohol acetate; HVA-GlcA, homovanillyl alcohol glucuronide. Different letters within a concentration indicate samples that were significantly different (p < 0.05).

(20−60 μM), all compounds, except HVA-GlcA, protected RBCs from oxidative hemolysis in a dose-dependent manner. Indeed, the homovanyllyl alcohol glucuronide presented the worst antioxidant performance among the six tested compounds, showing just a slight activity, independent of concentration. The ranking activity order at 20 μM was: hydroxytyrosol^{a} > hydroxytyrosol glucuronides^{b} = hydroxytyrosol acetate^{b} = homovanillyl alcohol^{bc} = homovanillyl actate^{c} > homovanillyl glucuronide^{c}. At the lowest concentration tested (10 μM), the hydroxytyrosol direct metabolites still had an important protective activity (Figure 4) being the ranking activity order hydroxytyrosol^{a} > hydroxytyrosol acetate^{b} = hydroxytyrosol glucuronides^{b}. The data obtained for hydroxytyrosol agree with those acquired in a similar system by Manna et al.[30] and Paiva-Martins et al.[31]

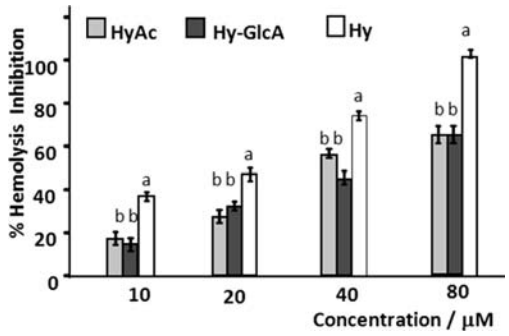

Figure 4. Inhibition of hemolysis of RBCs (mean ± SEM) at 2% hematocrit, incubated for 4 h with hydroxytyrosol derivative compounds at 10, 20, 40, and 80 μM and with 60 mM AAPH. Hy, hydroxytyrosol; HyAc, hydroxytyrosol acetate; Hy-GlcA, hydroxytyrosol glucuronides. Different letters within a concentration indicate samples that were significantly different (p < 0.05).

According to the literature, the concentration of hydroxytyrosol metabolites in the plasma rises after the consumption of VOO, with T_{max} around 1−2 h of ingestion.[14,15] Therefore, we also evaluated the protective effect of hydroxytyrosol metabolites against RBC-induced hemolysis after 2 h of incubation (Figure 5). At this time of incubation, Hy, HyAc

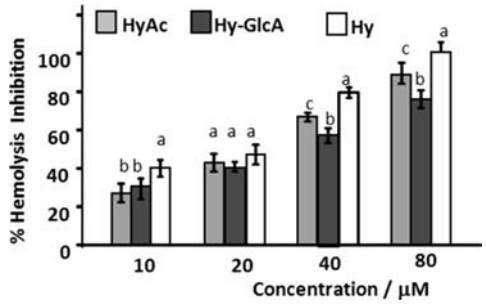

Figure 5. Inhibition of hemolysis of RBCs (mean ± SE) at 2% hematocrit, incubated for 2 h with hydroxytyrosol derivative compounds at 10, 20, 40, and 80 μM and with 60 mM AAPH. Hy, hydroxytyrosol; HyAc, hydroxytyrosol acetate; Hy-GlcA, hydroxytyrosol glucuronides. Different letters within a concentration indicate samples that were significantly different (p < 0.05).

and Hy-GlcA showed a quite similar protective activity at all concentrations tested and were able to significantly protect RBCs against the oxidative hemolysis, even at 10 μM.

The RBCs morphology before and after exposure to AAPH in the absence and presence of hydroxytyrosol and hydroxytyrosol glucuronides at 80 and 10 μM is illustrated in Figure 6. At these concentrations, the compounds were still able to protect RBC from hemolysis induced by AAPH. By using the same volume of the RBC suspensions, it was also possible to observe that the cellular density in the hydroxytyrosol glucuronides samples was lower than that in the hydroxytyrosol samples for the same concentration but still higher than the observed in the samples containing only AAPH. This observation was in accordance with the hemolysis study.

It was also investigated the nature of hemoglobin in the hemolysate of samples containing AAPH and several concentrations of hydroxytyrosol metabolites. When performing spectral scans (450−650 nm) of lysed RBC suspensions[28]

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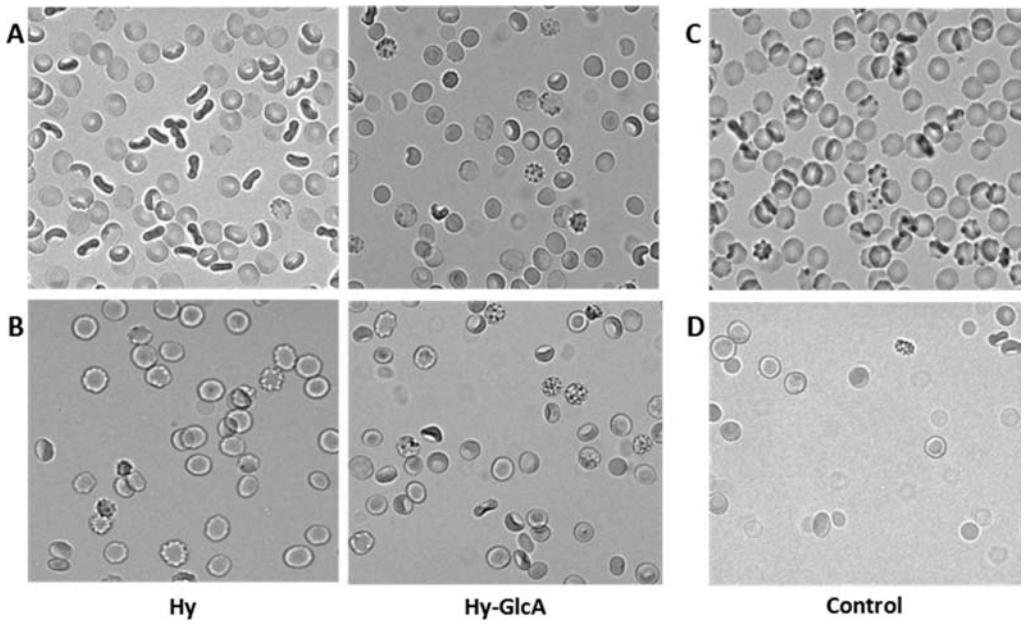

Figure 6. Optical microscopic evaluation of the erythrocyte morphology. (A) RBCs plus phenolic compounds at 80 μM and AAPH after 4 h of incubation. (B) RBCs plus phenolic compounds at 10 μM and 60 mM AAPH after 4 h of incubation (original magnification ×400). (C) Control just with RBCs. (D) Control with RBCs plus AAPH. Hy, hydroxytyrosol; Hy-GlcA, hydroxytyrosol glucuronides.

in the presence of phenolic compounds (lyses after 4 h of incubation, without AAPH), we did not observe any change in the oxy-hemoglobin peaks (540 and 578 nm) nor in its concentration[29] (data not shown), as compared to the control assay (RBCs without phenolic compounds). Furthermore, the oxy-hemoglobin disappeared in the presence of AAPH, but this was partially reversed by the addition of hydroxytyrosol, hydroxytyrosol acetate, and hydroxytyrosol glucuronides (Figure 7). Hydroxytyrosol glucuronides were apparently less effective in this protection at 80 μM than the other two compounds but showed a protection similar to that of hydroxytyrosol and hydroxytyrosol acetate at 20 and 10 μM.

The capacity of hydroxytyrosol and hydroxytyrosol glucuronides to protect RBC from oxidative hemolysis induced by H_2O_2, a physiological oxidizing agent in erythrocytes, was also evaluated. In this case, cells are attacked not only from the outside but also from the inside as H_2O_2 can easily cross the cell membrane. As expected, hydroxytyrosol protected RBC from oxidative injury in a dose-dependent manner. However, in the case of its glucuronides, the small protection observed was not dose dependent. In fact, the activity was very similar for all concentrations studied and similar to that of hydroxytyrosol for the lower concentrations tested (10 and 20 μM). Because glucuronides have not been described as crossing membranes, it can be hypothesized that some sort of saturation of specific RBC transporters could occur, thus leading to the same protection at higher concentrations. Alternatively, these glucuronides may have activity only after hydrolysis to hydroxytyrosol, which can then be absorbed by the cells. Some studies propose that glucuronides may act not only as detoxifing metabolites but also as bioactive agents, being precursors of more hydrophobic aglycones. Accordingly, aglycones may be assumed to emerge in the target site by the action of glucuronidases under oxidative stress.[32] The cardiovascular system and central nervous system seem to be the major targets of phenol glucuronides circulating in the human blood.[32] Therefore, the activity of glucuronides will be dependent on the availability of glucuronidases in a specific tissue or cell. It is also important to note that when RBCs are prepared, their content remains intact in the erythrocyte structure, but the exogenous antioxidant defenses are washed out during the manipulation, and there are no interactions with other blood cells and epithelium cells. These results are in agreement with those found for the hydroxytyrosol glucuronides in the protection of hemoglobin from the AAPH-induced injury (Figure 8). Indeed, because hemoglobin is located inside the RBC, it can only be protected from the oxidative injury if the compounds can cross the RBC membrane.

The present results suggest that hydroxytyrosol metabolites may play an important role in the protective activity of olive oil phenolic compounds. If 50 g of extra virgin olive oil with a high concentration in polyphenols is consumed, this would correspond to an intake of up to 32 mg per day.[9−11] This dose would lead to a relatively low plasma concentration (up to 5−10 μM),[15] but it is known that regular low doses, for example, of aspirin can confer cardiovascular health benefits.[33] In fact, hydroxytyrosol and its metabolites have been found not only in the plasma but also attached to lipoproteins,[34] other blood proteins, and cells.[31] Therefore, it is possible that the regular low lifetime intake of olive oil results in an overall protective effect. This phenomenon has been demonstrated by clinical trials showing that short-term consumption of olive oil in humans (50 mL/day) can change several oxidative stress markers,[35,36] although the concentrations of phenols are lower than those required to show in vitro biological activity. In fact, it should be taken in mind that food components are different than drugs and their actions on human physiology are usually moderate. While medicines are habitually employed for limited timeframes, food and its macro- and microcomponents are ingested throughout a lifetime, during which even modest effects, like those found for hydroxytyrosol glucuronides in the presence of H_2O_2, may become noteworthy.

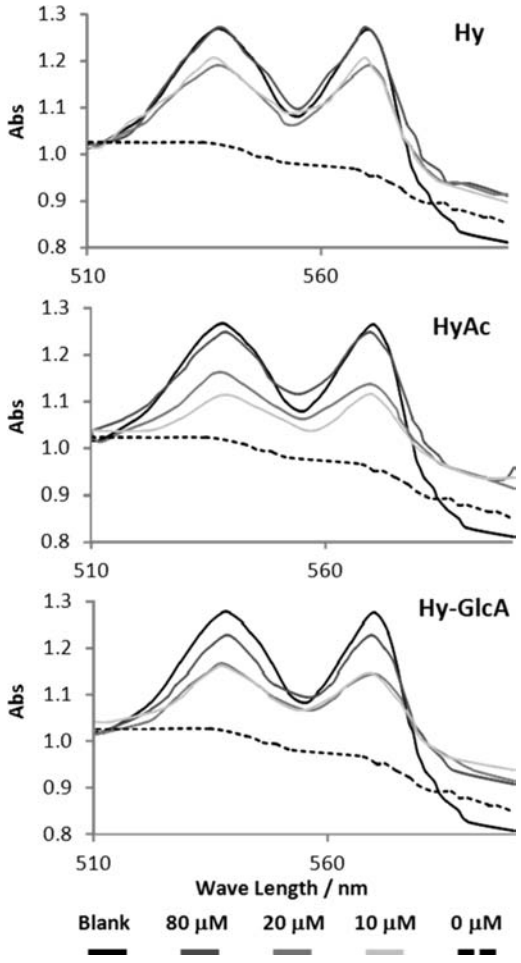

Figure 7. Spectral scans (500−600 nm) of lysed RBC suspensions, obtained after 3 h of incubation in the presence of hydroxytyrosol derivative compounds at 0, 10, 20, 40, and 80 μM with 60 mM AAPH. Hy, hydroxytyrosol; HyAc, hydroxytyrosol acetate; Hy-GlcA, hydroxytyrosol glucuronides.

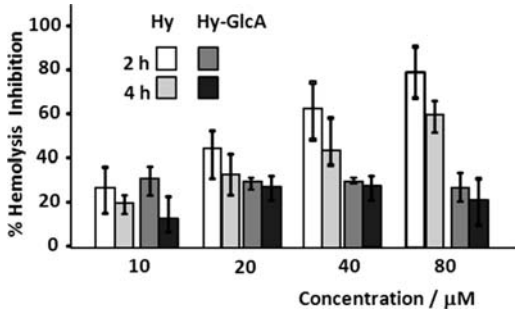

Figure 8. Inhibition of hemolysis of RBCs (mean ± SEM) at 2% hematocrit, incubated for 2 and 4 h with phenolic compounds at 10, 20, 40, and 80 μM and with 7.5 mM H_2O_2. Hy, hydroxytyrosol; Hy-GlcA, hydroxytyrosol glucuronides.

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Notes

The authors declare no competing financial interest.

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