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Phenolic compounds oleuropein and hydroxytyrosol exert differential effects on glioma development via antioxidant defense systems

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ABSTRACT

Oxidative stress is involved in many of the stages of tumorigenesis, and the administration of exogenous antioxidants seems to modulate them. Thus, it has been described that oleuropein and hydroxytyrosol exert important anti-cancer activities. We analyse *in vivo* the anti-tumor properties of both phenolic compounds in an animal model of glioma, and their effects on oxidative stress, enzymatic and non-enzymatic antioxidant defence systems and on several biochemical biomarkers. Hydroxytyrosol, but not oleuropein nor the mixture of both compounds, inhibits tumor growth through mechanisms that involve enzymatic and non-enzymatic antioxidant defences, as demonstrated by a decrease in lipid peroxidation and protein oxidation levels. Furthermore, hydroxytyrosol maintains the non-enzymatic antioxidants as in healthy animals, and positively modifies the enzymatic antioxidants. However, hydroxytyrosol probably acts not as an antioxidant, but through other mechanisms that only indirectly modify the redox status. Finally, these compounds yield few adverse effects related to changes in hepatic enzymes.

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1. Introduction

Tumorigenesis represents a multistage, multistep process involving a number of molecular and cellular events that lead to the transformation of a normal cell into a malignant cell. Free radicals seem to be involved in many steps of several of these stages, triggering lipid peroxidation of cellular membranes and oxidation of proteins and DNA, leading to changes in chromosome structure, genetic mutations and/or modulation of cell growth (Visioli, Bellomo, & Galli, 1998). In fact, tumor development has been associated with oxidative stress and reduced responses of antioxidant defense systems. However, reactive oxygen species (ROS) generation is a constant feature

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of oxygen metabolism in cells. An imbalance between ROS generation and the efficiency of antioxidant mechanisms leads to oxidative damage (Acharya, Das, Chandhok, & Saha, 2012; Herrera et al., 2012). In this manner, the central nervous system is especially vulnerable to free-radical damage (Liang & Patel, 2006). However, cancer cells may also be destroyed by ROS, which block key steps in the cell cycle and promote apoptosis through mechanisms that remain to be elucidated (Watson, 2013). Furthermore, several chemotherapy drugs that enhance apoptosis of cancer cells act by lowering antioxidant levels, and their actions cease if antioxidant compounds are administered concomitantly (Kirshner et al., 2008).

Therefore, although administration of exogenous antioxidants has received particular attention due to the potential of these agents to modulate oxidative stress and to act as putative antitumor compounds, an important controversy exists. A vast number of nutritional intervention trials have shown that antioxidants are not obviously effective in preventing cancer (Bjelakovic, Nikolova, Gluud, Simonetti, & Gluud, 2007; Watson, 2013), whereas other epidemiological evidence has indicated that increased consumption of fruits and vegetables containing antioxidants is associated with health improvements in terms of cancer risks (Charoenprasert & Mitchell, 2012).

Phenolic compounds derived from olives and virgin olive oils, especially oleuropein and its major metabolite hydroxytyrosol, exert important anti-inflammatory, cardioprotective and anticancer activities both *in vitro* and *in vivo* due to their antioxidant properties (Carluccio et al., 2007; Covas et al., 2006; Visioli et al., 1998; Visioli, Poli, & Gall, 2002), reducing the risk of mutagenesis and carcinogenesis (Waterman & Lockwood, 2007). Thus, in the present report, we analyzed the antitumor properties of the phenolic compounds oleuropein and hydroxytyrosol *in vivo* in an animal model of glioma. We also assessed their effects on oxidative stress biomarkers (lipid peroxidation and protein oxidation), on both non-enzymatic and enzymatic antioxidant defense systems and on several biochemical serum biomarkers to analyze their putative adverse effects on several physiological functions.

2. Materials and methods

2.1. Cell culture

C6 glioma cells were grown in 5% fetal bovine serum (FBS)-supplemented DMEM/HAM F-12 medium (Sigma-Aldrich, Madrid, Spain) without antibiotics. Cells were incubated at 37 °C in a modified atmosphere of 5% CO_2/95% air until confluence. Absence of mycoplasma contamination was assessed regularly using Hoechst 33258 (Invitrogen, Madrid, Spain).

2.2. Colorimetric cytotoxic assay

To set up a colorimetric cytotoxic assay (CCA), cells were trypsinized from a monolayer and diluted to 4×10^4 cells/mL. They were in the exponential phase of growth during the entire experiment. One-milliliter aliquots of cells were pipetted into wells of 24-well tissue culture plates and incubated for 24 h. Oleuropein and hydroxytyrosol were then added to the wells at a volume of 1 mL per well and a range of concentrations (25, 50, 100, 200, 400 and 500 mM), with each dose being used in at least four replicate wells. After 3 days of incubation, the medium was removed, and the cultures were washed with phosphate-buffered saline (PBS) prior to fixation with 10% trichloroacetic acid (TCA) for 30 min at 4 °C. Next, the cultures were washed with tap water to remove the TCA. The plates were air dried and then stored until use. TCA-fixed cells were stained for 20 min with 0.4% (w/v) sulforhodamine B (SRB) dissolved in 1% acetic acid. At the end of staining period, the SRB was removed, and the cultures were rinsed with 1% acetic acid to remove unbound dye. The cultures were air dried, and the bound dye was solubilized with 10 mM Tris base (pH 10.5). Optical density (OD) was measured with a Tecan Genios Plus plate reader at 492 nm. The photometer response was linear according to the dye concentration, and it was proportional to the cell numbers counted in parallel with an automated cell counter (TC10, BioRad). The fractions of cell growth affected (Fa) by the compound dosage were used to compute the doses required for the 50% inhibition of cell growth or IC_{50} (Dm) value, which is the coefficient of the sigmoidicity of the dose–effect curve (m) and the linear correlation coefficient of the median-effect plot (*r*).

2.3. Animals and treatments

Forty male adult Wistar rats (350 ± 3.24 g body weight) were used in this study. The animals were provided by Harlan Ibérica S.A. and maintained at the University of Jaen in an animal house in a controlled environment at a constant temperature (25 °C) with a 12 h-light/12 h-dark cycle. The rats were housed in cages and given free access to standard laboratory rat food and water. The experimental procedures for animal use and care were in accordance with the European Community Council directive (2010/63/EU). The protocols were approved by the Bioethical Committee of the University of Jaen (PEJA 4957M). The animals were randomly divided into five groups of eight rats each. Four groups were subjected to C6 glioma cell implantation (tumor groups), and one group remained as a non-tumor healthy control group. Ten days after C6 glioma cell implantation, animals in three of the tumor groups received daily subcutaneous injections of 100 μg oleuropein, 100 μg hydroxytyrosol or 100 μg oleuropein plus 100 μg hydroxytyrosol disolved in 500 μL saline solution for 5 days. The other tumor group and the non-tumor healthy control group received vehicle-only injections (saline solution) for the same time period.

2.4. Implantation of C6 glioma cells

Five million C6 glioma cells suspended in 25 μL of culture medium without FBS were injected subcutaneously in both dorsal flanks of the rats using a Hamilton syringe with a 26-gauge needle. The non-tumor group received the same procedure without cells. The characteristics of this glioma model have been previously described (Mayas, Ramirez-Exposito, Carrera, Cobo, & Martinez-Martos, 2012).

2.5. Measurement of tumor volume and sample collection

The size of the abdominal tumor was measured with slide calipers at 10 days after C6 glioma cell implantation, just before

the beginning and after 5 days of receiving the treatment. The tumor volume was defined as ½(a·b)^2 (a: long diameter and b: short diameter) (Navarro et al., 1999). The rats were anesthetized with equitensin (2 mL/kg body weight) by intraperitoneal injection. Blood samples, which were obtained from the left cardiac ventricle, were drawn into tubes without anticoagulant, allowed to clot, and then centrifuged for 10 min at 3000 × *g* to obtain serum, which was frozen and stored at −80 °C until use.

2.6. Oxidative stress parameter assays

2.6.1. Lipid peroxidation assay

Lipid peroxidation was measured by analyzing the amount of thiobarbituric acid-reactive substances (TBARS) as previously described by Mayas et al. (2005). Briefly, 25 µL of each sample were mixed with 100 µL of ice-cold 20% TCA. After centrifugation, a volume of supernatant was added to an equal volume of 0.67% 4,6-dihydroxypyrimidine-2-thiol (TBA), and the mixture was kept in a boiling water bath for 15 min. The samples were cooled to room temperature, and the absorbance at 532 nm was recorded after subtracting blanks containing equal volumes of TCA and TBA. The signal was measured against a malondialdehyde (MDA) standard curve, and the results were expressed as mg MDA equivalents/mL of serum.

2.7. Protein oxidation assay

Protein oxidation was measured by analyzing the carbonyl groups of proteins as previously described by Mayas et al. (2005). Briefly, 25 µL of sample were mixed with 100 µL of ice-cold 20% TCA and centrifuged. The protein precipitates were left to react with 10 mM 2,4-dinitrophenylhydrazine for 1 h at room temperature in the dark. After the reaction, the proteins were precipitated with 20% TCA, and the unreacted dye was washed twice with 10% TCA. The pellets were dissolved in 1 M NaOH, and the absorbance was recorded at 360 nm. The results were expressed as nmol per mg of protein using an extinction coefficient of $2.1 \times 10^4\ M^{-1} cm^{-1}$.

2.8. Determination of glutathione (GSH) and glutathione disulfide (GSSG)

GSH levels were measured according to Griffith (1980) with minor modifications. For this purpose, serum samples were treated with two volumes of buffer and centrifuged at 10000 × *g* for 5 min at 4 °C, and the pellets were discarded. Supernatants containing total GSH were mixed with 0.6 mg/mL of 5,5-dithiobis (2-nitrobenzoic acid) (DTNB) and 0.248 mg/mL nicotinamide adenine dinucleotide phosphate (NADPH). The reaction was initiated upon addition of 1 U/mL GSSG reductase. Absorbance was measured at 405 nm at 30 °C every 20 s for 2 min. For the GSSG determination, 2-vinylpyridine was used to derivatize the GSH. The data are presented as nmol of total GSH (GSH plus GSSG) per mg of total protein.

2.9. Catalase activity assay

Serum samples were processed and analyzed for catalase activity as described by Aebi (1984) with slight modifications by Cohen, Kim, and Ogwu (1996). Briefly, 10 µL of sample were added to 10 mM H_2O_2 in 20 mM potassium phosphate buffer (pH 7.0) and incubated at 30 °C for 1 min. The initial reaction rate was measured as the decrease in absorbance at 240 nm.

2.10. Superoxide dismutase (SOD) assay

SOD activity was measured according to Paoletti, Aldinucci, Mocali, and Caparrini (1986). Ten microliters of serum sample were mixed with a reaction buffer containing 100 mM triethanolamine-diethanolamine buffer (TDB) pH 7.4, 7.5 mM NADH and ethylenediaminetetraacetic acid (EDTA)/$MnCl_2$ (1:2 v/v). To initiate the reaction, 25 µL of 10 mM ß-mercaptoethanol were added. The absorbance was recorded at 340 nm for 2–15 min.

2.11. Glutathione peroxidase (GPx) activity assay

GPx activity was measured according to Ellerby and Bredesen (2000). The reaction mixture included 50 mM potassium phosphate (pH 7.4), 25 mM NADPH, 1 mmol/L GSH, and 100 U/mL yeast glutathione reductase. Ten microliters of serum sample were added and mixed with the reaction mixture in a 96-well dish. The hydroperoxide-independent NADPH consumption rate was recorded for 3 min at 37 °C at 340 nm. Then, 2.5 µL of tert-butyl hydroperoxide were added to start the reaction and mixed, and the overall rate at 340 nm was recorded. The same procedure was carried out in the same reaction volume without the sample, which allowed for the subtracting of the non-enzymatic rate of GSH oxidation.

2.12. Serum chemistry measurements

Electrolytes (sodium, potassium and chloride) were assayed using selective ion electrodes, according to Shibata, Maruizume, and Miyage (1992). The results were expressed as mEq/L; calcium was measured by a colorimetric assay, according to Farrel (1987), and phosphorus was also measured by a colorimetric assay, according to Tietz (1995). The results were expressed as mg/dL.

The nonprotein nitrogenous compounds uric acid, urea, creatinine, and glucose in the serum samples were assessed using commercial kits (Boehringer Mannheim, Madrid, Spain), according to the methods described by Praetorius and Poulsen (1953), Talke and Schubert (1965), Bartels, Bohmer, and Heierli (1972) and Peterson and Young (1968), respectively. The results were expressed as mg/dL.

Total cholesterol, HDL cholesterol and triacylglycerols were assayed using standard enzymatic colorimetric methods with commercially available kits according to Roeschlau, Bernt, and Gruber (1974), Sugiuchi et al. (1995) and Siedel, Schmuck, and Staepels (1993). The LDL cholesterol level was calculated according to the Friedewald formula. The results were expressed as mg/mL.

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were estimated by quantitative enzymatic colorimetric end-point methods using commercially available kits according to Tietz (1995) and Bergmeyer, Horder, and Rej (1986). The results were expressed as UI/L. Alkaline phosphatase (ALP) was determined by a colorimetric end-point

Table 1 – Dose-effect relationship parameters of oleuropein and hydroxytyrosol on growth of C6 glioma cells *in vitro*[a].

Drug dosage (μM)	*Fa*[b]	*m*[b]	*Dm* (mM)[b]	*r*[b]
Oleuropein				
25	0.087			
50	0.150			
100	0.190			
200	0.250			
400	0.390			
500	0.340	0.58 ± 0.05	1.17	0.982
Hydroxytyrosol				
25	0.071			
50	0.131			
100	0.147			
200	0.198			
400	0.264			
500	0.254	0.48 ± 0.05	3.70	0.978

[a] The cytotoxicity assay method is described in the Materials and methods section.
[b] The parameters *Fa*, *m*, *Dm* and *r* are the fractional inhibition, slope coefficient of the curve, dose at 50% inhibition (equivalent to the IC_{50} value), and the linear correlation coefficient of the median-effect plot.

method using commercially available kits, according to Belfield and Goldberg (1971). The results were expressed as UI/L.

The serum albumin concentration was determined by a colorimetric method using a commercial kit according to Doumas, Watson, and Biggs (1971). The results were expressed as g/dL. Total protein was estimated by the colorimetric method of Bradford (Bradford, 1976). The results were expressed as mg/mL.

2.13. *Statistical analysis*

All values represent the mean ± standard error of the mean (SEM). The data were analyzed by ANOVA plus Newman–Keul's test using IBM SPSS V.19 software. Values of $P < 0.05$ were considered significant.

3. Results

3.1. *Antiproliferative effects of oleuropein and hydroxytyrosol* in vitro

Table 1 shows the *in vitro* dose–effect relationship of oleuropein and hydroxytyrosol on the growth of C6 glioma cells, the doses of oleuropein and hydroxytyrosol required for the 50% inhibition of cell growth or IC_{50} (Dm), the coefficient of the sigmoidicity of the dose–effect curve (m) and the linear correlation coefficient of the median-effect plot (*r*). The IC_{50} values for oleuropein and hydroxytyrosol on the C6 cells after 72 h of exposure were 1.17 mM for oleuropein and 3.70 mM for hydroxytyrosol. Figure 1 shows the effects of both compounds on the C6 cultures as visualized by phase-contrast microscopy.

3.2. *Antitumor effects of oleuropein and hydroxytyrosol* in vivo

The *in vivo* effects of 5 days of administration of oleuropein, hydroxytyrosol and a mixture of both compounds on tumor growth in our experimental glioma model compared with the vehicle are presented in Fig. 2. The tumor volume in the non-treated animals increased significantly ($P < 0.001$) by ≈11-fold (from 0.68 ± 0.09 cm^3 to 7.47 ± 1.98 cm^3) over the course of the experiment. Similarly, the animals treated with oleuropein showed a significant increase in tumor volume ($P < 0.001$) by ≈9-fold (from 0.83 ± 0.08 to 7.39 ± 1.58 cm^3). In contrast, those treated with hydroxytyrosol showed a significant increase in tumor volume ($P < 0.05$) by only ≈2.5-fold (from 0.61 ± 0.09 to 1.51 ± 0.49 cm^3). Finally, the animals treated with both oleuropein and hydroxytyrosol showed a significant increase in tumor volume ($P < 0.001$) by ≈10-fold (from 0.58 ± 0.08 to 5.73 ± 1.01 cm^3).

3.3. *Effects of oleuropein and hydroxytyrosol administration on oxidative stress parameters*

We found increased levels of the oxidative stress parameters of lipid peroxidation (through the analysis of the TBARS concentration) and protein oxidation (through the analysis of the carbonyl- and diene-conjugate group concentrations) in the animals with glioma tumors treated with vehicle only compared with the non-tumor healthy control animals. Thus, those with tumors treated with the vehicle alone showed significantly increased levels of TBARS ($P < 0.001$) compared with the non-tumor healthy control group. Similarly, the animals treated with 100 μg/day oleuropein or hydroxytyrosol showed increased levels of TBARS ($P < 0.001$) compared with the non-tumor control group. However, the animals treated with both 100 μg/day oleuropein and 100 μg/day hydroxytyrosol showed significantly higher levels of TBARS compared with those with glioma tumors treated with the vehicle only, treated with oleuropein or treated with hydroxytyrosol ($P < 0.001$; Fig. 3A). Similarly, the animals with tumors treated with the vehicle alone showed significantly increased levels of protein oxidation ($P < 0.01$) compared with the non-tumor healthy control group. Furthermore, those treated with 100 μg/day oleuropein or oleuropein plus hydroxytyrosol also showed increased levels of protein carbonyls ($P < 0.001$) compared with the non-tumor control group. However, the animals treated with 100 μg/day hydroxytyrosol showed a significant decrease in the protein carbonyl concentration compared with those with glioma tumors treated with the vehicle only, with oleuropein or with oleuropein plus hydroxytyrosol, reaching similar levels as the non-tumor healthy control animals (Fig. 3B).

3.4. *Effects of administration of oleuropein and hydroxytyrosol on the non-enzymatic antioxidant defense system*

Figure 4 shows the levels of reduced GSH (GSHr) and GSSG in the serum of the healthy animals (control group) treated with the vehicle and those with glioma treated with the vehicle only, with 100 μg/day oleuropein, with 100 μg/day hydroxytyrosol or with 100 μg/day oleuropein plus hydroxytyrosol. The animals with tumors treated with the vehicle alone showed significantly decreased levels of GSHr ($P < 0.001$) compared with the non-tumor healthy control group. In contrast, those treated with 100 μg/day oleuropein or hydroxytyrosol also showed decreased levels of GSHr ($P < 0.01$) compared with the non-tumor control group but showed significantly higher levels of

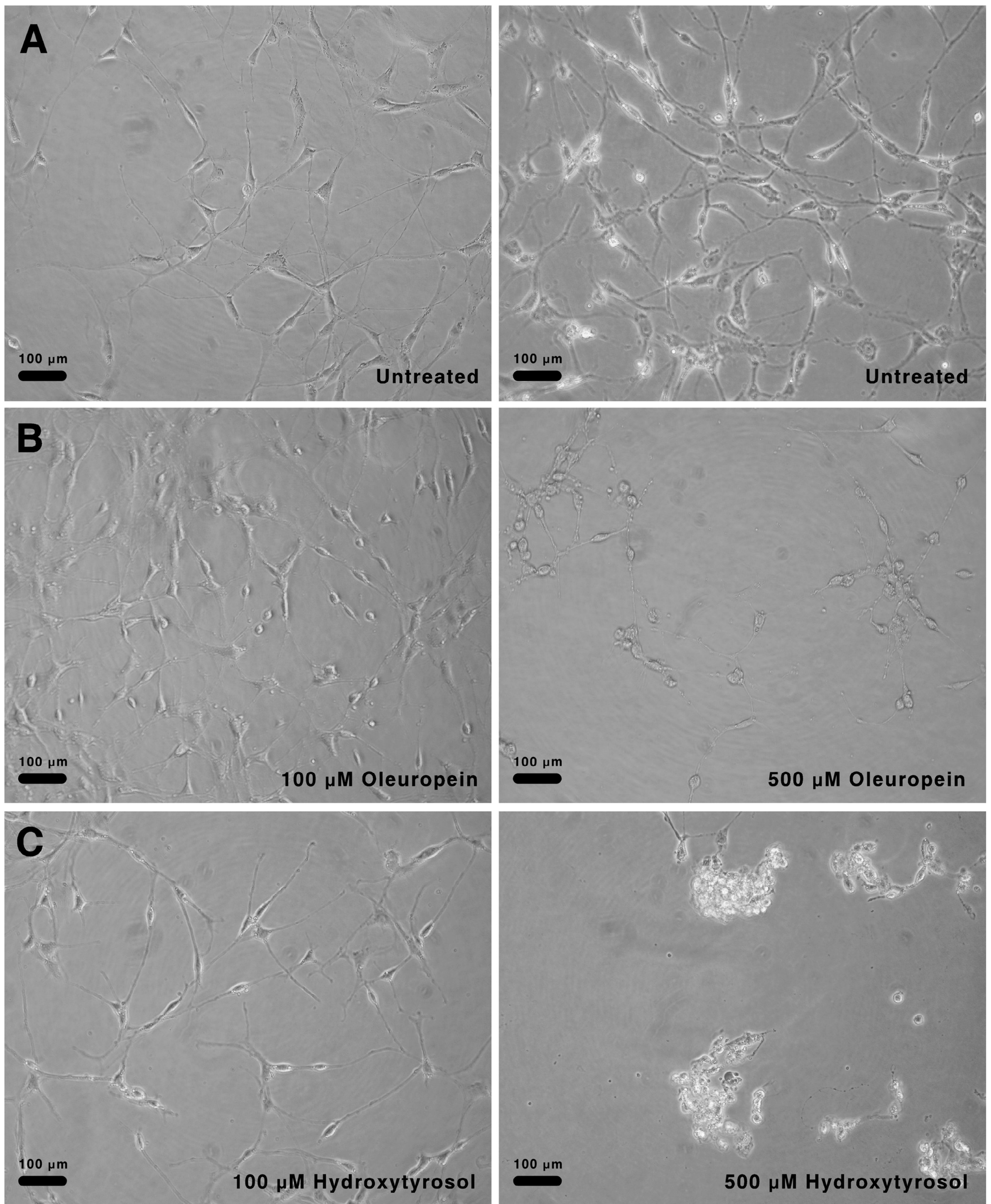

Fig. 1 – Phase-contrast microphotography of C6 glioma cell cultures untreated (A) or treated with 100–500 μM oleuropein (B) or hydroxytyrosol (C). Original magnification 200×.

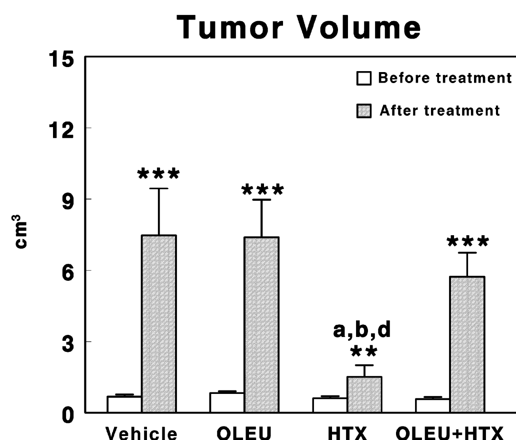

Fig. 2 – Tumor volumes following implantation of C6 glioma cells into the subcutaneous region before and after the treatments with the vehicle (Control), 100 μg/day oleuropein (OLEU), 100 μg/day hydroxytyrosol (HTX) and 100 μg oleuropein plus 100 μg of hydroxytyrosol/day (OLEU+HTX) for 5 days. The results are expressed as cm^3 (mean ± SEM; *n* = 8; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{a}P < 0.001$ vs. vehicle-treated group; $^{b}P < 0.001$ vs. OLEU group; $^{d}P < 0.001$ vs. OLEU+HTX group).

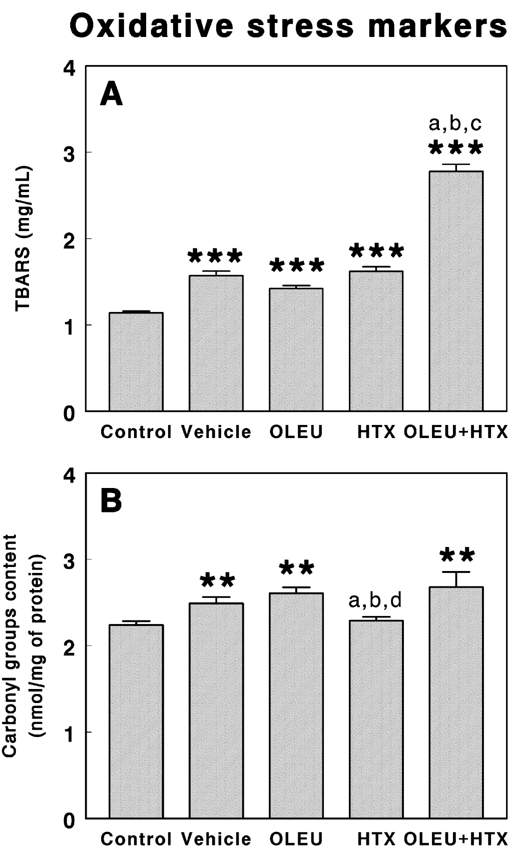

Fig. 3 – Concentrations of thiobarbituric acid-reactive substances (TBARS) (A) and carbonyl groups (B) in the sera of non-tumor healthy control animals treated with the vehicle (Control) and the animals with C6 glioma implanted into the subcutaneous region treated with the vehicle or with 100 μg of oleuropein (OLE), 100 μg of hydroxytyrosol (HTX) and 100 μg oleuropein plus 100 μg of hydroxytyrosol/day (OLEU+HTX) for 5 days. The results are expressed as mg/mL for TBARS and as nmol/mg of protein for the protein carbonyls (mean ± SEM; *n* = 8; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{a}P < 0.001$ vs. vehicle-treated group; $^{b}P < 0.001$ vs. OLEU group; $^{c}P < 0.001$ vs. HTX group; $^{d}P < 0.001$ vs. OLEU+HTX group).

GSHr ($P < 0.01$) compared with those with tumors treated with the vehicle only. Furthermore, the animals treated with both 100 μg/day oleuropein plus 100 μg/day hydroxytyrosol showed significantly lower levels of GSHr compared with the healthy control animals or with those with glioma tumors treated with the vehicle only, with oleuropein or with hydroxytyrosol ($P < 0.001$; Fig. 4A).

The animals with tumors treated with the vehicle alone showed significantly increased levels of GSSG ($P < 0.001$) compared with the non-tumor healthy control group. The animals treated with 100 μg/day oleuropein or oleuropein plus hydroxytyrosol showed GSSG levels similar to those observed in the healthy control animals. However, those treated with 100 μg/day hydroxytyrosol showed a significant decrease in GSSG levels compared with the healthy control animals, with the animals with glioma tumors treated with vehicle only, and with those with glioma tumors treated with oleuropein or with oleuropein plus hydroxytyrosol (Fig. 4B).

3.5. *Effects of administration of oleuropein and hydroxytyrosol on the enzymatic antioxidant defense system*

Figure 5 shows the serum SOD, CAT and GPx activities under the different experimental procedures. The animals with tumors treated with the vehicle alone showed significantly increased SOD activity ($P < 0.01$) compared with the non-tumor healthy control group. Similarly, those treated with 100 μg/day oleuropein or 100 μg/day oleuropein plus hydroxytyrosol showed increased SOD activity ($P < 0.001$) compared with the non-tumor control group or with the animals with glioma tumors treated with the vehicle only. In contrast, those treated with 100 μg/day hydroxytyrosol did not show any alterations in SOD activity compared with the non-tumor healthy animals, and they possessed significantly lower levels of SOD activity compared with the animals with glioma tumors treated with the vehicle only, treated with oleuropein or treated with both oleuropein plus hydroxytyrosol ($P < 0.05$ and $P < 0.001$, respectively; Fig. 5A).

Regarding CAT activity, no changes were detected in the animals with tumors treated with the vehicle alone or with 100 μg/day hydroxytyrosol compared with the non-tumor healthy control animals. In contrast, those treated with 100 μg/day oleuropein showed increased CAT activity ($P < 0.001$) compared with the non-tumor control group or with the animals with glioma tumors treated with the vehicle, with 100 μg/day hydroxytyrosol or with a mixture of both. The animals treated with 100 μg/day oleuropein plus hydroxytyrosol showed a significant decrease in CAT activity compared with the non-tumor healthy control group or with the animals with glioma tumors treated with the vehicle, with 100 μg/day

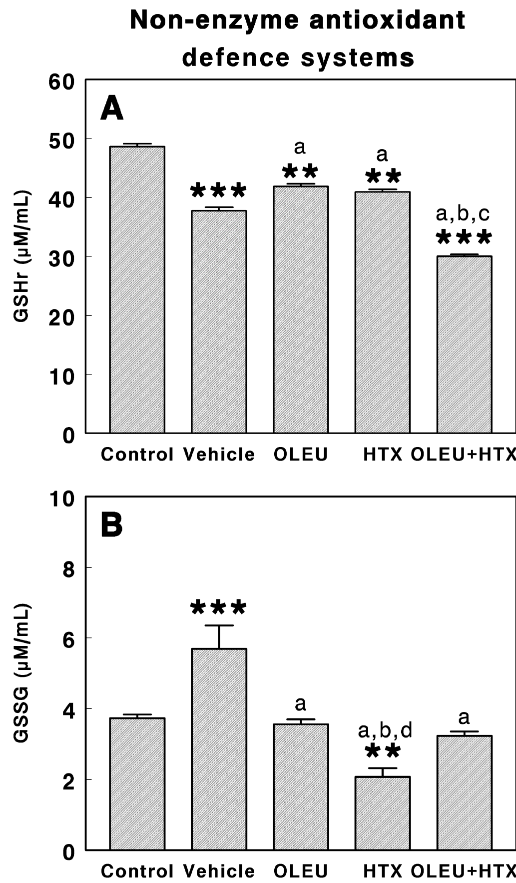

Fig. 4 – Reduced glutathione (GSHr) (A) and oxidized glutathione (GSSG) (B) concentrations in the serum of the non-tumor healthy control animals treated with the vehicle (Control) and the animals with C6 glioma cells implanted into the subcutaneous region treated with the vehicle or with 100 μg of oleuropein (OLEU), 100 μg of hydroxytyrosol (HTX) and 100 μg oleuropein plus 100 μg of hydroxytyrosol/day (OLEU+HTX) for 5 days. The results are expressed as μM/mL (mean ± SEM; $n = 8$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{a}P < 0.001$ vs. vehicle-treated group; $^{b}P < 0.001$ vs. OLEU group; $^{c}P < 0.001$ vs. HTX group; $^{d}P < 0.001$ vs. OLEU+HTX group).

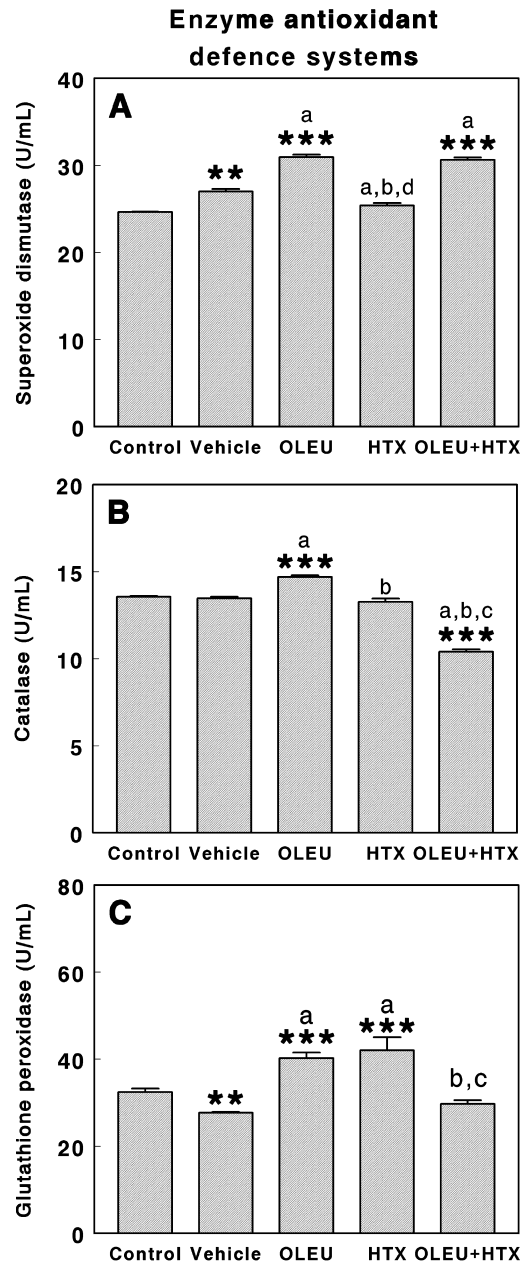

Fig. 5 – Superoxide dismutase (SOD) (A), catalase (CAT) (B) and glutathione peroxidase (GPx) (C) activities in the sera of animals treated with the vehicle (Control) and the animals with C6 glioma cells implanted into the subcutaneous region treated with the vehicle or with 100 μg of oleuropein (OLEU), 100 μg of hydroxytyrosol (HTX) and 100 μg oleuropein plus 100 μg of hydroxytyrosol/day (OLEU+HTX) for 5 days. The results are expressed as U/mL (mean ± SEM; $n = 10$; $^{***}P < 0.001$; $^{a}P < 0.01$).

oleuropein or with 100 μg/day hydroxytyrosol ($P < 0.001$ in all cases; Fig. 5B).

Finally, the animals with tumors treated with the vehicle alone showed significantly decreased GPx activity ($P < 0.01$) compared with the non-tumor healthy control animals. In contrast, those treated with 100 μg/day oleuropein or 100 μg/day hydroxytyrosol showed increased GPx activity ($P < 0.001$) compared with the non-tumor control animals or with those with glioma tumors treated with the vehicle only. The animals treated with 100 μg/day oleuropein plus hydroxytyrosol did not show any alterations in GPx activity compared with the non-tumor healthy animals or with those with glioma tumors treated with the vehicle only, although they did show significantly decreased levels of GPx activity compared with the animals with glioma tumors treated with oleuropein or with hydroxytyrosol ($P < 0.001$ in both cases; Fig. 5C).

3.6. *Effects of oleuropein and hydroxytyrosol administration on blood serum chemistry*

Table 2 shows the serum levels of electrolytes (calcium, phosphorus, sodium, potassium and chloride), glucose, non-protein nitrogenous compounds (urea, creatinine and uric acid), proteins (total protein and albumin), enzymes (alanine

Table 2 – Serum levels of electrolytes, glucose, non-protein nitrogenous compounds, proteins, enzymes and lipid profile of the sera of non-tumor healthy control animals treated with the vehicle (Control) and the animals with C6 glioma cells implanted into the subcutaneous region treated with the vehicle or with 100 μg/day of oleuropein, 100 μg/day of hydroxytyrosol and 100 μg oleuropein plus 100 μg of hydroxytyrosol/day for 5 days.

Parameter	Control	Vehicle	Oleuropein	Hydroxytyrosol	Oleuropein + Hydroxytyrosol	Significance level
Electrolytes						
Calcium (mg/dL)	10.4 ± 0.1	10.7 ± 0.1	10.6 ± 0.1	10.36 ± 0.1	10.6 ± 0.1	N.s.
Phosphorus (mg/dL)	8.3 ± 0.2	9.1 ± 0.5	9.5 ± 0.4	8.8 ± 0.4	8.2 ± 0.4	N.s
Sodium (mEq/L)	141.7 ± 0.9	142.5 ± 0.9	141.1 ± 0.6	139.2 ± 0.8[a]	139.5 ± 0.3[a]	[a]P < 0.001
Potassium (mEq/L)	5.7 ± 0.1	5.7 ± 0.2	5.9 ± 0.1	6.2 ± 0.2	7.0 ± 0.4[a]	[a]P < 0.001
Chloride (mEq/L)	100.4 ± 0.9	100.0 ± 0.7	99.8 ± 0.7	97.2 ± 0.5[a]	99.2 ± 0.5	[a]P < 0.05
Glucose (mg/dL)	219 ± 12	220 ± 6	211 ± 7	251.4 ± 4.9[a]	270.4 ± 7.2[a]	[a]P < 0.01
Non-protein nitrogenous compounds						
Urea (mg/dL)	41 ± 2	39 ± 2	32 ± 1[a]	39 ± 3	39 ± 1	[a]P < 0.01
Creatinine (mg/dL)	0.47 ± 0.02	0.47 ± 0.02	0.47 ± 0.03	0.58 ± 0.04[a]	0.60 ± 0.03[a]	[a]P < 0.001
Uric acid (mg/dL)	0.81 ± 0.04	1.20 ± 0.12[a]	1.20 ± 0.05[a]	1.28 ± 0.19[a]	1.34 ± 0.31[a]	[a]P < 0.01
Proteins and enzymes						
Total protein (mg/mL)	59.0 ± 0.1	59.5 ± 0.1	58.8 ± 0.3	59.8 ± 0.3	59.2 ± 0.3	N.s
Albumin (g/dL)	2.88 ± 0.03	2.95 ± 0.04	2.78 ± 0.03	3.04 ± 0.04	2.98 ± 0.04	N.s
Alanine aminotransferase (UI/L)	49 ± 6	44 ± 2	46 ± 4	48 ± 5	54 ± 5[a]	[a]P < 0.05
Aspartate aminotransferase (UI/L)	146 ± 11	240 ± 23[a]	203 ± 23[a]	210 ± 23[a]	255 ± 23[a]	[a]P < 0.01
Alkaline phosphatase (UI/L)	105 ± 10	157 ± 15[a]	203 ± 13[a,b]	138 ± 9[a]	130 ± 13[a]	[a]P < 0.01, [b]P < 0.05
Lipid profile						
Total cholesterol (mg/dL)	60 ± 2	81 ± 4[a]	88 ± 6[a]	61 ± 4	65 ± 4	[a]P < 0.001
HDL-cholesterol (mg/dL)	30 ± 1	42 ± 3[a]	45 ± 2[a]	29 ± 1	28 ± 1	[a]P < 0.001
LDL-cholesterol (mg/dL)	4 ± 1	10 ± 2[a]	11 ± 2[a]	13 ± 3[a]	3 ± 1	[a]P < 0.001
Total cholesterol/HDL-cholesterol	1.9 ± 0.03	2.0 ± 0.1	1.9 ± 0.1	2.0 ± 0.05	2.3 ± 0.1	N.s
Triacylglycerols (mg/dL)	135 ± 13	155 ± 16	168 ± 18	157 ± 17	164 ± 14	N.s

The data are expressed in the indicated units as the mean ± SEM; $n = 8$ animals per group.
[a, b] Statistical significance.

aminotransferase, aspartate aminotransferase and alkaline phosphatase) and the lipid profiles (total cholesterol, HDL-cholesterol, LDL-cholesterol, total cholesterol/HDL-cholesterol ratio and triacylglycerols) in the non-tumor healthy control animals treated with the vehicle (control) and the animals with subcutaneously implanted C6 glioma treated with the vehicle or with 100 μg oleuropein, 100 μg hydroxytyrosol or 100 μg oleuropein plus 100 μg hydroxytyrosol/day for 5 days. No significant changes in the levels of calcium, phosphorous, sodium, potassium or chloride were observed in the animals with glioma tumors treated with the vehicle compared with the non-tumor healthy control animals. Similarly, no changes were observed after treatment with oleuropein. The hydroxytyrosol treatment, however, significantly decreased the sodium and chloride levels ($P < 0.001$ and $P < 0.05$, respectively). Finally, the sodium level remained low after treatment with oleuropein plus hydroxytyrosol, but no changes were observed in the chloride level. However, a significant increase in the potassium level ($P < 0.001$) was observed after this treatment.

No significant changes in the glucose concentration were found between the non-tumor healthy control animals and those with glioma tumors treated with the vehicle only or with oleuropein. In contrast, the glucose levels were significantly increased in the animals treated with hydroxytyrosol ($P < 0.01$) or with oleuropein plus hydroxytyrosol ($P < 0.01$).

The non-protein nitrogenous compounds urea and creatinine did not show any significant changes between the control animals and those with glioma tumors treated with the vehicle only. Uric acid, however, was significantly increased ($P < 0.01$) in the animals with glioma tumors treated with the vehicle alone compared with the non-tumor healthy animals. However, those with glioma tumors treated with oleuropein showed significantly decreased urea levels ($P < 0.01$), although the uric acid levels remained high. The animals with glioma tumors treated with hydroxytyrosol or with oleuropein plus hydroxytyrosol did not show any alterations in their urea levels, but they did show significantly increased creatinine ($P < 0.001$) and also maintained high levels of uric acid ($P < 0.01$).

With regard to proteins and enzymes, no significant changes in the serum levels of total protein and albumin were found between the control animals and those with glioma tumors with or without treatment. However, alanine aminotransferase slightly increased in the animals with glioma tumors treated with both oleuropein and hydroxytyrosol ($P < 0.05$) compared with the other groups. Aspartate aminotransferase activity increased significantly in the animals with glioma tumors independent of the treatment received. Finally, alkaline phosphatase activity also increased significantly in the animals with glioma tumors compared with the non-tumor healthy control animals, but to different degrees depending on the treatment received, and the animals treated with oleuropein showed the highest activity level.

Finally, significant changes were found in the lipid profile between the control animals and those with glioma tumors. The animals with glioma tumors treated with the vehicle or with oleuropein showed significantly increased levels of total, HDL- and LDL- cholesterol ($P < 0.001$ in all cases). However, the treatment with hydroxytyrosol only significantly increased the

LDL-cholesterol level ($P < 0.001$). Furthermore, the animals with glioma tumors treated with oleuropein plus hydroxytyrosol showed a lipid profile similar to that of the non-tumor healthy control animals. No changes were observed between the groups in the total cholesterol/HDL-cholesterol ratio or triacylglycerol level.

4. Discussion

Several reports have shown that antioxidants may be useful in treating primary brain tumors because a dynamic relationship exists between oxidative stress and brain tumor appearance. In fact, the tumor microenvironment has been found to be a key player in the neoplastic process. However, cancer cells can also be killed by ROS, which block key steps in the cell cycle and promote apoptosis through mechanisms that remain to be elucidated (Watson, 2013). Cancer cells largely driven by the *RAS* and *MYC* oncogenes are among the most difficult to treat due to their high levels of ROS-destroying antioxidants. Furthermore, several chemotherapy drugs that enhance the apoptotic killing of cancer cells act by lowering antioxidant levels, and their actions cease if they are administered along with antioxidant compounds (Kirshner et al., 2008). Therefore, a very precise balance must occur between pro-oxidant and antioxidant activities, which is the key to promoting either cell proliferation or cell death. In the present report, we described that the IC_{50} values of oleuropein and hydroxytyrosol for C6 glioma cells *in vitro* were comparable with those of other well-known natural antioxidant substances, such as resveratrol or melatonin (at a millimolar level), with oleuropein being more potent than hydroxytyrosol. Furthermore, this is the first report of the inhibition of tumor growth by hydroxytyrosol but not by oleuropein or by a mixture of both phenolic compounds using an experimental model of glioma *in vivo*. We also provided support for the notion that hydroxytyrosol does not act as an antioxidant but that it actually acts via other mechanisms that only indirectly modify the redox status. Indeed, the *in vivo* effects found here do not correspond with the effects found *in vitro*.

4.1. *Treatments and tumor growth*

One of the most important findings of the present report is that although the treatments were performed over a short period of time (5 days), the hydroxytyrosol treatment decreased the tumor volumes. However, treatment with oleuropein or oleuropein plus hydroxytyrosol did not have any effect on tumor growth. With regard to the *in vivo* effects of oleuropein, we may not have used an adequate amount of oleuropein, or we may have used it for too short of a duration. Therefore, we also carried out experiments using higher doses of oleuropein and/or longer administration periods. However, no inhibition of tumor growth was found. Moreover, we observed a stimulatory effect on tumor growth under those conditions (data not shown). The mechanisms underlying the stimulatory effects of oleuropein on tumor growth remain to be defined. Oleuropein has been described as a potent scavenger of oxygen free radicals (Manna et al., 2002) and nitrogen-based free radical species (de la Puerta et al., 2001). In addition, it plays an important role in the prevention of DNA damage, thus inhibiting mutagenesis and carcinogenesis (Valko et al., 2004). Furthermore, Hamdi and Castellon (2005) have demonstrated that the antitumor effect of oleuropein may be exerted by the disruption of actin filaments in tumor cells. However, to date, the antitumor effects of oleuropein and hydroxytyrosol have been mainly described by *in vitro* studies. We propose three hypotheses to explain the stimulatory effects of oleuropein in our *in vivo* glioma model: (1) oleuropein inhibits the immune response against tumors (very potent in this animal model); (2) oleuropein has structural similarities with a growth factor or other type of stimulator, acting as an agonist and thus leading to an increase in tumor growth by mechanisms not related to its antioxidant properties; and (3) inhibition of tumor growth by oleuropein occurs via its potent antioxidant properties, inhibiting destruction of cancer cells via ROS and promotion of tumor growth. In fact, tumor cells with high levels of ROS-destroying antioxidants are among the most difficult to treat. Additionally, several chemotherapy drugs that act by lowering antioxidant levels cease their actions if antioxidant compounds are concomitantly administered (Kirshner et al., 2008; Watson, 2013). Therefore, further research is necessary to solve these issues.

With regard to the inhibitory effects of hydroxytyrosol administration on tumor growth, a growing number of *in vitro* studies have demonstrated that it has antiproliferative effects against several tumor cell lines, likely involving inactivation of the AKT and nuclear-factor-kappa B (NFκB) pathways (Zhao et al., 2014). *In vivo*, hydroxytyrosol seems to induce cell cycle arrest and apoptosis (Li et al., 2014). Our results also showed that the *in vivo* administration of a mixture of both compounds is inefficient to prevent tumor development, suggesting the opposing functions of oleuropein and hydroxytyrosol. The *in vivo* effects of hydroxytyrosol are highly dependent on the dose used (Kotronoulas et al., 2013), and some competition between both compounds can occur, resulting in the blocking of the effects of hydroxytyrosol. Furthermore, the antioxidant potency of oleuropein, which may also promote tumor growth, could be stronger than the antitumor and/or antioxidant-related properties of hydroxytyrosol.

4.2. *Treatments and oxidative stress parameters*

Several authors have shown that oleuropein and hydroxytyrosol inhibit oxidative stress (Carluccio et al., 2007; Visioli et al., 1998; 2002; Waterman and Lockwood, 2007). Therefore, we studied the effects of oleuropein, hydroxytyrosol and oleuropein plus hydroxytyrosol on the oxidative stress parameters of lipid peroxidation and protein oxidation in an *in vivo* model of glioma tumors. We found increased levels of both lipid peroxidation and protein oxidation in the animals with glioma tumors treated with the vehicle only compared with the non-tumor healthy control animals. The animals treated with oleuropein or hydroxytyrosol showed similar levels of lipid peroxidation compared with the vehicle-treated animals, although the hydroxytyrosol-treated animals did not show any changes in protein oxidation compared with the healthy control animals. Interestingly, the combination of oleuropein and hydroxytyrosol produced a large increase in lipid peroxidation, which was also

reflected in the protein oxidation level. Therefore, only hydroxytyrosol administration led to a decrease in the oxidative stress biomarker of protein oxidation, but neither oleuropein nor hydroxytyrosol modified the level of lipid peroxidation. Lipid peroxidation is an early biomarker of oxidative damage because of the increased propagation of free radicals associated with it. Elevations in oxidative stress in cells can lead to modifications of a number of cellular targets, causing cellular damage and a subsequent lack of cellular repair processes associated with carcinogenesis (Federico et al., 2007). Cancer cells are under increased and persistent oxidative stress due to the elevated generation of intracellular free radicals, leading to promotion of the carcinogenic process through mechanisms such as oncogenic stimulation, increased metabolic activity and mitochondrial malfunction (Pelicano et al., 2004). The magnitude of this oxidative damage depends not only on free radical levels but also on the efficiencies of the antioxidant mechanisms. The disruption of this delicate oxidant/antioxidant balance seems to play a causative role in carcinogenesis (Acharya et al., 2012). Therefore, high levels of oxidative stress result in peroxidation of membrane lipids and generation of peroxides that can decompose into multiple mutagenic aldehyde products, mainly MDA, which is involved in cancer progression (Zhang et al., 2002). The results of our study agree with those of Zengin, Atukeren, Kokoglu, Gumustas, and Zengin (2009), who have observed increased TBARS levels in tumor tissue samples compared with those obtained from peritumoral areas, which could have been attributed to an increased formation or inadequate clearance of free radicals by cellular antioxidants. In astrocytomas, meningiomas, metastatic tumors and other tumor types, TBARS levels were significantly higher compared with the corresponding peritumoral adjacent tissues. In addition, differences were observed when the astrocytoma tumor group was compared with other tumor groups. When the TBARS levels of the low-grade and high-grade tumors were compared, lipid peroxidation was significantly higher in the high-grade tumors. Elevated levels of lipid peroxidation products support the hypothesis that tumor cells produce a large amount of free radicals and that a relationship between free radical activity and carcinogenesis exists. Furthermore, previous studies have also shown that the lipid peroxidation state depends on the tumoral area studied. An estimation of lipid peroxidation in low-grade and high-grade astrocytomas (Zengin et al., 2009) has revealed that TBARS levels in low-grade astrocytomas are significantly elevated compared with those in malignant lesions, particularly at the tumor surface. Further, examinations of energetic and oxidant metabolic processes in low-grade gliomas obtained from the centers and peripheries of tumors (Louw, Bose, Sima, & Sutherland, 1997) have shown that lipid peroxidation increases at the periphery compared with the center. Cirak, Inci, Palaoglu, and Bertan (2003) have studied lipid peroxidation levels in serum as well as in tissue samples obtained from patients with high- and low-grade glial tumors, revealing that those with high-grade tumors have higher MDA levels in both their sera and tissues compared with patients with low-grade tumors and controls. Several products of lipid peroxidation are responsible for protein oxidation (Guo & Prokai, 2011), and only the hydroxytyrosol treatment inhibited protein oxidation in our study, which correlated with the inhibition of tumor growth.

4.3. *Treatments and non-enzymatic antioxidant defense systems*

Cellular antioxidants and free-radical scavengers protect the cell against toxic levels of oxygen radicals and include GSH, which is an important non-protein thiol. A significant depletion of GSH has been found in astrocytomas, meningiomas, metastatic tumors and other types of brain tumors compared with their peritumoral tissues. Zengin et al. (2009) have reported a significant decrease in GSH in high-grade compared with low-grade tumors. Here, we also showed a decrease in reduced GSH and an increase in its oxidized form, GSSG. GSH depletion is probably related to an enhanced pro-oxidant milieu, which correlates with the increase in lipid peroxides observed. Our results also agree with those of Navarro et al. (1999), who have shown that changes in GSH status and the antioxidant system in blood and cancer cells are associated with tumor growth *in vivo*. Higher GSSG levels are due to increased H_2O_2 production by tumors, as well as to changes in GPx activity. With regard to our results, the treatment with oleuropein, hydroxytyrosol or the mixture of both had important effects on the non-enzymatic antioxidant defense system. Thus, the animals treated with oleuropein or hydroxytyrosol showed increased levels of GSH compared with the vehicle-treated animals, although the administration of both compounds decreased GSH to levels below those found in the vehicle-treated animals. These findings confirmed that both compounds increased the activities of the non-enzymatic antioxidant defense systems, although only hydroxytyrosol significantly decreased GSSG levels. In fact, oleuropein did not inhibit tumor growth. The animals treated with both oleuropein and hydroxytyrosol showed a greater decrease in total GSH and similar changes in GSSG levels compared with those treated with the individual compounds alone. The combined treatment of oleuropein plus hydroxytyrosol did not inhibit tumor growth, confirming that oleuropein inhibited hydroxytyrosol.

4.4. *Treatments and enzymatic antioxidant defense systems*

During prolonged oxidative stress, changes in the activities of the antioxidant enzymes SOD, CAT and GPx occur. These enzymes normally act to prevent or decrease the tissue damage caused by free radicals. Thus, SOD metabolizes free radicals and dismutates superoxide anions ($O_2^{\bullet -}$) to H_2O_2 and protects cells against $O_2^{\bullet -}$-mediated lipid peroxidation, CAT converts H_2O_2 into H_2O and O_2, and GPx reduces hydrogen peroxide and other organic peroxides (Gilca et al., 2009). We found that the vehicle-treated animals with glioma tumors showed higher levels of SOD activity than the healthy control animals. Furthermore, the highest levels of SOD activity were found in the animals with glioma tumors treated with oleuropein and the mixture of oleuropein plus hydroxytyrosol. Only the treatment with hydroxytyrosol alone led to SOD activity levels similar to those of the vehicle-treated animals. Levels of this enzyme are decreased in brain tumors compared with normal controls (Aggarwal et al., 2006; Popov et al., 2003). However, Del Maestro, McDonald, and Anderson (1983) have reported relatively higher SOD activity levels in human glioma cells compared with other tumor types, which seems to contradict the general

observation of low SOD activity in tumor cells. This exception may be explained by the unique characteristics of the brain, which is a high oxygen-consuming organ. There is an increased production of superoxides during normal aerobic metabolism in brain cells. Thus, relatively high levels of SOD and other antioxidant enzymes are required to remove high levels of free radicals to protect against damage to brain tissues. Although SOD plays an important role in protecting against oxidative damage, it is likely that a balance of antioxidant enzymes is more important than their levels, which may influence intracellular oxidative states. In any case, most studies have indicated a significant reduction in SOD activity in several brain tumor types, such as gliomas, meningiomas and metastatic tumors. Levchenko and Demchuk (1991) have reported a reduction in SOD activity with an increase in MDA levels in both the blood and tumor tissues of meningioma patients. An increase in SOD activity levels, therefore, should play a protective role against tumor growth, but our results showed that the animals with high tumor growth had high SOD levels, which also seemed to be promoted by oleuropein but not by hydroxytyrosol. This could have been an indicator of the existence of high levels of superoxide radicals that promoted the development and proliferation of glioma tumors in this animal model.

In contrast, we did not find any differences in catalase activity between the non-tumor healthy control animals and those with glioma tumors treated with the vehicle only. However, oleuropein increased catalase activity and the mixture of oleuropein plus hydroxytyrosol decreased it, whereas no changes were observed following the hydroxytyrosol treatment. Several authors have described increased CAT activity in brain tumors (Yilmaz et al., 2006; Popov et al. (2003), although it did not occur in our model. However, following the treatment with oleuropein or with the mixture of oleuropein and hydroxytyrosol, we found increased or decreased levels of CAT activity, respectively, suggesting that these treatments enhanced or blocked the conversion of H_2O_2 to H_2O by CAT, respectively, which are both situations related to unchanged tumor growth.

The vehicle-treated animals with glioma tumors showed decreased levels of GPx activity compared with the healthy control animals. In contrast, higher levels of GPx activity were found after treatment with oleuropein or hydroxytyrosol, although no changes were detected after the treatment with the mixture of both compounds, also indicating interference between the compounds. These data also explained the lower antioxidant capacities of the antioxidant defense systems against free radicals, the low levels of GSH and the high levels of GSSG in the vehicle-treated animals with glioma tumors and following the treatment with oleuropein, hydroxytyrosol or the mixture of both compounds. Furthermore, SOD was found to mediate the increased activity of the enzymatic antioxidant defense system. Therefore, the ability to scavenge oxygen free radicals seems to have been impaired in the animals with glioma tumors because of the reduced levels of antioxidants, which predisposed them to cancer progression. Several other authors have found diminished levels of GPx in brain tumors (Aggarwal et al., 2006; Rao, Rao, Raja, Rao, & Rao, 2000). Moreover, when the authors separated the cases on the basis of histopathological tumor type, GPx markedly decreased as tumors became more malignant (Aggarwal et al., 2006). The low levels of antioxidants in brain tumors could be as a result of this increased oxidative damage, or these low levels may have aggravated the free-radical damage and increased the chance of developing cancer, supporting the role of antioxidants in the prevention of and the role of oxidative injury in the development of cancer. However, the putative role of free radicals in the destruction of cancer cells must also be considered as well as the importance of an adequate balance between pro-oxidants and antioxidants. Depending on the imbalance present, either protection against tumor growth or its promotion was favored.

4.5. *Effect of treatments on other physiological functions*

We also measured blood chemistry parameters (electrolytes, biomarkers of renal and hepatic function and the lipid profile) to analyze the potential adverse effects of oleuropein, hydroxytyrosol and the mixture of both compounds on several physiological processes.

The animals with tumors treated with the vehicle alone or with oleuropein did not show any significant changes in the levels of calcium, phosphorus, sodium, potassium and chloride electrolytes compared with the non-tumor healthy control animals. However, those treated with hydroxytyrosol alone or in combination with oleuropein had altered sodium, potassium and chloride levels (Table 2). The changes found in the electrolytic balance, mainly hyperkalemia, may have been due to the death of tumor cells due to the hydroxytyrosol treatment. This tumor lysis syndrome typically follows administration of chemotherapy, and malignancies associated with large tumor burdens and rapid turnover, as well as their rapid breakdown following chemotherapy, are susceptible. However, all electrolytes were maintained at values considered to be normal.

Neither the animals with tumors nor those treated with the vehicle or with oleuropein showed significant differences in the levels of glucose, urea and creatinine compared with the healthy control animals (Table 2). In contrast, increased levels of uric acid were found in the animals with tumors, and these levels remained unchanged following the different treatments. Similarly, higher levels of glucose, urea and creatinine were found as a consequence of the hydroxytyrosol treatment, alone or in combination with oleuropein. However, all values were within the normal limits. Nutrient concentrations in tumors are different than those in normal tissues, and cancer cells *in vivo* may have metabolic dependencies that are not present in normal cells. In particular, tumor glucose concentrations are frequently 3- to 10-fold lower than those in non-transformed tissues, and this is likely a result of a high rate of glucose consumption by cancer cells and poor tumor vasculature. Here, we found increased glucose levels in the animals treated with hydroxytyrosol and with hydroxytyrosol plus oleuropein but not in those treated with oleuropein alone. Therefore, high glucose levels seem more strongly related to hydroxytyrosol administration than to tumor growth. To our knowledge, no information is currently available regarding the effects of hydroxytyrosol on circulating glucose levels; thus, these effects remain to be elucidated.

We also revealed interesting information pertaining to uric acid. It has been hypothesized that the antioxidant properties of serum uric acid may play a crucial role in cancer etiology

by preventing the formation of oxygen radicals, thereby protecting against carcinogenesis (Fridovich, 1999). However, several reports have provided contrasting evidence of the proposed antioxidant effect of serum uric acid against cancer, indicating that high levels are independently associated with disease outcome and that they may reflect a worse prognosis (Strasak et al., 2007). In fact, it has been shown that astrocytoma patients' uric acid levels are significantly increased in neoplastic tissues compared with non-neoplastic tissues, and levels are even higher in necrotic tissues (Landolt, Langemann, Probst, & Gratzl, 1994). Therefore, our results agree with those reported by several authors (Pietila et al., 2009; Ueda, Wakisaka, Kinoshita, & Adachi, 1984). However, after the treatment of the glioma tumors with oleuropein, hydroxytyrosol or the mixture of both compounds, uric acid levels remained high. Therefore, the use of serum uric acid as a biomarker of tumor progression requires further research.

In our glioma model, aspartate aminotransferase increased in the animals with tumors treated with the vehicle, with oleuropein, with hydroxytyrosol or with the mixture of both compounds, indicating that this increase was exclusively due to the tumor process. Several authors have described increases in transaminases in cancer patients and during cancer treatments. Transaminases, such as aspartate aminotransferase and alanine aminotransferase, are intracellular enzymes that exist in only small amounts in the serum. Damage to liver cells may result in the leakage of enzymes into the plasma due to a high concentration gradient. Consequently, tumor-related processes may lead to the release of these enzymes into the plasma as a result of autolytic breakdown or cellular necrosis. Furthermore, increases in the activities of these enzymes in the serum may be a result of impaired function of tissues with subsequent liberation of enzymes into circulation from the damaged tissue.

Similar results have been obtained with alkaline phosphatase, which has been shown to increase as a consequence of tumors themselves but not treatments, although we found that the oleuropein treatment enhanced its increase. Alkaline phosphatase partially reflects osteoblastic activity, which is likely to be more pronounced in patients with larger tumors or aggressive bony metastatic disease (Sonpavde et al., 2012). Serum alkaline phosphatase is a relatively nonspecific biomarker that can be elevated due to sources other than bone (*e.g.,* the liver). In patients with bone metastases and elevated baseline alkaline phosphatase, the bone is the dominant source of this enzyme, and patients are likely to have liver metastasis that cause alkaline phosphatase elevation. In any case, we did not detect the presence of metastasis in our model, although the presence of micrometastases in several other organs/tissues, including the liver, could have explained the increase in this enzymatic activity.

With regard to the serum lipid profile, we found increased levels of total cholesterol, HDL-cholesterol and LDL-cholesterol in the animals with glioma tumors treated with the vehicle compared with the healthy control animals, whereas the oleuropein treatment did not affect any of the parameters. In contrast, the hydroxytyrosol treatment decreased total cholesterol via the decrease in HDL-cholesterol, and the treatment with oleuropein plus hydroxytyrosol returned the values to those of the healthy control animals. However, no changes were found in the total cholesterol/HDL-cholesterol ratio or the triacylglycerols between the groups. Altered lipid profiles are associated with malignancy because lipids play pivotal roles in the maintenance of cell integrity. Patients with oral cancer have lower levels of serum cholesterol and other lipid constituents; this phenomenon is thought to be due to an increased usage of lipids for new membrane biogenesis in tumor cells (Ghosh et al., 2011). To our knowledge, no information is currently available on the serum lipid profiles of brain tumors, although cholesterol-lowering drugs are effective in inhibiting cancer cell proliferation (Fagherazzi et al., 2010). Therefore, further research is necessary to better understand the mechanisms underlying the regulation of serum cholesterol concentrations in patients with cancer. In any case, the antitumor effects of hydroxytyrosol, but not the mixture of oleuropein plus hydroxytyrosol, did not seem to be related to the changes in the serum lipid profile.

We conclude that the treatment with hydroxytyrosol, but not with oleuropein or with the mixture of both compounds, for short durations led to the significant inhibition of tumor growth in our *in vivo* glioma model via mechanisms involving endogenous enzymatic and non-enzymatic antioxidant defense systems, as demonstrated by the decreases in oxidative stress biomarkers, such as TBARS and protein oxidation. Thus, the hydroxytyrosol treatment maintained the non-enzymatic antioxidant defense systems similar to those in the healthy animals and positively modified the enzymatic antioxidant defense systems. In contrast, oleuropein did not possess these antitumor effects and even promoted tumor growth despite being a more potent antioxidant than hydroxytyrosol, supporting the notion that modification of antioxidant defense systems is an indirect effect of hydroxytyrosol, which may act as an antitumor compound through other unknown mechanisms. Finally, the use of these compounds yields few adverse effects related to liver damage.

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