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Gender differences in the antioxidant response of oral administration of hydroxytyrosol and oleuropein against N-ethyl-N-nitrosourea (ENU)-induced glioma

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ABSTRACT

Brain tumorigenesis has been associated not only with oxidative stress, but also with a reduced response of non-enzyme and enzyme antioxidant defense systems. In fact, the imbalance between free-radical production and the efficiency of the antioxidant defense systems triggers the process because the central nervous system (CNS) is very sensitive to free-radical damage. Phenolic compounds, mainly oleuropein and its major metabolite hydroxytyrosol, derived from olives and virgin olive oil, have been shown to exert important anticancer activities both in vitro and *in vivo* due to their antioxidant properties. The present study analyzes the effects of the oral administration of oleuropein, hydroxytyrosol and the mixture of both phenolic compounds in rats with trans-placental N-ethyl-N-nitrosourea (ENU)-induced brain tumors to analyze their potential effect against brain tumorigenesis through the modification of redox system components. Oxidative stress parameters, non-enzyme and enzyme antioxidant defense systems and blood chemistry were assayed in the different experimental groups. The treatment with oleuropein, hydroxytyrosol and/or the mixture of both phenolic compounds promotes a limited beneficial effect as anticancer compounds in our ENU-induced animal model of brain tumor. These effects occur via redox control mechanisms involving endogenous enzymatic and non-enzymatic antioxidant defense systems, and are highly dependent on the gender of the animals.

1. Introduction

Free radicals are molecules characterized by a very short half-life and a high reactivity due to their instability. To achieve stability, free radicals react with numerous molecules of great biological importance, such as lipids, proteins, and DNA, causing modifications that have been associated with numerous pathological processes, including cancer (Ramirez-Exposito & Martinez-Martos, 2019; Rinaldi et al., 2016). Numerous data relate this oxidative stress caused by free radicals with modifications in cell cycle (Droge, 2002), apoptosis, expression of oncogenes, activation of different intracellular signaling pathways, mitochondrial failures and increases in cellular metabolism (Rinaldi et al., 2016), all these processes are related to the promotion and initiation of different tumor types. In addition, the tumor cells themselves, through the production of free radicals, can also favor the damage of other cells and tissues and, therefore, can even facilitate tumor growth and invasion (Liou & Storz, 2010).

Brain tumorigenesis (such as other tumor types) has been associated not only with oxidative stress, but also with a reduced response of non-enzyme (reduced glutathione, GSH) and enzyme antioxidant systems (superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx)) (Illan-Cabeza et al., 2013). In fact, the imbalance between free-radical production and the efficiency of the antioxidant defense systems triggers the process because the central nervous system (CNS) is very sensitive to free-radical damage (Ramirez-Exposito & Martinez-Martos, 2019). Gliomas are the most common primary intraparenchymal brain tumors and have a very poor prognosis. They represent approximately 26% of all primary brain and other CNS tumors and 81% of the malignant tumors. Glioblastoma accounts for the majority of gliomas (56.6%). Relative survival estimates for glioblastoma are quite low with only 5.6% of patients who survived five years post diagnosis (Gittleman et al., 2018; Ostrom et al., 2018).

Phenolic compounds derived from olives and virgin olive oil, mainly oleuropein and its major metabolite hydroxytyrosol, have been shown to

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exert important anticancer activities both in vitro and *in vivo* due to their antioxidant properties (Carrera-Gonzalez, Ramirez-Exposito, Mayas, & Martinez-Martos, 2013; Kouka et al., 2020). We have previously shown (Martínez-Martos et al., 2014) in an animal model of C6 glioma implanted at the subcutaneous region that the subcutaneous administration of hydroxytyrosol, but not oleuropein or the mixture of both compounds, lead to the significant inhibition of tumor growth through 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 that modification of antioxidant defense systems is an additional effect of hydroxytyrosol, which may act as an antitumor compound through other unknown mechanisms. In the present study, we analyzed the effects of the oral administration of the phenolic compounds oleuropein, hydroxytyrosol and the mixture of both phenolic compounds in rats with transplacental N-ethyl-N-nitrosourea (ENU)-induced brain tumors to analyze their potential effect against brain tumorigenesis through the modification of redox system components. Models of ENU-induced tumors of the nervous system in rodents have been widely used (Doi et al., 2015; McNeill, Irvin, & Miller, 2016; Schiffer et al., 2018; Wang et al., 2016) and this animal model has shown a high rate of tumor induction (100%) and the appearance of many tumors per brain. In addition, the profile and time course of tumor progression in this experimental model have been extensively documented (Bulnes & Lafuente, 2007; Kokkinakis et al., 2004; Mahlke et al., 2011; Wang et al., 2016). Moreover, we have previously described in this model several carcinogenesis and oxidative stress parameters, along the response of enzyme and non-enzyme antioxidant defense systems (Ramirez-Exposito, Mayas, Carrera-Gonzalez, & Martinez-Martos, 2019).

2. Material and methods

2.1. Animals and treatments

Female (n = 17) and male (n = 9) Wistar rats were obtained from Harlan laboratories (Spain). The animals were maintained in a controlled environment under constant temperature (25 °C) with a 12 h-light/12 h-dark cycle. Rats were housed in cages and given free access to a standard rat chow and water. The experimental procedures for animal use and care were in accordance with the European Community Council directive (2010/63/EU). Protocols were approved by the Bioethical Committee of the University of Jaen (Reference number CVI09-4957 M). Female Wistar rats weighing 200–250 g were caged overnight with males, and the day when the sperm was confirmed in vaginal smears was designated as day 1 of gestation. On day 18 of gestation, pregnant rats were injected intravenously (i.v.) with a single dose of ENU, 75 mg/kg body weight dissolved in saline solution. Female and male offspring from ENU-treated rats were used in these experiments. A total of 158 offspring were obtained. The offspring were naturally delivered and weaned at 22 days old. At this time, males and females were housed separately and randomly divided into four groups. Group one (OLEU group, consisted of 34 animals, 20 females and 14 males) received oleuropein solution (50 mg/L) in drinking water; group two (HTX group, consisted of 35 animals, 20 females and 15 males) received hydroxytyrosol solution (25 mg/L) in drinking water; group three (OLEU + HTX group, consisted of 35 animals, 19 females and 16 males) received oleuropein plus hydroxytyrosol solution (50 mg/L oleuropein and 25 mg/L hydroxytyrosol) in drinking water; and finally, the fourth group (Untreated group, 54 animals, 29 females and 25 males) received tap water. All animals were allowed *ad libitum* access to drink and food, and were kept under weekly observation for any sign of neurological or health problems. After 30 weeks of treatment, surviving rats were killed. Tumor and plasma samples were obtained. Table 1 shows the mean amount of oleuropein, hydroxytyrosol and oleuropein plus hydroxytyrosol intake during the time of the treatment for the different experimental groups.

2.2. Magnetic resonance imaging (MRI) analysis

Magnetic resonance images were acquired at 9.4 Tesla (Bruker Biospec, Ettlingen, Germany) at the Foundation IDICHUS (Santiago de Compostela, Spain). The ENU-exposed rats were imaged at 30 weeks of age using a volume coil for transmission and a surface coil for reception. Anesthetized rats were placed in a MRI probe in a supine position in a cradle and their head was maintained with ear bars and a bite bar. For each animal, 20 contiguous 1 mm thick slices were acquired using a T2-weighted spin echo sequence (repetition time/echo time (TR/TE) = 2000/80 ms, matrix = 256 × 192 or 128 × 128, two accumulations, Field of View = 30 × 30 mm^2). To aid in tumor visualization, animals were injected intraperitoneally (i.p.) with 1.5 mL/kg body weight of gadolinium (Gd-DTPA) (Magnevist, Schering). Tumor volume was calculated using Image J software. Tumor surface (millimeter square) was measured on MRI images according to MRI resolution. Tumor volume was calculated by analyzing the surface of the tumor on successive MRI slices multiplied by slice thickness (1 mm). Images were analyzed and processed by a scientist blinded to the study by using Bruker's Paravision 5.1 software and Image-J (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, MD, USA, https://imagej.nih.gov/ij/).

2.3. Plasma and tissue collection

Rats were anesthetized with equithesin (2 mL/Kg body weight) by intraperitoneal injection and then shaved and sterilized with 10% povidone-iodine. Blood samples were obtained from the left cardiac ventricle, drawn into tubes with heparin as anticoagulant, allowed to clot, and then centrifuged for 10 min at 3000*g* to obtain the plasma, which was frozen and stored at −80 °C until use. Tissue samples were homogenized in 10 volumes of 10 mM HCl-Tris buffer (pH 7.4) and centrifuged for 30 min at 4 °C. Samples of tumors were quickly removed and prepared for histopathological examination or frozen at −80 °C until use.

2.4. Oxidative stress assays

2.4.1. Metabolism behavior assay

Metabolic activity of brain tissue was assayed by using tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Martinez-Martos, Ramirez-Exposito, Mayas-Torres, Garcia-Lopez, & Ramirez-Sanchez, 2000). Briefly, samples were mixed with 100 μL of 1 mM MTT in HCl-Tris buffer and incubated for 30 min at 37 °C. Then, 100 μL of HCl in isopropanol (0.04 M) was added to each

Table 1
Mean amount of oleuropein, hydroxytyrosol and oleuropein plus hydroxytyrosol intake during the 30 weeks or oral treatment in male and female rats with N-ethyl-N-nitrosourea (ENU)-induced gliomas.

Experimental group	OLEU	HTX	OLEU + HTX	
	Oleuropein intake	Hydroxytyrosol intake	Oleuropein intake	Hydroxytyrosol intake
Male animals	4.63 ± 0.18	2.95 ± 0.14	5.66 ± 0.19	2.82 ± 0.09
Female animals	5.43 ± 0.17	4.04 ± 0.13	6.20 ± 0.23	3.10 ± 0.11

Data are expressed as mean ± SEM in mg/Kg/day of the corresponding compound.

well and the mixture was shaken vigorously to dissolve the dark blue crystals. MTT is hydrolyzed by the mitochondrial enzyme succinate dehydrogenase, which produces a dark blue tetrazolium salt that can be measured spectrophotometrically using a test wavelength of 550 nm and a reference wavelength of 620 nm. The resulting values were expressed in optical density units/mg of protein.

2.4.2. Protein oxidation assay

Protein oxidation was measured by analyzing the carbonyl groups content of proteins. Briefly, samples were mixed with 100 μL of ice-cold 20% trichloroacetic acid (TCA) and centrifuged. Protein precipitates were left to react with 10 mM 2,4-dinitrophenylhydrazine for an hour at room temperature in the dark. After the reaction, proteins were precipitated with 20% TCA and unreacted dye was washed twice with 10% TCA. The pellets were dissolved in 1 M NaOH and absorbance was recorded at 360 nm. The results were expressed as nmol/mg of protein using an extinction coefficient of $2.1x10^4$ M^{-1} cm^1.

2.5. Non-enzyme antioxidant defense systems

2.5.1. Total glutathione assay

Total glutathione was measured using a commercial kit from Biovision, according to the manufacturer's instructions. Data are expressed as nmol of GSH/mg of protein.

2.6. Enzyme antioxidant defense systems

2.6.1. Superoxide dismutase assay

Samples were mixed with reaction buffer containing 100 mM triethanolamide-diethanolamide buffer (TDB) pH 7.4, 7.5 mM NADH and relation 1:2 EDTA/$MnCl_2$. To start the reaction, 10 mM ß-mercaptoethanol were added. The absorbance was recorded at 340 nm for 2–15 min. Results are expressed in U/mg of protein. One unit of SOD activity is defined as the amount of enzyme necessary to produce a 50% inhibition of the NADH oxidation rate under the assay conditions.

2.6.2. Catalase activity assay

Samples were added to 10 mM H_2O_2 in 20 mM potassium phosphate buffer (pH 7.0) and incubated at 30 °C for one minute. Initial reaction rate was measured from the decrease in absorbance at 240 nm. Results are expressed in U/mg of protein. One unit of CAT activity is defined as 1 μmol of H_2O_2 decomposed per minute under the assay conditions.

2.6.3. Glutathione peroxidase activity assay

Samples were added and mixed with 50 mM potassium phosphate (pH 7.4), 25 mM NADPH, 1 mmol/L GSH and 100 U/mL of yeast glutathione reductase in a 96-well plate. The hydroperoxide-independent NADPH consumption rate was recorded for 3 min at 37 °C at 340 nm. Then, *tert*-butyl hydroperoxide was added to start the reaction, mixed, and the overall rate at 340 nm was recorded. The same procedure was performed in the same reaction volume without the sample. This allows subtracting the non-enzymatic rate of GSH oxidation. Results were expressed in U/mg of protein. One unit of GPx activity is defined as 1 μmol of NADH oxidized per minute under the assay conditions.

2.7. Western blotting

Samples were treated with RIPA buffer. Fifty micrograms of total protein were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Amersham GE-Healthcare, Buckinghamshire, UK). Protein concentration was determined according to the Bradford's method. Proteins were loaded per lane onto a 15% SDS-PAGE gel for SOD-1 (23 kDa), or a 10% SDS-PAGE gel for CAT (64 kDa) and 7.5% SDS-PAGE gel for GPx-1 (92 kDa). After blocking with 5% skim milk in TBS, the membrane was incubated with goat anti-SOD-1 antibodies(1:200, Santa Cruz Biotechnology, Inc.), mouse monoclonal anti-CAT antibodies (1:200, Santa Cruz Biotechnology, Inc.) and goat anti-GPx-1 antibodies (1:200, Santa Cruz Biotechnology, Inc.) overnight at 4 °C. Blots probed with tubulin (1:10000; Santa Cruz Biotechnology, Inc.) were used as loading controls. The membranes were washed three times with TBS-T. Immunoreactive polypeptide was visualized using horseradish peroxidase conjugated secondary antibodies (anti-goat IgG peroxidase conjugated 1:2000, 1:5000 Santa Cruz Biotechnology, Inc. and anti-mouse IgG peroxidase conjugated 1:2000, Amersham Bioscience) and enhanced-chemiluminescence detection reagents (Amersham Bioscience) following manufacturer-supplied protocols. Immunoblots were analyzed by using the Image Lab ™ Software version 2.0.1 (Bio-Rad) to provide quantitative values for relative expression of each protein (all normalized to their own loading control). The optical densities of the bands were measured by using Image Lab software.

2.8. RT-PCR

For first strand cDNA synthesis, 1 μg of total RNA was reverse transcribed using random hexamers (Roche Diagnostic) as primers and Transcriptor Reverse Transcriptase (Roche Diagnostic). Gene expression was assessed by RT-PCR using MiniOpticon technology (BioRad) with SYBER Green detection. A standard curve was created with serial dilutions of a PCR fragment from RNA of brain tissue (Clontech Laboratories, Inc., Mountain View, CA). For quantification purposes, mRNA levels were always reported to β-actin and GAPDH levels, constitutively expressed genes. All samples were quantified in duplicate and positive and negative controls were included in all the reactions. The reaction was performed following the protocol of manufacturers in a final volume of 25 μL. The cycle program consisted of an initial denaturing of 10 min at 95 °C, then 40 cycles of 15 s denaturizing phase at 95 °C and 1 min annealing and extension phase at 60 °C. Primers design is shown in table 2.

2.9. Blood chemistry measurements

Electrolytes (sodium, potassium and chloride), calcium and phosphorus were assayed using selective ion electrodes. Results are expressed in mEq/L; calcium and phosphorus were assayed by colorimetric methods. Results are expressed in mg/dL. The non-protein nitrogenous compounds, uric acid, urea, creatinine, and glucose in samples were assessed using commercial kits (Boehringer Mannheim) with the automated Roche-Hitachi 917 system. Total cholesterol, high-density lipoprotein (HDL) cholesterol and triglycerides were assayed with the use of standard enzymatic colorimetric methods using commercially available kits. The low-density lipoprotein (LDL) cholesterol level was calculated according to the Friedewald formula. Results are expressed in mg/mL. Plasma activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were estimated by quantitative enzymatic colorimetric, end point methods using commercially available kits. Results are expressed in UI/L. Alkaline phosphatase (ALP) was determined by colorimetric, end point method using commercially available kits. Results are expressed in UI/L. The albumin content was determined by colorimetric method using a commercial kit. Results are

Table 2
Primers used for quantitative real-time PCR.

Wistar Rat Gene	Forward Primer 5́ → 3́	Reverse primer 5́ → 3́
SOD-1	AATGTGTCCATTGAAGATCGTGTGA	GCTTCCAGCATTTCCAGTCTTTGTA
CAT	GAATGGCTATGGCTCACACA	CAAGTTTTTGATGCCCTGGT
GPx-1	AGTTCGGACATCAGGAGAATGGCA	TCACCATTCACCTCGCACTTCTCA
ß-Actin	CTCTCTTCCAGCCTTCCTTC	GGTCTTTACGGATGTCAACG
GAPDH	GCACCGTCAAGGCTGAGAAC	ATGGTGGTGAAGACGCCAGT

expressed in g/dL. Total protein level was estimated by the colorimetric method of Bradford. Results are expressed in mg/mL.

2.10. Statistical analysis

All values represent the mean ± standard error of the mean (SEM). Data were analyzed by multiple analysis of variance (MANOVA) plus Newman-Keuĺs test. Kaplan–Meier's statistics and log-rank tests were performed to estimate the significance of differences in survival times. IBM Pass V.24 software was used. Values of $P < 0.05$ were considered significant.

3. Results

3.1. Parameters of the carcinogenesis

Fig. 1 shows several examples of ENU-induced gliomas on MRI, in untreated male and female rats and in male and female rats of the OLEU, HTX and OLEU + HTX groups, as well as histopathological sections of tumors from animals of the different experimental groups.

Kaplan-Meier survival curves demonstrated that OLEU, HTX and OLEU + HTX male rats had a significantly higher survival rate than untreated male rats (57.1%, 60% and 50%, respectively, vs. 24.1% in 30-wk survival, log-rank test, $P < 0.05$ in all cases; Fig. 2A). No significant differences were found between treatments. In female rats, survival curves demonstrate that the OLEU + HTX treatment promoted a significantly higher survival rate than untreated female rats (52.6% vs. 24.1% in 30-wk survival, log-rank test, $P < 0.05$; Fig. 2B) or borderline significance than OLEU or HTX groups (35% vs. 24.1% in 30-wk survival, log-rank test, $P = 0.0516$).

ENU-treated animals showed a tumor incidence, defined as the percentage of rats bearing at least one malignant tumor at sacrifice, of 100% in both male and female rats. In addition, males showed a range from 1.9 ± 0.05 to 2.0 ± 0.12 tumors per animal, without significant

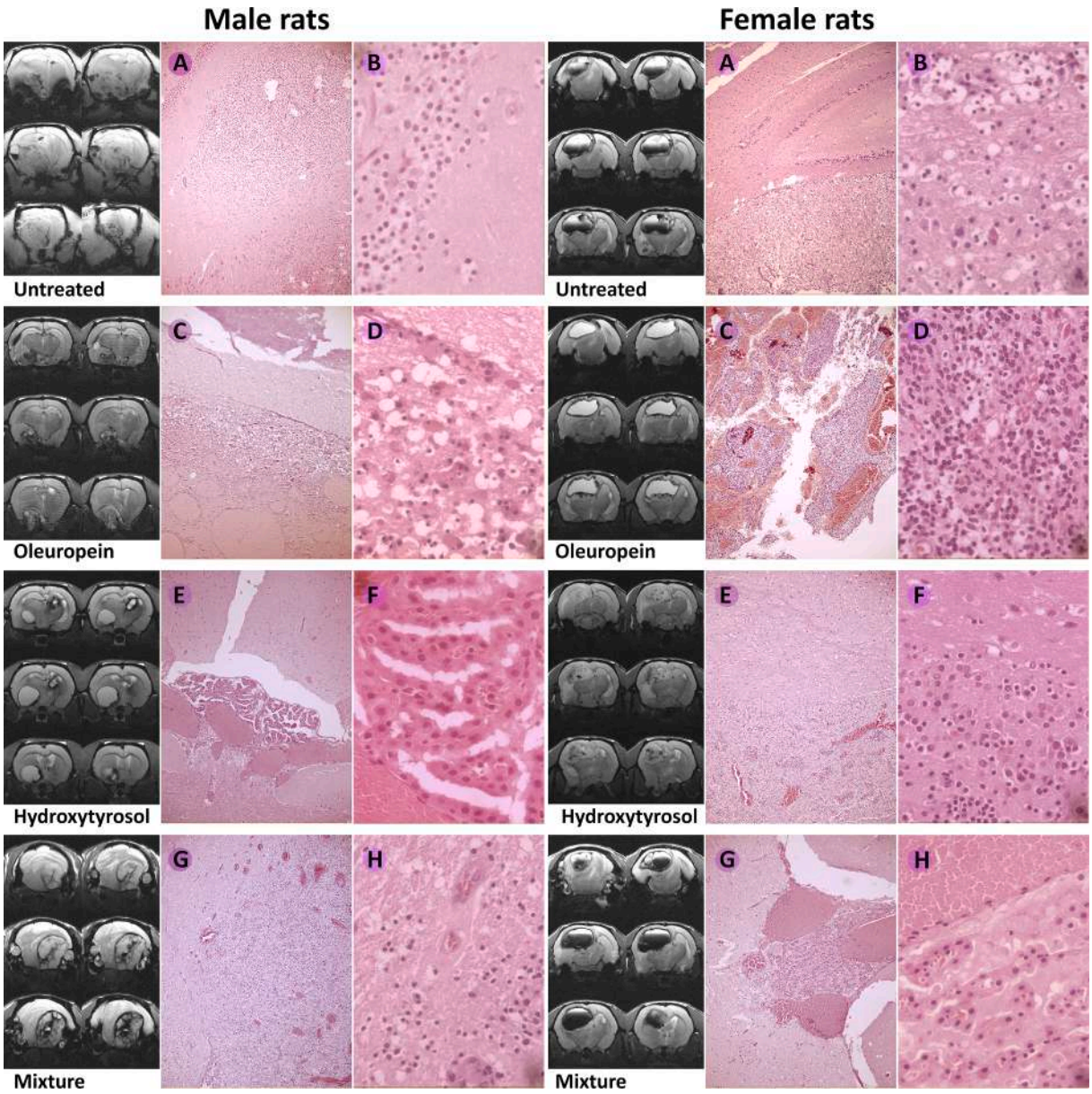

Fig. 1. Typical T2-weighted MRI images of ENU-induced tumors in male (left pane) and female (right pane) untreated animals and in animals treated with oleuropein, hydroxytyrosol and the mixture of oleuropein plus hydroxytyrosol. Histopathological H&E sample sections from untreated animals (A, 10X; B, 80X) and animals treated with oleuropein (C, 10X; D, 80X), hydroxytyrosol (E, 10X; F, 80X) and the mixture of oleuropein plus hydroxytyrosol (G, 10X; H, 80X) are shown for male and female rats.

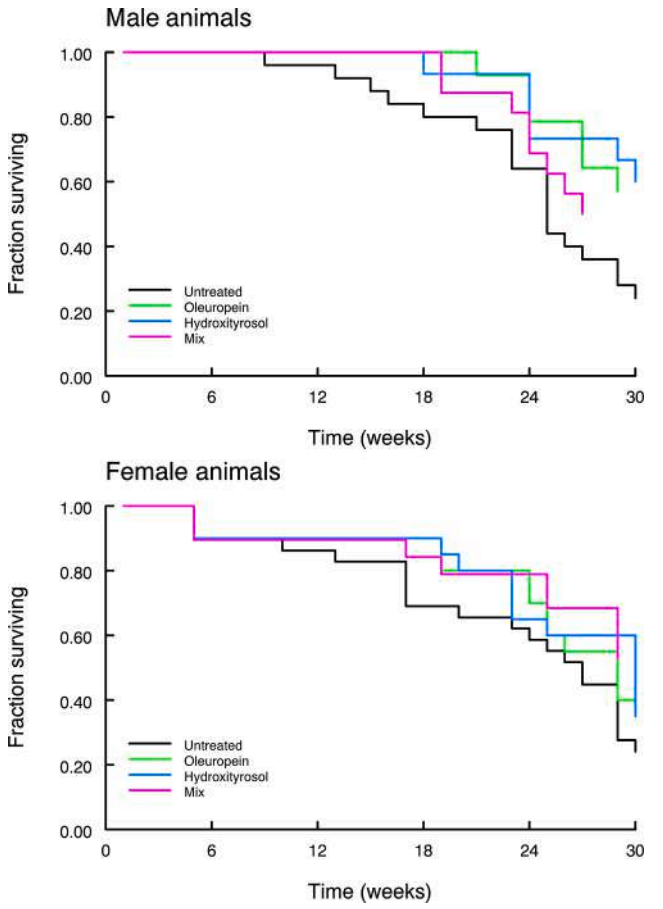

Fig. 2. Kaplan-Meier estimates of survival in (A) male and (B) female untreated animals with ENU-induced gliomas and animals with ENU-induced gliomas treated with oleuropein, hydroxytyrosol and the mixture of oleuropein plus hydroxytyrosol.

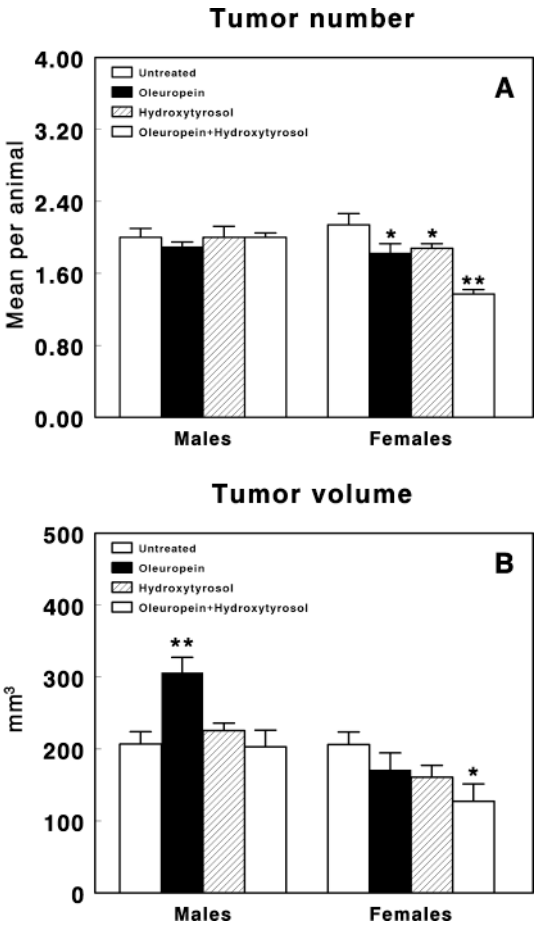

Fig. 3. Tumor number (A) and tumor volume (B) in male and female untreated animals with transplacental N-ethyl-N-nitrosourea (ENU)-induced gliomas and animals with ENU-induced gliomas treated with oleuropein, hydroxytyrosol and the mixture of oleuropein plus hydroxytyrosol. Results are expressed as Mean ± SEM (***P < 0.001; **P < 0.01; *P < 0.05).

differences between OLEU, HTX and OLEU + HTX in males. In contrast, untreated females showed a mean tumor number per animal of 2.14 ± 0.12, whereas OLEU and HTX female groups showed a significant decrease ($P < 0.05$ in both cases) by ≈1.2-fold and ≈1.1-fold, respectively, up to 1.83 ± 0.1 and 1.88 ± 0.05 tumors per animal, respectively. Finally, OLEU + HTX female group showed the lowest mean number of tumors per animal (by ≈1.6-fold, $P < 0.01$), with a value of 1.37 ± 0.05 (Fig. 3A). Regarding tumor volume, no significant difference was found between untreated male rats and HTX or OLEU + HTX males, ranging from 203.3 ± 23.26 to 222.55 ± 10.44 mm^3. However, OLEU males showed a significant increase in tumor volume by ≈1.5-fold, up to 306.32 ± 21.03 mm^3 (Fig. 3B). In female rats, a significant tumor volume decrease ($P < 0.05$) by ≈1.6-fold, from 206.18 ± 17.52 mm^3 to 127.43 ± 24.09 mm^3 was found in OLEU + HTX compared with untreated animals, whereas only a borderline significance was found in OLEU or HTX females t, with values ranging from 206.18 ± 17.52 mm^3 to 160.76 ± 16.55 mm^3.

3.2. Metabolism behavior

We found significantly decreased levels of metabolism behavior (assayed through mitochondrial succinate dehydrogenase activity) in OLEU, HTX and OLEU + HTX male and female groups with ENU-induced gliomas a (Fig. 4). Thus, in male rats, oleuropein significantly decreased ($P < 0.01$) metabolism behavior by ≈2-fold, whereas hydroxytyrosol and the mixture of oleuropein plus hydroxytyrosol only decreased it by ≈1.4–1.5-fold ($P < 0.05$ in both cases). In contrast, both oleuropein and hydroxytyrosol significantly decreased ($P < 0.01$)

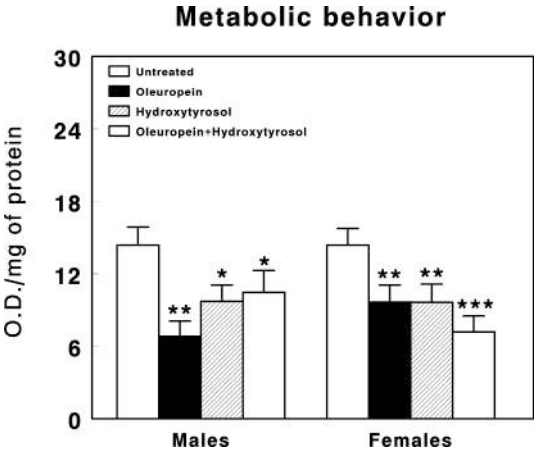

Fig. 4. Metabolism behavior of tumoral tissue in male and female untreated animals with transplacental N-ethyl-N-nitrosourea (ENU)-induced gliomas and animals with ENU-induced gliomas treated with oleuropein, hydroxytyrosol and the mixture of oleuropein plus hydroxytyrosol. Results are expressed in optical density units per mg of protein (Mean ± SEM; ***P < 0.001; **P < 0.01; *P < 0.05).

metabolism behavior in female rats by ≈1.5-fold, whereas the mixture of oleuropein and hydroxytyrosol decreased it by ≈2-fold ($P < 0.001$) compared with untreated rats.

3.3. Oxidative stress parameters

3.3.1. Protein oxidation

The analysis of protein oxidation (assayed as carbonyl and diene conjugate groups content) in the OLEU, HTX and OLEU + HTX male and female groups, in addition to the untreated group, is shown in Fig. 5. OLEU and HTX male rats t showed a significant increase in protein oxidation by ≈1.2-fold ($P < 0.01$) and ≈1.3-fold ($P < 0.001$), respectively. In the same way, the OLEU + HTX group has also shown a significant increase in protein oxidation ($P < 0.05$) compared with untreated animals, but only by ≈1.1-fold. Similarly, OLEU and HTX female rats have shown a significant increase in protein oxidation by ≈1.5-fold ($P < 0.001$ in both cases) compared with untreated animals. However, no significant differences in protein oxidation were found between OLEU + HTX female rats and the untreated group (Fig. 5).

3.4. Antioxidant defense systems

3.4.1. Non-enzymatic antioxidant defense system

The analysis of total glutathione in untreated, OLEU, HTX, OLEU + HTX groups of both male and female animals l is shown in Fig. 6. In male animals, OLEU, HTX and OLEU + HTX showed a significant higher levels of total glutathione by ≈2.2-fold ($P < 0.001$), ≈2.8-fold ($P < 0.001$) and ≈2.6-fold ($P < 0.001$), respectively, compared with untreated animals. In contrast, no changes in total glutathione content of tumoral tissue were found in female OLEU vs. untreated group. However, HTX and OLEU + HTX females showed a significant higher levels of total glutathione by ≈2.3-fold ($P < 0.001$) and ≈1.8-fold ($P < 0.01$), respectively, compared with untreated female animals (Fig. 6).

3.4.2. Enzymatic antioxidant defense system

3.4.2.1. Superoxide dismutase (SOD). Fig. 7A shows the analysis of SOD activity in both male and female untreated animals having ENU-induced gliomas and animals with ENU-induced gliomas treated with oleuropein, hydroxytyrosol and the mixture of oleuropein plus hydroxytyrosol. Male animals of OLEU, HTX and OLEU + HTX groups showed a significant increase in SOD activity by ≈2.2-fold ($P < 0.001$), ≈2.3-fold ($P < 0.001$) and ≈2.3-fold ($P < 0.001$), respectively, compared with untreated animals. In the same way, OLEU and HTX female groups s

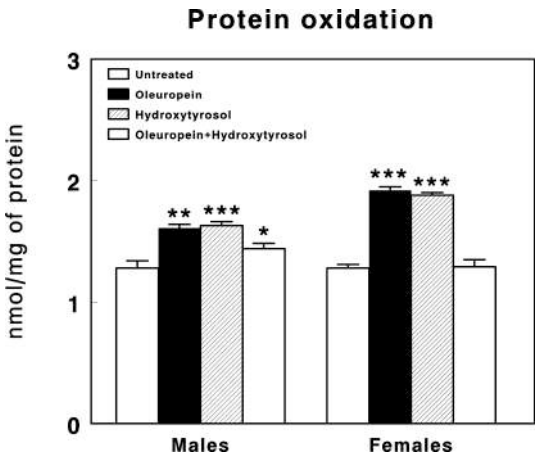

Fig. 5. Carbonyl groups content in tissue (A) and plasma (B) of male and female control animals and animals with gliomas induced by transplacental N-ethyl-N-nitrosourea (ENU) exposure. Results are expressed in nmol per mg of protein (Mean ± SEM; ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$).

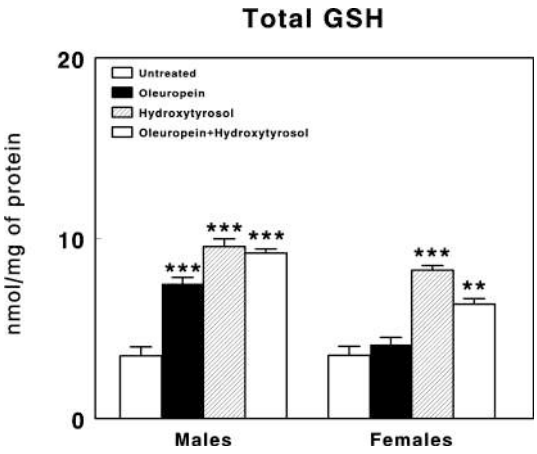

Fig. 6. Total glutathione (GSH) content in tissue (A) and plasma (B) of male and female control animals and animals with gliomas induced by transplacental N-ethyl-N-nitrosourea (ENU) exposure. Results are expressed in nmol per mg of protein (Mean ± SEM; ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$).

showed a significant increase in SOD activity by ≈2.5-fold ($P < 0.001$) and by ≈2.2-fold ($P < 0.001$), respectively compared with the untreated group; whereas OLEU + HTX female group showed a significant increase in SOD activity only by ≈1.4-fold ($P < 0.05$) compared with untreated female animals (Fig. 7A). On the contrary, mRNA expression analysis (Fig. 7B) showed significantly decreased levels in OLEU males by ≈1.7-fold, by ≈1.7-fold in HTX males and by ≈3.2-fold in OLEU + HTX compared with untreated males ($P < 0.001$ in all cases), whereas no significant changes were found in females after the different treatments. Similarly, SOD-1 protein levels significantly decreased in OLEU males by ≈1.7-fold ($P < 0.01$), in HTX males by ≈1.6-fold ($P < 0.01$) and in OLEU + HTX by ≈2.6-fold compared with untreated males ($P < 0.001$). No significant changes were found in females after the different treatments compared with untreated females (Fig. 7C).

3.4.2.2. Catalase. Fig. 8A shows the analysis of CAT activity in both male and female untreated animals with ENU-induced gliomas and animals with ENU-induced gliomas treated with oleuropein, hydroxytyrosol and the mixture of oleuropein plus hydroxytyrosol. Neither in male nor female OLEU, HTX and OLEU + HTX groups showed significant differences compared with control animals. On the contrary, mRNA expression analysis (Fig. 8B) showed significantly increased levels in OLEU males by ≈2.5-fold ($P < 0.001$) a and OLEU + HTX by ≈1.6-fold compared to the untreated ($P < 0.001$), whereas no changes were found in HTX vs. untreated males. In female animals, OLEU, HTX, OLEU + HTX showed a significant decrease in CAT mRNA levels by ≈1.2-fold ($P < 0.05$), ≈1.5-fold ($P < 0.01$) and ≈2-fold ($P < 0.001$), respectively, compared with untreated animals. In the same way, CAT protein levels showed significant increased levels in OLEU males by ≈1.3-fold ($P < 0.05$) and OLEU + HTX by ≈1.2-fold ($P < 0.05$) compared with untreated animals, whereas no significant differences were found in HTX vs. untreated males. In female OLEU, HTX and OLEU + HT animals showed a significant decrease in CAT protein levels by ≈1.3-fold, ≈1.5-fold and ≈1.8-fold ($P < 0.05$ in all cases), respectively, compared with the untreated group (Fig. 8C).

3.4.2.3. Glutathione peroxidase. Fig. 9A shows the analysis of GPx activity in both male and female untreated animals with ENU-induced gliomas and animals with ENU-induced gliomas treated with oleuropein, hydroxytyrosol and the mixture of oleuropein plus hydroxytyrosol. In male animals, OLEU and OLEU + HTX showed a significant increase in GPx activity by ≈1.2-fold ($P < 0.001$) and ≈1.5-fold ($P < 0.001$ in both cases), respectively, compared with untreated animals. However, no significant changes were found in HTX males vs. untreated.

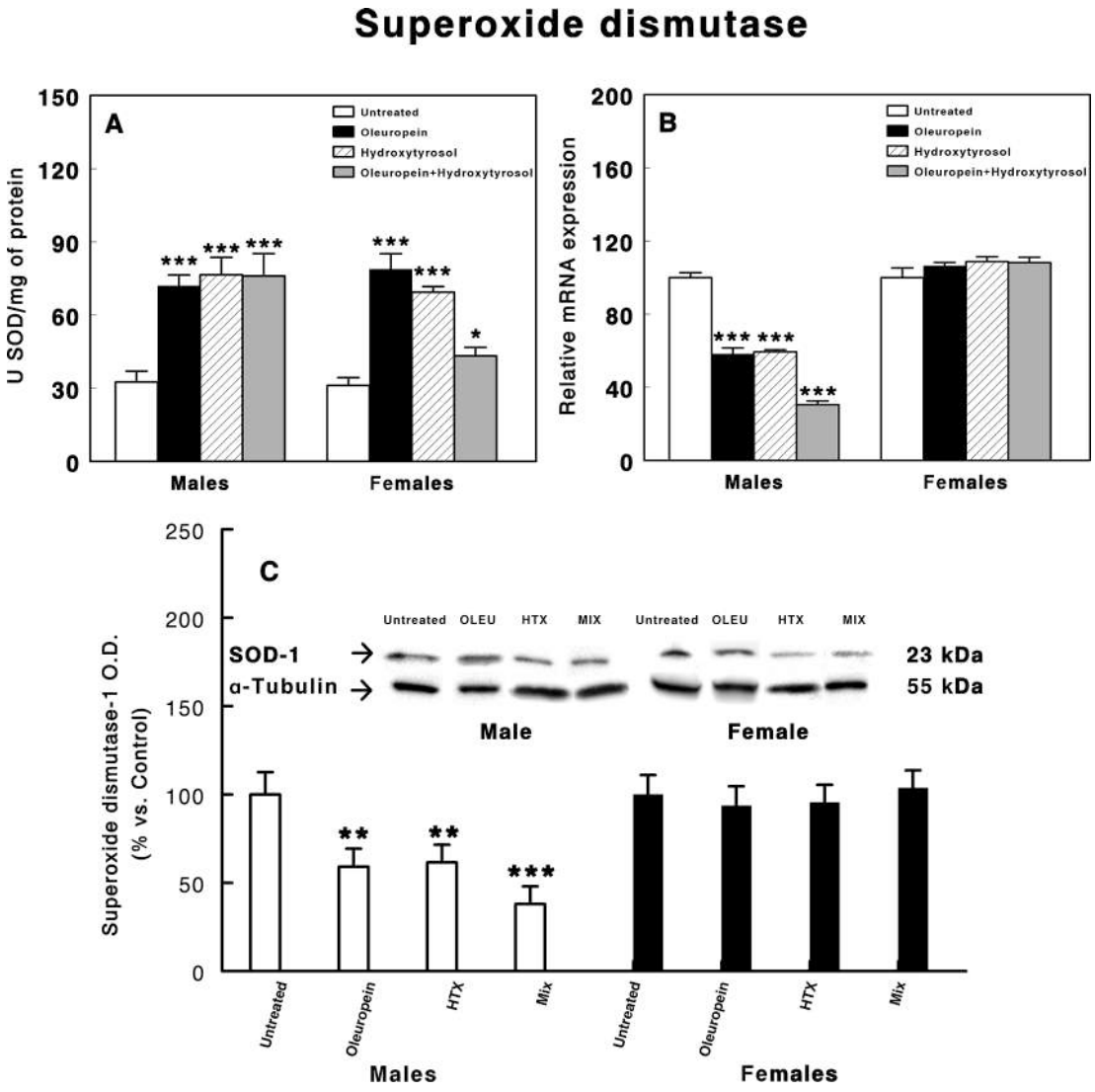

Fig. 7. Superoxide dismutase (SOD) activity in tissue (A) and plasma (B) of male and female control animals and animals with gliomas induced by transplacental N-ethyl-N-nitrosourea (ENU) exposure. Results are expressed in units per mg of protein. Figure (C) shows a representative western blot showing Cu-Zn SOD-1 (23 KDa) protein levels in tissue. Densitometry analyses are presented as a relative ratio of SOD-1 to α-tubulin. Figure (D) shows tissue SOD-1 mRNA expression normalized to ß-actin and GAPDH levels (Mean ± SEM; ***P < 0.001; **P < 0.01; *P < 0.05).

In female animals, no significant differences GPx activity were found in OLEU and HTX compared with untreated animals, whereas OLEU + HTX showed a significant increase in GPx activity by ≈1.3-fold ($P < 0.001$) compared with untreated female animals (Fig. 9A). Similarly, mRNA expression analysis (Fig. 9B) showed significantly increased levels in OLEU males by ≈1.3-fold ($P < 0.01$) and in OLEU + HTX by ≈1.7-fold ($P < 0.001$) compared with untreated males; whereas no significant differences were found in HTX males vs. untreated. In females, GPx mRNA expression significantly increased in OLEU + HTX by ≈1.2-fold ($P < 0.01$) a, whereas no differences were observed in OLEU or HTX vs. untreated. In the same way, OLEU males showed significant increased levels of GPx-1 protein by ≈1.7-fold ($P < 0.001$) and OLEU + HTX by ≈2-fold ($P < 0.001$) compared with untreated, no significant differences in GPx-1 protein levels were found in HTX males vs. untreated animals. No significant differences were found in GPx-1 protein expression levels in OLEU or HTX females compared with untreated females, although a significant increase was found in OLEU + HTX by ≈1.3-fold ($P < 0.05$) compared with untreated females (Fig. 9C).

3.5. Blood chemistry

Table 3 shows the level of several electrolytes (calcium, phosphorus, sodium, potassium and chloride) analyzed in plasma of untreated animals with ENU-induced gliomas and animals with ENU-gliomas treated with oleuropein, hydroxytyrosol and the mixture of oleuropein plus hydroxytyrosol. No significant changes were found in calcium, phosphorus, sodium, potassium or chloride after the administration of the different treatments. All parameters showed values within normal levels.

Table 4 shows plasma levels of glucose and non-protein nitrogenous compounds (urea, creatinine and uric acid) in untreated ENU-animals and after the treatment with oleuropein, hydroxytyrosol and the mixture of oleuropein plus hydroxytyrosol.

A significant ($p < 0.01$) increase in glycemia was found in OLEU and OLEU + HTX males and females, but no increase was found in HTX groups compared to the untreated group. In contrast, HTX females, showed significant increased levels of urea ($P < 0.01$), creatinine ($P < 0.01$) and uric acid ($P < 0.01$) compared to untreated animals, but no changes were found in OLEU or OLEU + HTX females compared to untreated females. No significant differences were found in any of these

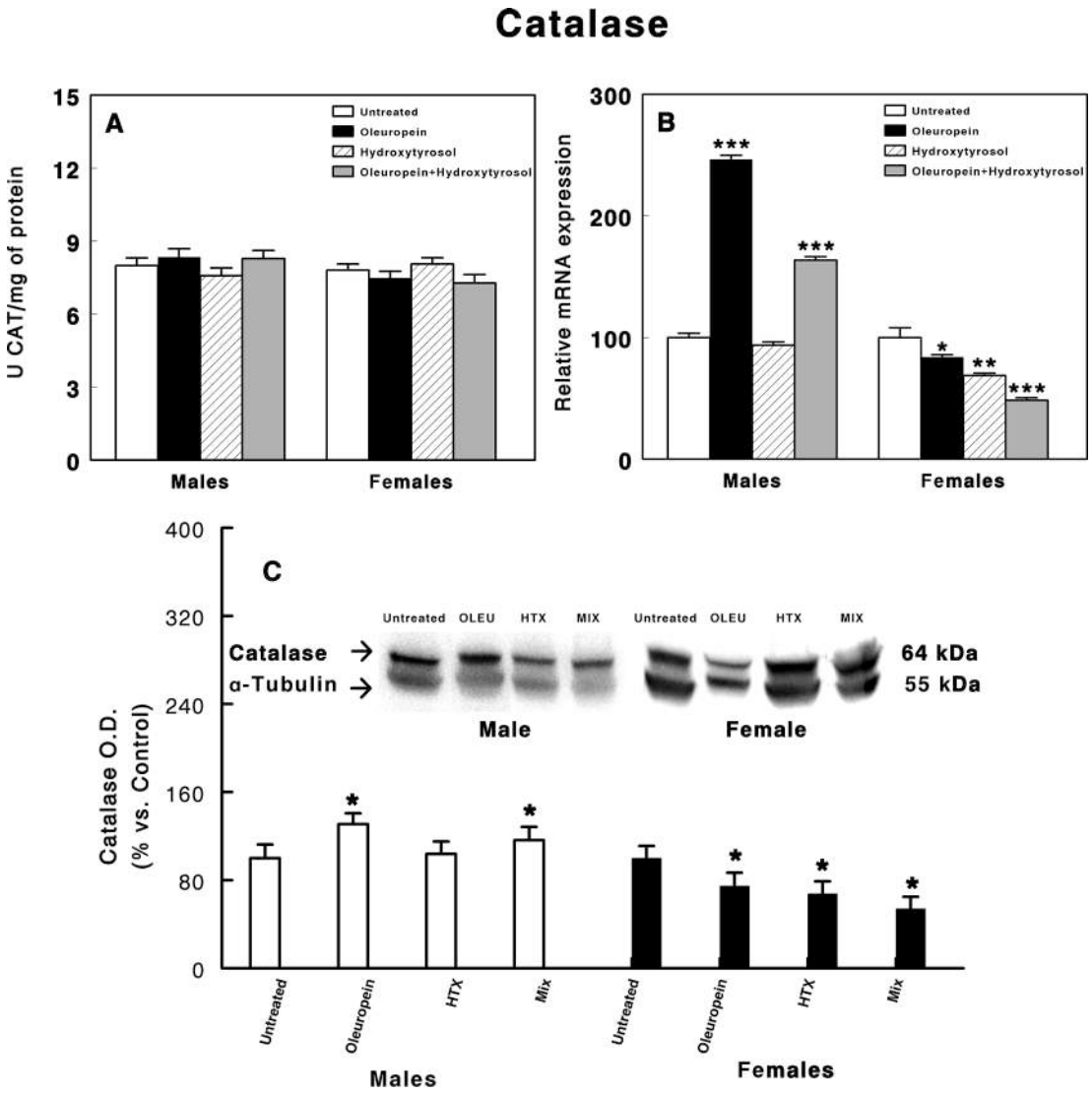

Fig. 8. Catalase (CAT) activity in tissue (A) and plasma (B) of male and female control animals and animals with gliomas induced by transplacental N-ethyl-N-nitrosourea (ENU) exposure. Results are expressed in units per mg of protein. Figure (C) shows a representative western blot showing CAT (64 kDa) protein levels in tissue. Densitometry analyses are presented as a relative ratio of CAT to α-tubulin. Figure (D) shows tissue CAT mRNA expression normalized to ß-actin and GAPDH levels (Mean ± SEM; ***P < 0.001; **P < 0.01; *P < 0.05).

parameters in male animals.

Table 5 shows plasma lipid profile (total cholesterol, HDL-cholesterol, LDL-cholesterol, total cholesterol/HDL-cholesterol ratio and triglycerides) in untreated animals with ENU-induced gliomas and animals with ENU-gliomas treated with oleuropein, hydroxytyrosol and the mixture of oleuropein plus hydroxytyrosol. The OLEU + HTX male group showed a significant decrease in total cholesterol levels ($P < 0.01$) whereas no significant changes were found in females. In contrast, HDL-cholesterol was significantly lower in OLEU male and female groups ($P < 0.01$ in both cases) and in the OLEU + HTX groups ($P < 0.01$ in males and $P < 0.05$ in females). The treatment with hydroxytyrosol significantly decreased LDL-cholesterol levels in males ($P < 0.01$), but increased its levels in females ($P < 0.01$). Furthermore, OLEU + HTX showed a significant decreas in LDL-cholesterol in males ($P < 0.01$), whereas no changes were found in females compared to untreated animals. In the same way, the total cholesterol/HDL-cholesterol ratio significantly decreased in both male and female HTX animals ($P < 0.01$ in both cases), whereas no changes were found in OLEU or OLEU + HTX. Lastly, significant increased levels of triglycerides where found in male and female OLEU animals . This increase also occurs in females, but not in males, after the treatment with the mixture of oleuropein plus hydroxytyrosol ($P < 0.01$). In addition, the HTX female group showed a significant decrease in triglycerides ($P < 0.01$), whereas no changes were found in HTX males compared with the untreated group.

Table 6 shows plasma levels of protein (total protein and albumin) and other enzymes (alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase) analyzed in untreated animals with ENU-induced gliomas and animals with ENU-gliomas treated with oleuropein, hydroxytyrosol and the mixture of oleuropein plus hydroxytyrosol. No significant differences were found in plasma levels of total protein and albumin after the administration of the different experimental treatments. On the contrary, significant increased levels of alanine aminotransferase where found in OLEU or OLEU + HTX males ($P < 0.01$), but not in females. Similarly, significant increased levels of alkaline phosphatase were found in OLEU males ($P < 0.01$), in OLEU females ($p < 0.05$), and in OLEU + HTX females ($P < 0.01$). In contrast, the administration of hydroxytyrosol alone decreased significantly alkaline phosphatase activity in females ($P < 0.01$). Finally, no significant differences in plasma levels of aspartate aminotransferase were found either in males or females of the OLEU, HTX and OLEU + HTX groups compared with the untreated group.

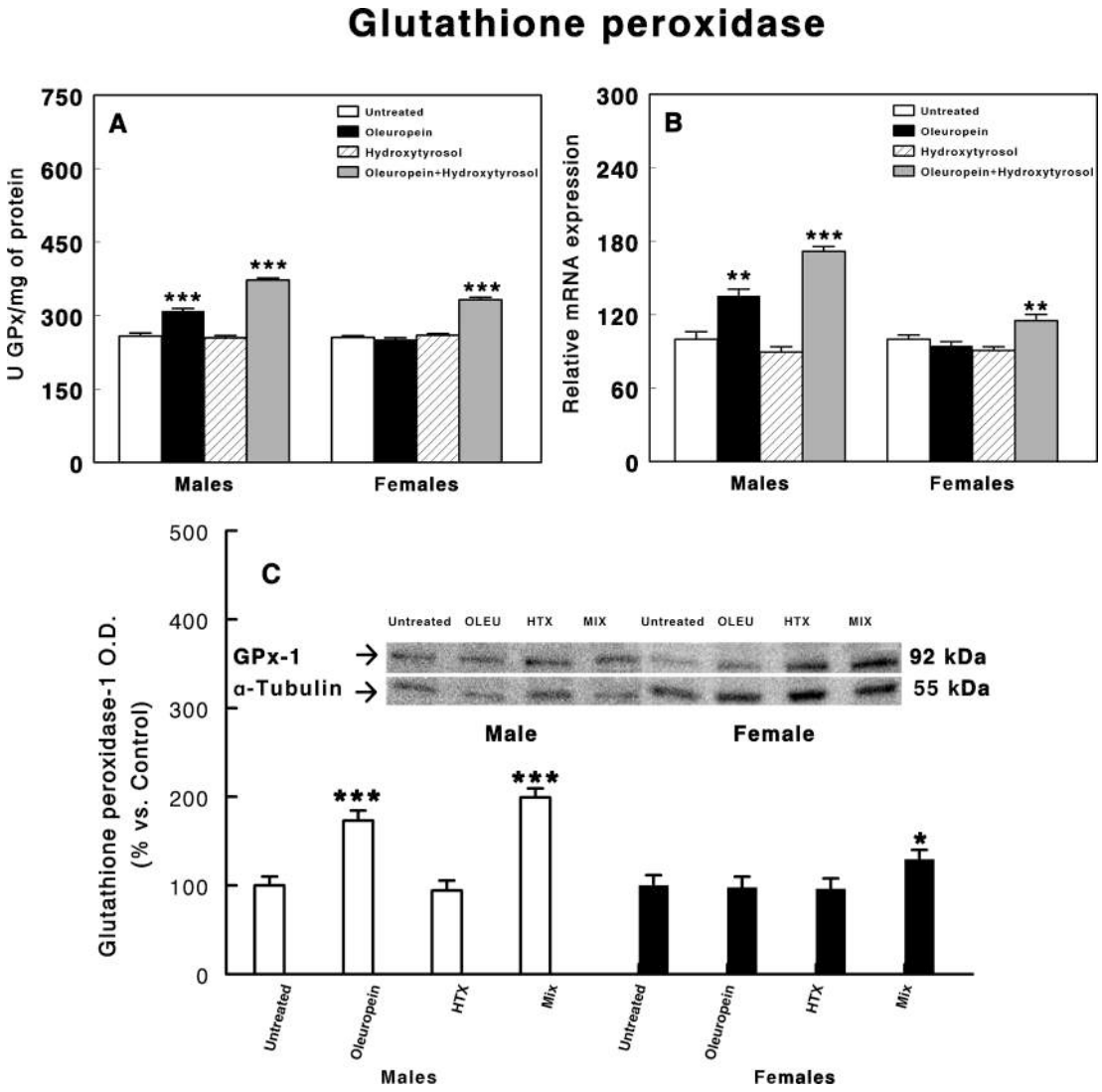

Fig. 9. Glutathione peroxidase (GPx) activity in tissue (A) and plasma (B) of male and female control animals and animals with gliomas induced by transplacental N-ethyl-N-nitrosourea (ENU) exposure. Results are expressed in units per mg of protein. Figure (C) shows a representative western blot showing GPx-1 (92 kDa) protein levels in tissue. Densitometry analyses are presented as a relative ratio of GPx-1 to α-tubulin. Figure (D) shows tissue GPx-1 mRNA expression normalized to ß-actin and GAPDH levels (Mean ± SEM; ***P < 0.001; **P < 0.01; *P < 0.05).

Table 3
Plasma levels of electrolytes in untreated animals with ENU-induced gliomas and animals with ENU-induced gliomas treated with oleuropein, hydroxytyrosol and the mixture of oleuropein plus hydroxytyrosol.

Parameter	Untreated		Oleuropein		Hydroxytyrosol		Oleuropein + Hydroxytyrosol		Significance level
	Male	Female	Male	Female	Male	Female	Male	Female	
Calcium (mg/dL)	10.33 ± 0.27	10.58 ± 0.16	10.63 ± 0.20	10.58 ± 0.16	9.96 ± 0.17	10.35 ± 0.15	10.07 ± 0.08	10.63 ± 0.11	n.s.
Phosphorus (mg/dL)	7.13 ± 0.56	5.05 ± 0.35	7.00 ± 0.99	4.90 ± 0.44	6.06 ± 0.47	5.85 ± 1.03	7.37 ± 0.69	6.18 ± 0.48	n.s
Sodium (mEq/L)	142.00 ± 1.73	141.50 ± 0.34	143.66 ± 0.76	142.83 ± 0.87	144.40 ± 0.40	144.33 ± 0.76	144.28 ± 0.80	142.62 ± 0.37	n.s.
Potassium (mEq/L)	5.30 ± 0.25	4.85 ± 0.15	5.48 ± 0.24	4.91 ± 0.21	5.10 ± 0.22	4.90 ± 0.17	5.97 ± 0.15	5.42 ± 0.23	n.s.
Chloride (mEq/L)	98.00 ± 2.52	100.50 ± 0.89	100.66 ± 0.80	101.00 ± 0.57	100.60 ± 0.67	99.33 ± 0.33	100.42 ± 0.86	100.87 ± 0.29	

Data are expressed in the indicated units as mean ± SEM. n.s., non-significant.

4. Discussion

The CNS is highly sensitive to reactive oxygen species (ROS), and tumorigenesis in nervous tissue has been highly related to oxidative stress. In fact, the transformation of a normal cell into a malignant one involves multiple cellular and molecular events that seem triggered by oxidative damage. Thus, free radicals would promote lipid peroxidation of cellular membranes and oxidation of proteins and DNA, the latter inducing alterations in chromosome structure, genetic mutations and/or disturbances in cell growth (Ramirez-Exposito & Martinez-Martos, 2019; Visioli, Bellomo, & Galli, 1998; Visioli, Poli, & Gall, 2002). Oxidative damage is also promoted by a lower response of the

Table 4
Plasma levels of glucose and non-protein nitrogenous compounds in untreated animals with ENU-induced gliomas and animals with ENU-induced gliomas treated with oleuropein, hydroxytyrosol and the mixture of oleuropein plus hydroxytyrosol.

Parameter	Untreated		Oleuropein		Hydroxytyrosol		Oleuropein + Hydroxytyrosol		Significance level
	Male	Female	Male	Female	Male	Female	Male	Female	
Glucose (mg/dL)	171.33 ± 10.84	193.50 ± 8.79	231.66 ± 40.7[a]	245.50 ± 19.6[a]	203.80 ± 22.72	196.16 ± 11.05	234.57 ± 40.2[a]	234.00 ± 9.29[a]	[a]P < 0.05
Urea (mg/dL)	49.33 ± 5.04	47.50 ± 7.21	48.33 ± 2.61	42.66 ± 6.08	47.80 ± 2.24	65.00 ± 2.73[a]	47.57 ± 8.86	43.37 ± 4.24	[a]P < 0.01
Creatinine (mg/dL)	0.67 ± 0.03	0.58 ± 0.02	0.63 ± 0.05	0.58 ± 0.01	0.66 ± 0.04	0.78 ± 0.06 [a]	0.70 ± 0.04	0.63 ± 0.02	[a]P < 0.01
Uric acid ((mg/dL)	1.43 ± 0.09	1.20 ± 0.06	1.90 ± 0.55	1.48 ± 0.22	1.40 ± 0.10	1.73 ± 0.19 [a]	1.87 ± 0.37	1.26 ± 0.13	

Data are expressed in the indicated units as mean ± SEM; [a]Significance level when compared with untreated ENU animals.

Table 5
Plasma lipid profile in untreated animals with ENU-induced gliomas and animals with ENU-induced gliomas treated with oleuropein, hydroxytyrosol and the mixture of oleuropein plus hydroxytyrosol.

Parameter	Untreated		Oleuropein		Hydroxytyrosol		Oleuropein + Hydroxytyrosol		Significance level
	Male	Female	Male	Female	Male	Female	Male	Female	
Total cholesterol (mg/dL)	71.00 ± 8.74	71.67 ± 4.65	68.00 ± 4.97	61.50 ± 5.94	67.00 ± 7.32	65.66 ± 10.12	58.42 ± 6.69[a]	64.75 ± 8.58	[a]P < 0.01
HDL-cholesterol (mg/dL)	33.67 ± 5.90	35.33 ± 2.79	27.83 ± 2.03 [a]	29.16 ± 3.46 [a]	35.00 ± 3.71	34.00 ± 5.22	32.57 ± 5.49[b]	[a]P < 0.01; [b]P < 0.05	
LDL-cholesterol (mg/dL)	25.33 ± 1.92	15.84 ± 4.00	23.00 ± 4.69	18.15 ± 5.25	20.50 ± 5.25[a]	24.10 ± 4.28[a]	27.28 ± 3.27[a]	17.80 ± 5.25	[a]P < 0.01
Total cholesterol/HDL-c	2.11 ± 1.48	102.50 ± 29.29	2.47 ± 0.09	2.15 ± 0.09	1.91 ± 0.09[a]	1.94 ± 0.09[a]	2.16 ± 0.08	2.03 ± 0.07	[a]P < 0.01
Triglycerides (mg/dL)	60.00 ± 4.62		108.66 ± 20.28[a]	167.66 ± 39.49[a]	79.00 ± 13.25	37.83 ± 3.63[a]	70.57 ± 13.04	135.87 ± 20.77[a]	[a]P < 0.01

Data are expressed in the indicated units as mean ± SEM; [a]Significance level when compared with untreated ENU animals.

Table 6
Plasma levels of proteins and enzymes in untreated animals with ENU-induced gliomas and animals with ENU-induced gliomas treated with oleuropein, hydroxytyrosol and the mixture of oleuropein plus hydroxytyrosol.

Parameter	Untreated		Oleuropein		Hydroxytyrosol		Oleuropein + Hydroxytyrosol		Significance level
	Male	Female	Male	Female	Male	Female	Male	Female	
Total protein (mg/mL)	6.43 ± 0.27	6.77 ± 0.22	6.18 ± 0.20	6.53 ± 0.12	6.84 ± 0.17	7.06 ± 0.27	6.02 ± 0.20	6.52 ± 0.13	n.s.
Albumin (g/dL)	3.30 ± 0.20	3.75 ± 0.14	3.23 ± 0.076	3.66 ± 0.080	3.28 ± 0.080	3.63 ± 0.13	2.97 ± 0.089	3.33 ± 0.07	n.s
Alanine aminotransferase (UI/L)	38.67 ± 5.90	48.50 ± 4.43	65.33 ± 12.32[a]	50.83 ± 6.49	39.20 ± 8.69	46.50 ± 22.59	105.14 ± 29.52[a]	45.37 ± 5.40	[a]P < 0.01
Aspartate aminotransferase (UI/L)	189.6 ± 25.78	185.83 ± 22.62	202.16 ± 56.92	160.66 ± 11.12	147.20 ± 13.20	191.50 ± 63.56	214.00 ± 30.08	201.37 ± 31.83	n.s.
Alkaline phosphatase (UI/L)	54.33 ± 7.69	41.17 ± 4.51	82.50 ± 9.97[a]	51.66 ± 8.50[b]	54.80 ± 4.36	28.16 ± 5.39[a]	58.00 ± 12.06	86.75 ± 19.51[a]	[a]P < 0.01;[b]P < 0.05

Data are expressed in the indicated units as mean ± SEM; [a]Significance level when compared with untreated ENU animals.

antioxidant defense systems, which also allow further tumor development. In fact, the imbalance between free radical generation (mainly ROS) and the efficiency of the antioxidant mechanism maintains the oxidative damage (Acharya, Das, Chandhok, & Saha, 2010; Herrera et al., 2012). However, cancer cells can also be destroyed by ROS, which block key steps in the cell cycle and promote apoptosis (Watson, 2013). In fact, cancer cells largely driven by *RAS* and *MYC* oncogenes are among the most difficult to treat due to their high levels of ROS-destroying antioxidants. In addition, several chemotherapy treatments enhance apoptosis of tumoral cells by lowering antioxidant levels, being their action decreased if antioxidant compounds are administrated concomitantly (Kirshner et al., 2008). Therefore, a delicate equilibrium must occur between pro-oxidant and antioxidant actions to promote either cell proliferation or cell death (Ramirez-Exposito & Martinez-Martos, 2019).

In the last few years, there is an increased body of knowledge about the utility of antioxidants in treating primary brain tumors, and those of natural origin have been extensively analyzed. The established or putative beneficial effects of extra-virgin olive oil on several diseases including cancer, in the context of the Mediterranean diet, have been mainly attributed not only to its high levels in monounsaturated fatty acids (MUFA) (de la Torre, 2008) but also to its content in minor but highly bioactive components (Crespo, Tome-Carneiro, Davalos, & Visioli, 2018; Pignatelli et al., 2006). These minor components are present in about 2% of extra-virgin olive oil weight and include > 230 chemical compounds. Their presence almost exclusively in virgin olive oil, and mainly in extra-virgin olive oil is due to the refining process expunges these compounds. Thus, the final proportion of minor compounds depend on the manufacturing processes of oil. Because these processes vary by oil mill, it is difficult to quantify the dietary intake of these components, although Mediterranean countries tend to consume important amounts of extra-virgin olive oil, which is much richer in phenolic compounds than refined oils. In any case, the main antioxidants in olives are carotenoids and polyphenolic compounds. The primary polyphenols are oleuropein (methyl (4S,5E,6S)-4-[2-[2-(3,4-dihydroxyphenyl)ethoxy]-2-oxoethyl]-5-ethylidene-6-

[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy-4H-pyran-3-carboxylate), hydroxytyrosol (2-(3,4-dihydroxyphenyl)-ethanol) and ß-tocopherol. The oleuropein is hydrolyzed to the catechol hydroxytyrosol and functions as a hydrophilic phenolic antioxidant that is oxidized to its catechol quinone during redox cycling (Martínez-Martos et al., 2014). In the present report, we analyzed the effects of the phenolic compounds oleuropein, hydroxytyrosol and the mixture of both phenolic compounds on brain tumorigenesis, using the ENU experimental model of glioma *in vivo*. ENU-induced tumorigenesis is an experimental brain tumor model extensively described (Druckrey, Ivankovic, & Preussmann, 1966; Ikeno, Shimokawa, Higami, & Ikeda, 1993; Schiffer, Giordana, Pezzotta, Lechner, & Paoletti, 1978) in which rats exposed in utero to a single dose of a mutagen, ENU, preferentially develop brain tumors (Kish et al., 2001; Slikker, Mei, & Chen, 2004; Zook, Simmens, & Jones, 2000). Many authors, have described some benefits of this model, as its high rate of tumor induction (100%) and the appearance of multiple tumors per brain (Mahlke et al., 2011). In fact, after ENU exposure, 100% rats used in our study showed tumors. In addition, other studies have indicated a role for oxidative stress in this type of induced carcinogenesis (Bartsch, Hietanen, & Malaveille, 1989; Hietanen & Bartsch, 1992). Thus, the ENU-induced tumorigenesis model allows to test the oxidative processes/redox status at brain tissue level involved in the development of tumors, and to analyze the anticancer properties of antioxidant compounds. Furthermore, this model will also allow us to study gender differences in the several processes involved.

4.1. Effects of oral administration of the phenolic compounds oleuropein, hydroxytyrosol and the mixture of oleuropein plus hydroxytyrosol

4.1.1. Effects on survival

The analysis of the Kaplan-Meier survival curves demonstrates,: 1. the differential effect of oleuropein, hydroxytyrosol and the mixture of both compounds on the survival of animals with tumors induced by ENU, and 2. gender differences in these effects. Thus, OLEU, HTX or OLEU + HTX male rats showed a significantly higher survival rate than untreated male rats, although no significant differences were found between treatments. On the contrary, in female rats, survival curves demonstrate that the treatment with the mixture of oleuropein plus hydroxytyrosol promoted a significantly higher survival rate than untreated female rats, whereas borderline significance was found in those OLEU and HTX females. However, no gender-related differences were found in the survival rate in untreated animals. Therefore, the differences found can be related to the differential effects of the phenolic compounds on their corresponding targets in male and female animals. Although the assessment of gender differences in brain tumorigenesis and growth has received little attention, other authors have also found significant gender-dependent differences in the study of pathological indicators, such asedema, inflammation, cellularity and microvasculature, suggesting higher cell proliferative rate, inflammation and vasogenic edema and or necrosis in glioma-bearing male rats. Several hemodynamic parameters indicated a major disruption of the blood brain barrier (BBB), confirmed postmortem, in male rats. Metabolomics and energy metabolism activity data are consistent with a higher malignancy and aggressiveness of this cancer model on males (Perez-Carro, Cauli, & Lopez-Larrubia, 2014). In the same way, gender-related differences have been found in glioma patients indicating that genetic alterations influence glioma biology in synergy with essential aspects of sex determination (Sui et al., 2018). Therefore, the gender differences found in the present study could be related to the several different mechanisms affected in males and females. In fact, as explained below, several other gender differences have been found between our experimental groups. In addition, total phenolic components intake has been relatively higher in female animals than in males (table 1), this must be also taken into account to rule out a possible effect in females of a poor intake of these polyphenols. Furthermore, several clinical and animal studies have provided evidence that phenolic compounds are absorbed, metabolized and distributed though the blood stream to almost all parts of the body, even across the BBB, to exert their biological effects in a dose-dependent manner (Carrera-Gonzalez et al., 2013; Visioli et al., 2002).

4.1.2. Effects on carcinogenesis parameters

Our results have also shown gender differences in the effects of oleuropein, hydroxytyrosol and the mixture of oleuropein plus hydroxytyrosol in the number and volume of tumors. Thus, none of the phenolic compounds alone or in combination modified the number of tumors in male animals, but decreased it in female animals. In the same way, no changes in tumor volume were found in males, with the exception of OLEU, whereas a decrease in tumor volume was found in female animals, with the lowest value in OLEU + HTX. Regarding the effects found for oleuropein in male animals, where an important increase in tumor volume was found, consistent results were also found in a previous study using the C6-glioma implanted in the subcutaneous region, where oleuropein was administered subcutaneously (Martínez-Martos et al., 2014). The mechanisms by which oleuropein stimulates tumor growth remain unresolved. Oleuropein has been described as a potent scavenger of oxygen free radicals (Manna et al., 2002) and nitrogen free radical species (de la Puerta, Martinez Dominguez, Ruiz-Gutierrez, Flavill, & Hoult, 2001). In addition, oleuropein plays an important role in the prevention of DNA damage by inhibiting mutagenesis and carcinogenesis (Valko, Izakovic, Mazur, Rhodes, & Telser, 2004). Furthermore, Hamdi and Castellon (Hamdi & Castellon, 2005) have demonstrated that the antitumor effect of oleuropein may be exerted by the disruption of the actin filaments in tumor cells. Moreover, our own results suggest a beneficial effect of oleuropein intake on survival despite the increase in tumor volume promoted by oleuropein intake. We had proposed 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 the C6-glioma implanted in the subcutaneous region); (2) oleuropein has structural similarities with a growth factor or other type of stimulator, acting as an agonist and thus producing an increase in tumor growth by mechanisms not related to its antioxidant properties; (3) stimulation of tumor growth due to its potent antioxidant properties that inhibits the killing of cancer cells via ROS. In this sense, it has been described that tumor cells with high levels of ROS-destroying antioxidants are among the most difficult to treat. Similarly, when antioxidants are administered together with some antineoplastic drugs, with have an antioxidant mechanism of action, a decrease in the anticancer effect has been found (Kirshner et al., 2008; Watson, 2013). Therefore, further research is necessary to solve these issues.

Regarding the inhibitory effects of phenolic compounds in female animals in tumor number and tumor volume, a growing number of studies have demonstrated that both oleuropein and mainly hydroxytyrosol have antiproliferative effects against several tumor cell lines acting at various levels. For example, inactivating the AKT and nuclear-factor-kappa B (NFκB) pathways (Zhao et al., 2014). *In vivo*, these compounds seem to induce cell cycle arrest and apoptosis (Li et al., 2014). The female hormonal status is highly related to these effects. Our results also show that the *in vivo* administration of a mixture of both compounds is the most efficient against tumor development and growth, but also in survival rate. This data are inconsistent with previous results found in the subcutaneous C6-glioma model, although those experiments were performed in male rats only and with a different amount, duration and route of administration of the compounds (Martínez-Martos et al., 2014). It is well known that the *in vivo* effects of phenolic compounds are highly dependent on the dose used (Kotronoulas et al., 2013). However, we have to consider that the aromatic ring present in oleuropein and hydroxytyrosol is a feature common to estradiol (E_2). This common structure might suggest a putative mechanism of action of these polyphenols correlated to their capacity to compete with estrogens for estrogen receptor (ER) binding sites. In highly hormonal-dependent tumors, such as those of the breast, the growth is stimulated by E_2 and,

therefore, polyphenols of natural origin as oleuropein and hydroxytyrosol could interfere with tumor cell proliferation. In this context, it has been described in human MCF-7 breast cancer cells that the presence of oleuropein and hydroxytyrosol interfere with E_2-dependent MCF-7 cells proliferation in a dose-dependent manner. However, concentrations higher than 100 μM of the two compounds show cytotoxic effects (Sirianni et al., 2010). Thus, the two molecules possess antiestrogen activity. The data point out to an inhibition of E_2-dependent rapid signaling pathways, which are able to induce, within a few minutes, molecules involved in proliferation events, such as mitogen-activated protein kinase (MAPK). These studies are consistent with recent results obtained by Elamin et al. (Elamin et al., 2013). These authors showed that oleuropein is cytotoxic and induces apoptosis in breast cancer cells, being this effect more pronounced on ER-negative breast cancer cells than on ER-positive cells. Similarly, oleuropein is a potent inhibitor of cell proliferation by delaying the cell cycle at S phase and down-regulated two major breast cancer-related onco-proteins, NF-κß and cyclin D1. Although with differences in nervous tissue, the interaction of oleuropein and/or hydroxytyrosol with different intracellular signaling routes in which ER are involved could explain the differential effects found in the present study between male and female animals, in addition or concomitantly with their antioxidant effects and in relation to the amount of polyphenols intake.

4.1.3. Effects on oxidative stress parameters

4.1.3.1. Metabolism behavior and protein oxidation. We had previously described an increased mitochondrial activity in brain tissue of both male and female animals with ENU-induced tumors compared to healthy controls, but no gender differences were found in this regard (Ramirez-Exposito et al., 2019). These results indicated an increased oxidative activity in the brain of male and female animals with ENU-induced tumors. In the present study, we found that oral administration of phenolic compounds decreases mitochondrial activity to varying extents depending on the compounds, in both male and female animals with ENU-induced tumors, also no gender differences were found and producing levels of mitochondrial activity comparable to those observed in healthy animals. The decrease in the mitochondrial activity could also favor a lower production of free radicals. Of particular interest is that oleuropein promotes the lowest mitochondrial activity in male rats, which are those with the highest tumor volume. Similarly, in female animals a synergistic effect of oleuropein and hydroxytyrosol was found when administered together, no synergy was found in male animals. Therefore, an imbalance between oxidants and antioxidants could also explain this situation in these particular conditions. In the same way, the mixture of oleuropein plus hydroxytyrosol administration to female animals showed the highest decrease in mitochondrial activity, which is associated with the lowest tumor number and tumor volume in female animals, but also with the highest survival rate in these animals. The particular hormonal status of female animals seems to be related with the putative effects of phenolic compounds and with effects that go beyond those limited exclusively to their antioxidant effects.

Therefore, we had also reported the level of protein oxidation (measured as carbonyl and diene conjugate group content) in animals with ENU-induced tumors. Significantly increased concentrations of carbonyl groups were found in brain tissue of both male and female rats, although no gender differences were found in these concentrations. After phenolic compounds administration, we found few gender differences in protein oxidation levels. The administration of both phenolic compounds alone (in males and females) or in combination (only in males) increased even more the levels of protein oxidation in both male and female animals. Only in females, the administration of both polyphenols prevented the increase in protein oxidation. These results suggest that the oxidative damage of proteins in the carcinogenesis process is not prevented by the administration of these compounds, with the exception of their effects when administered to female animals in combination. This phenomenon could be explained as a consequence of the longtime of evolution of the tumor process in this animal model, but also considering the putative effects not associated with the antioxidant functions mentioned above. However, these results are consistent with those obtained in the C6-glioma implanted in the subcutaneous region previously reported (Martínez-Martos et al., 2014).

4.1.3.2. Effects on non-enzyme antioxidant defense systems. The magnitude of the oxidative damage depends not only on free radicals, but also on the efficiency of the antioxidant mechanisms. As previously reported, the balance between oxidant and antioxidant systems plays a key role in carcinogenesis (Amstad, Moret, & Cerutti, 1994; Amstad et al., 1991; Ramirez-Exposito & Martinez-Martos, 2019). Cellular antioxidants and free-radical scavengers protect the cell against toxic levels of oxygen radicals. One of them is GSH, an important non-protein thiol. In humans, a significant depletion in GSH levels has been found in astrocytoma, meningioma, metastatic and other types of brain tumor compared with their peritumoral tissues. Zengin et al. (Zengin, Atukeren, Kokoglu, Gumustas, & Zengin, 2009) reported a significant decrease in GSH in high-grade tumors compared with low-grade tumors. GSH depletion is probably related to the enhanced pro-oxidant milieu, and correlates with the increased level of lipid peroxides found. We had previously described a significant decrease in GSH at brain tissue level in male and female rats with ENU-induced tumors compared with their control groups (Ramirez-Exposito et al., 2019). It is well documented that tumor cells have lower levels of GSH than healthy cells (Sun, St Clair, Xu, Crooks, & St Clair, 2010) and previous studies have described a significant decrease in GSH in astrocytomas, meningiomas and other types of brain tumors (Shi et al., 2015; Zengin et al., 2009). Similarly, we found a decrease in GSH content in animals with subcutaneously implanted C6 glioma (Illan-Cabeza et al., 2013). All these results are consistent with those described by Navarro et al. (Navarro et al., 1999) who have shown that those changes in GSH status and the antioxidant system in blood and cancer cells are associated with tumor growth *in vivo*. Probably, the decrease in GSH is related to an increased pro-oxidant milieu correlating with the increase in lipid peroxidation and protein oxidation found.

Regarding the treatments with oleuropein, hydroxytyrosol or the mixture of both phenolic compounds, we demonstrate in the present study their important effects on this non-enzyme antioxidant defense system and the existence to a certain degree of gender differences. Thus, male animals treated with oleuropein, hydroxytyrosol or the mixture of both compounds showed increased levels of GSH compared to untreated animals, and these compounds restored the levels of GSH found in healthy animals (Ramirez-Exposito et al., 2019). In females, only hydroxytyrosol or the mixture of both phenolic compounds induce an increase in GSH levels to restore the values found in healthy animals, whereas oleuropein has no such effect. It also indicates a main effect of hydroxytyrosol but not of oleuropein on female animals. Once again, this data supports the putative alternative or concomitant mechanism of action of these compounds other than the antioxidant one. In fact, in the C6-glioma implanted in the subcutaneous region model, none of the phenolic compounds, alone or in combination, was able to restore GSH levels to those observed in control healthy animals, demonstrating the importance of the concentration of compounds used and, probably, the route of administration.

4.1.3.3. Effects on enzyme antioxidant defense systems. Redox status is also controlled by multiple antioxidant enzymes such as SOD, CAT and GPx. These enzymes normally act to prevent or decrease the tissue damage caused by free radicals to macromolecules, such as lipids, proteins and nucleic acids. 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 H_2O_2 and other organic peroxides (Gilca et al., 2009). During prolonged

or excessive oxidative stress, changes in SOD, CAT and GPx activities may occur, regardless they are enough or not to prevent the oxidative damage, these changes play a significant role in the pathogenesis of several diseases, including cancer.

4.1.3.3.1. Superoxide dismutase. We had previously described a significant decrease in SOD activity in brain tissue of both male and female animals with ENU-induced tumors compared to healthy controls (Ramirez-Exposito et al., 2019). These results corroborate that SOD has important roles in cancer (Che, Wang, Li, Wang, & Zheng, 2016). Several authors have described lower levels of SOD in tumor tissue compared with normal tissue (Aggarwal, Subberwal, Kumar, & Sharma, 2006; Popov, Gadjeva, Valkanov, Popova, & Tolekova, 2003). Furthermore, it has been described several forms of SOD in eukaryotic cells (different in their metal binding characteristics and intracellular distribution) which have different roles in cancer due to their different cellular localizations, tissue distribution and biological functions (Che et al., 2016). In this way, SOD-1 is located mainly in the cytoplasm (Crapo, Oury, Rabouille, Slot, & Chang, 1992), SOD-2 in the mitochondrial matrix (Kienhofer et al., 2009; MacMillan-Crow & Thompson, 1999) and SOD-3 is the secreted form of SOD with expression restricted mainly to the lung, kidney and adipose tissue (Che et al., 2016; Lund, Chu, Miller, & Heistad, 2009). An emerging role of SOD-1 form in cancer biology has been actually considered (Papa, Hahn, Marsh, Evans, & Germain, 2014; Papa, Manfredi, & Germain, 2014; Ramirez-Exposito et al., 2019). Whereas the contribution of SOD-1 in the cytoplasm is well known, many authors suggest that the inter-membrane space fraction of SOD-1 may also play a role supporting the viability of cancer cells and that SOD-1 may potentially become a therapeutic target for cancers (Che et al., 2016; Papa, Hahn, et al., 2014; Papa, Manfredi, et al., 2014). Huang et al. (Huang, Feng, Oldham, Keating, & Plunkett, 2000) identified SOD-1 as a target of an anti-cancer drug in leukemia and supported the hypothesis that SOD-1 may be necessary for the adaptation of cancer cells to elevated oxidative stress (Papa, Hahn, et al., 2014; Papa, Manfredi, et al., 2014), although the mechanism by which the inhibition of SOD-1 acts on cancer cells is unclear. The malfunction of the antioxidant machinery of the mitochondria seems to play a critical role because it promotes the production of free radicals in mitochondrial matrix. Thus, the overexpression of SOD-1 in the cytoplasm, the intermembrane space, and the nucleus probably maintains low ROS levels in these cellular compartments.

In our previous study (Ramirez-Exposito et al., 2019), we also determined the levels of SOD-1 protein and mRNA expression levels in animals with ENU-induced tumors and healthy controls. No changes were found in these parameters. However, lower SOD activity was found in brain samples from rats with ENU-induced tumors. Taken together, these results may indicate that although there are no differences in SOD-1 production and expression, a possible loss of enzymatic function may occur as a consequence of tumor process and therefore, its antioxidant effectiveness would be compromised as it is reflected by the increased oxidative activity found in rats with ENU-induced tumors.

However, as demonstrated in the present study, both male and female animals with ENU-induced tumors treated with oleuropein, hydroxytyrosol and the mixture of both phenolic compounds showed higher levels of SOD than untreated animals. Furthermore, the levels of SOD activity found in male ENU animals were independent of the type of treatment. On the contrary, in female animals, the highest levels were found in OLEU and HTX groups, and the lowest in OLEU + HTX female group, also indicating a putative antagonistic effect among both compounds, or a synergistic response due to the high concentration reached as the sum of both polyphenols. In both male and female animals with ENU-induced tumors, the treatment with phenolic compounds return SOD activity levels to those found in healthy control animals (Ramirez-Exposito et al., 2019). However, Del Maestro et al. (Del Maestro, McDonald, & Anderson, 1983) reported that human glioma cells generally had relatively higher SOD activity than other tumor types, which seems inconsistent with the general observation of low SOD activity in tumor tissue. This exception can probably be explained by the special situations of the brain, which is a high oxygen-consuming organ. There is high production of superoxides during normal aerobic metabolism in the brain cells. Thus, relatively high levels of SOD and other antioxidant enzymes are required to remove high levels of free radicals to protect brain tissues against damage. Although the levels of SOD are important in protecting against oxidative damage, a balance of antioxidant enzymes is probably more important than their levels, which may influence intracellular oxidative states. Most studies have shown a significant decrease in SOD activity in several brain tumor types, such as glioma, meningioma and metastatic tumors. Levchenko and Demchuk (Levchenko & Demchuk, 1991) reported a decrease in SOD activity with an increase in malondialdehyde (MDA) levels in meningioma patients, both in blood and tumor tissues. The increase in SOD activity levels found after the treatment with the phenolic compounds, therefore, should have a protective role against tumor growth, although gender differences must exist in the mechanisms of action of these compounds. In fact, we have found an important decrease in mRNA and SOD-1 protein expression with the treatments in male animals, whereas no changes have been found in female ones.

4.1.3.3.2. Catalase. CAT is an antioxidant enzyme responsible for the conversion of H_2O_2 to H_2O. Changes in CAT activity have been described in different brain tumors. Although many authors have described significant increases in CAT activity in both glial and meningioma tumors (Popov et al., 2003; Yilmaz et al., 2006), we have previously described lower levels of CAT activity in brain tissue samples of animal with ENU-induced tumors. Furthermore, no gender differences were found. These results suggest that tumor induction inhibits the role of CAT in converting H_2O_2 to H_2O and, therefore, its antioxidant effect would be compromised. However, these results could also indicate that due to the lower SOD activity observed, H_2O_2 production could be decreased and therefore a lower CAT activity may be necessary to catalyze the reaction from H_2O_2 to H_2O and O_2. Although, the analysis of protein and mRNA expression levels showed significant increases in both male and female animals with ENU-induced tumors, also indicating that even though the enzyme production is increased, its effectiveness is diminished as a consequence of tumor process; but could also indicate that as a result of the loss of effectiveness, the amount of enzyme is increased as a compensatory mechanism.

In the same way, we found no differences in the levels of CAT activity in OLEU, HTX or OLEU + HTX groups, males or females, compared with untreated animals, indicating that CAT activity remains under the values found in healthy animals. However, oleuropein and the mixture of oleuropein plus hydroxytyrosol increased mRNA and protein expression levels in male animals (supporting a main effect of oleuropein but not of hydroxytyrosol), whereas all treatments decreased mRNA and protein expression levels in female animals. The effects found here clearly indicate again the interaction of other gender-related mechanisms and the differential effects of oleuropein and hydroxytyrosol on their targets in addition to their antioxidant effects, as above mentioned.

4.1.3.3.3. Glutathione peroxidase. GPx is another important enzyme responsible for the removal of hydrogen peroxide. Although many authors have described lower levels of GPx in various brain tumors (Aggarwal et al., 2006; Gauchez, Riondel, Jacrot, Calop, & Favier, 1995; Rao, Rao, Raja, Rao, & Rao, 2000), and we have also described lower GPx activity in the C6 glioma model implanted in the subcutaneous region (Martínez-Martos et al., 2014), we had found a significant increase in GPx activity in brain tissue samples of both male and female animals with ENU-induced tumors (Ramirez-Exposito et al., 2019). In the same way, higher GPx-1 mRNA and protein expression levels were found without gender differences. This increase in GPx activity could compensate the effect of lower CAT activity in the removal of H_2O_2. In the present study, we show even higher GPx activity in OLEU and OLEU + HTX male animals, supporting again a main role for oleuropein but not hydroxytyrosol, and in OLEU + HTX female animals, which supports a differential effect and/or a dependence of the concentration of

polyphenols. Similarly, the same pattern was found in mRNA and protein expression. In any case, the gender differences found regarding the treatment with both polyphenols were evident for GPx.

Although, several forms of GPx have been cloned (Chu, 1994; Esworthy, Doan, Doroshow, & Chu, 1994; Hall, Williams, Perry, Frayne, & Jury, 1998) to study GPx mRNA expression and protein levels, we have determined the levels of the GPx-1 isoform because is it a crucial antioxidant enzyme, which catalyzes the reduction of H_2O_2 and a large variety of hydroperoxides (such as DNA hydroperoxides and lipid hydroperoxides) into water and alcohols, respectively (Freeman & Crapo, 1982; Meister, 1994), to protect cells under oxidative stress. In fact, it has been reported that GPx-1 protein and mRNA expression levels are up-regulated in carcinoma tissues to protect against DNA damage (Baliga et al., 2008), which are also associated with increased risk of cancer (Lei, Cheng, & McClung, 2007; Yagublu et al., 2011; Zhuo & Diamond, 2009).

Although CAT and GPx both act removing H_2O_2, their behavior in animals with ENU-induced tumors is different as well as their response to the administration of oleuropein, hydroxytyrosol or the combination, showing OLEU + HTX group gender differences. Antioxidant proteins with similar enzymatic activities may have different effects after modulation due to the different localizations within cells. Both GPx and CAT remove H_2O_2, but their contribution varies depending on the amount and the localization of H_2O_2 production (Chandra, Samali, & Orrenius, 2000).

The overall effects of the antioxidant system depend on the intracellular balance between the several antioxidant enzymes rather than a single component (Amstad et al., 1994; Amstad et al., 1991). The imbalance in the adequate expression and/or activity of antioxidant enzymes can promote the generation of oxidative stress (Amstad et al., 1994; Amstad et al., 1991). This evidence imply that the balance of SOD, CAT and GPx is more important than the level of each one to prevent or to induce the tumor growth or its promotion. Therefore, in the present study, the ability to scavenge oxygen free radicals seems to be impaired in the animals with tumors because of the reduced levels of antioxidants, which may predispose them to cancer progression. The treatment with the different polyphenols, alone or in combination, modifies this balance to different extent and in a gender-dependent manner to promote some beneficial anticancer effects (Fig. 10). The existence of gender differences also imply that the different hormonal status for males and females, including gender-specific intracellular signaling routes, clearly influences the process of tumorigenesis and the response to possible treatments, or at least those treatments aimed at manipulating the redox status.

4.1.4. *Effect on other physiological functions*

Finally, we have also measured several blood chemistry parameters (electrolytes, biomarkers of renal and hepatic functions and lipid profile) to analyze the potential adverse effects of the long treatment with oleuropein, hydroxytyrosol or the mixture of oleuropein plus hydroxytyrosol in male and female animals with ENU-induced tumors in several physiological processes. Previously, we had described that slight modifications occurring when we compared between animals with ENU-induced tumors and healthy controls, but all of them were considered within the normal range. Thus, ENU administration itself does not seem to cause important changes in other physiological functions. In contrast, several alterations in blood chemistry have been found as consequence of the treatments, some of them with gender differences.

One of the effects of all the treatments has been the increase in the glycemia. Nutrient concentrations in tumors are different to those in normal tissues, and cancer cells *in vivo* may have metabolic dependencies that are not in common with normal cells. In particular, tumor glucose concentrations are frequently 3- to 10-fold lower than those in non-transformed tissues, probably as a result of the high rate of glucose consumption by cancer cells and the poor tumor vasculature. Here, we have found increased glucose levels in animals treated with oleuropein, hydroxytyrosol and with the mixture of oleuropein plus hydroxytyrosol. To our knowledge, no information is available about the effects of these polyphenols on circulating glucose levels. Further research is necessary to elucidate this issue.

Another finding it that, an impaired renal function was found in female rats treated with hydroxytyrosol, with higher plasma levels of urea, creatinine and uric acid. However, all values were within the normal limits. In this regard, 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 contain evidence contrary to the proposed antioxidant effect of serum uric acid against cancer, and instead indicate high levels to be independently associated with outcome, possibly reflecting a worse prognosis (Strasak et al., 2007). In fact, it has been shown that uric acid levels in astrocytoma patients were significantly increased in neoplastic tissue compared with non-neoplastic tissue, and levels were even higher in necrotic tissue (Landolt, Langemann, Probst, & Gratzl, 1994). Therefore, our results are consistent with those reported by several authors (Pietila et al., 2009; Ueda, Wakisaka, Kinoshita, & Adachi, 1984).

Regarding the serum lipid profile, we have also found changes in total cholesterol, HDL-cholesterol, LDL-cholesterol and triglycerides in male and female animals with ENU-induced tumors treated with oleuropein, hydroxytyrosol and/or the mixture of both phenolic compounds, with some gender differences. Altered lipid profile patterns are associated with malignancies because lipids play a pivotal role in the maintenance of cell integrity. Patients with cancer usually have modified levels of cholesterol and other lipid constituents, and this is thought to be due to increased usage of lipids by tumor cells for new membrane biogenesis (Ghosh, Jayaram, Patil, & Malik, 2011). To our knowledge, no information is available to date about the lipid profile of patients with brain tumors, although cholesterol-lowering drugs are effective in inhibiting cancer cell growth proliferation (Fagherazzi, Fabre, Boutron-Ruault, & Clavel-Chapelon, 2010). Therefore, further research is necessary to better understand the underlying mechanisms of the regulation of blood lipids in cancer and how the oral administration of phenolic compounds could be related to lipid profile, considering the several pharmacological properties described for both compounds in lipid metabolism (Fki et al., 2020).

Alanine aminotransferase is also increased in OLEU and OLEU + HTX male animals with ENU-tumors, indicating that this increase is mainly due to oleuropein administration. Several authors have described increases in transaminases with cancer and cancer treatments. Transaminases, such as aspartate aminotransferase and alanine aminotransferase, are intracellular enzymes that exist in only a small amount 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 the result of autolytic breakdown or cellular necrosis. Furthermore, the increase in the activities of these enzymes in the serum may be a result of the impairment of the function of tissues with subsequent liberation of the enzymes into the circulation from the damaged tissue.

Similar results have been obtained with alkaline phosphatase, which was increased in OLEU male animals and in OLEU + HTX females, although hydroxytyrosol alone decreased this activity. Alkaline phosphatase partly reflects osteoblastic activity, which is likely to be more significant in patients with larger tumors or aggressive bony metastatic disease (Sonpavde et al., 2012). Alkaline phosphatase is a relatively nonspecific biomarker and can be originated from sources other than bone (*e.g.* the liver). In patients with bone metastases and elevated baseline alkaline phosphatase, bone is the dominant source of the enzyme, and patients are likely to have liver metastases causing alkaline phosphatase elevations. In any case, we did not analyze the putative presence of metastases in other organs/tissues.

Finally, we must indicate that differences in the metabolism of

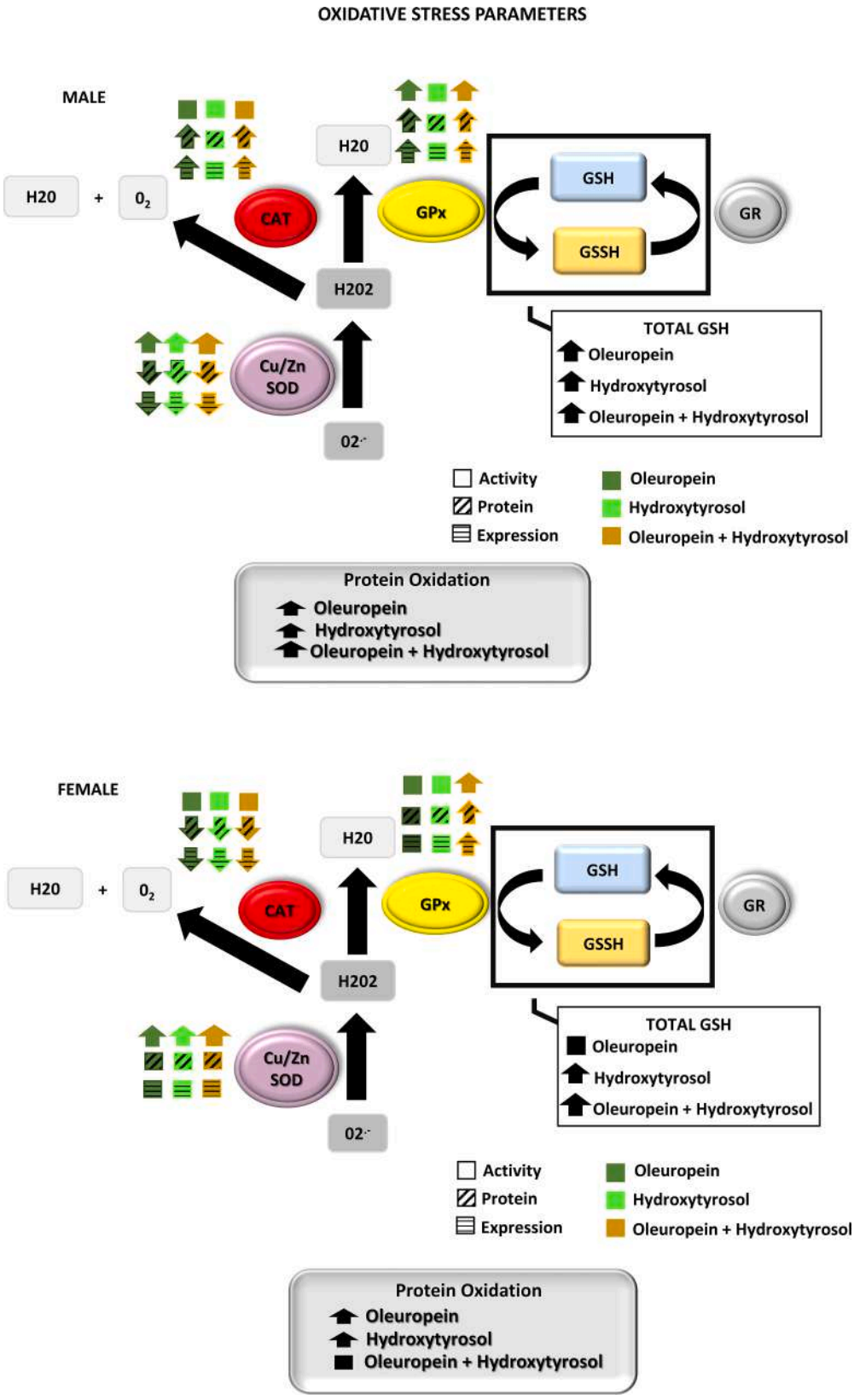

Fig. 10. Summary of the alterations promoted in male and female animals with ENU-induced glioma in oxidative stress parameters and the enzymatic and non-enzymatic antioxidant defense systems, as well as the effects of the treatments with oleuropein, hydroxytyrosol and the mixture of both polyphenols in ENU-induced glioma animal model.

hydroxytyrosol between human and rats have already been described (Visioli et al., 2003). Therefore, this limitation of our study must also be considered.

5. Conclusions

We conclude that the treatment with oleuropein, hydroxytyrosol and/or the mixture of both phenolic compounds promotes a limited beneficial effect as anticancer compounds in our ENU-induced animal model of brain tumor. These effects occur via redox control mechanisms involving endogenous enzymatic and non-enzymatic antioxidant defense systems, and are highly dependent on the gender of the animals. Therefore, the existence of gender differences in brain tumor-related processes -such as the redox status management showed in the present study- indicates that brain cancer research need to consider gender differences in preclinical studies, screening and prevention programs, and also in the therapeutic approaches.

CRediT authorship contribution statement

M.J. Ramírez-Expósito: Conceptualization, Methodology, Validation, Investigation, Resources, Writing - original draft, Supervision, Funding acquisition. **M.P. Carrera-González:** Investigation, Resources, Visualization, Writing - original draft. **M.D. Mayas:** Investigation, Resources, Visualization. **J.M. Martínez-Martos:** Conceptualization, Methodology, Validation, Investigation, Formal analysis, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

None.

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