RESEARCH ARTICLE

Gender Differences in the Antioxidant Response to Oxidative Stress in Experimental Brain Tumors

María Jesús Ramírez-Expósito[a], María Dolores Mayas[a], María Pilar Carrera-González[b] and José Manuel Martínez-Martos[a,*]

[a]*Department of Health Sciences, Faculty of Health Sciences, University of Jaén, Jaén, Spain;* [b]*Department of Nursing, Faculty of Medicine and Nursing, University of Córdoba, Córdoba, Spain*

Abstract: ***Background*:** Brain tumorigenesis is related to oxidative stress and a decreased response of antioxidant defense systems. As it is well known that gender differences exist in the incidence and survival rates of brain tumors, it is important to recognize and understand the ways in which their biology can differ.

***Objective*:** To analyze gender differences in redox status in animals with chemically-induced brain tumors.

***Methods*:** Oxidative stress parameters, non-enzyme and enzyme antioxidant defense systems are assayed in animals with brain tumors induced by transplacental N-ethyl-N-nitrosourea (ENU) administration. Both tissue and plasma were analyzed to know if key changes in redox imbalance involved in brain tumor development were reflected systemically and could be used as biomarkers of the disease.

***Results*:** Several oxidative stress parameters were modified in tumor tissue of male and female animals, changes that were not reflected at plasma level. Regarding antioxidant defense system, only glutathione (GSH) levels were decreased in both brain tumor tissue and plasma. Superoxide dismutase (SOD) and catalase (CAT) activities were decreased in brain tumor tissue of male and female animals, but plasma levels were only altered in male animals. However, different protein and mRNA expression patterns were found for both enzymes. On the contrary, glutathione peroxidase (GPx) activity showed increased levels in brain tumor tissue without gender differences, being protein and gene expression also increased in both males and female animals. However, these changes in GPx were not reflected at plasma level.

***Conclusion*:** We conclude that brain tumorigenesis was related to oxidative stress and changes in brain enzyme and non-enzyme antioxidant defense systems with gender differences, whereas plasma did not reflect the main redox changes that occur at the brain level.

ARTICLE HISTORY

Received: April 29, 2018
Revised: August 13, 2018
Accepted: September 15, 2018

DOI:
10.2174/1568009618666181018162549

Keywords: Lipid peroxidation, Total antioxidant capacity, Glutathione, Superoxide dismutase, Catalase, Glutathione peroxidase.

1. INTRODUCTION

Brain tumors are central nervous system (CNS) neoplasias difficult to treat because of their infiltrative nature and their resistance to chemotherapy and other treatments [1-3]. As in other types of cancer, the development of these tumors is the result of multistage, multistep processes that involve several multiple molecular and cellular changes that promote the transformation of normal cells into malignant ones, being oxidative stress frequently associated with triggering the process [4, 5]. In fact, the imbalance between free radical production and the efficiency of the antioxidant defense systems promotes different reactions which induce oxidative damage and contribute to the development of carcinogenesis [3, 6, 7]. Also, initiation, promotion and progression stages are controlled by polymorphic genes which can be altered by free radicals, leading to their dysfunctions [8, 9]. Under these non-physiological conditions, free radicals are formed in excess from both endogenous sources (mitochondria, peroxisomes, inflammatory cell activation or neurotransmitters oxidation) and exogenous sources (environmental and chemical agents, drugs or irradiation) [3, 10]. These large amounts of free radicals and specially those derived from oxygen (reactive oxygen species –ROS- such as superoxide anion, hydrogen peroxide and hydroxyl groups) lead to lipid peroxidation of cellular membranes, oxidation of proteins and DNA and promoting changes in chromosome structure and genetic mutations that alter the modulation of cell growth [3, 11, 12]. In the same way, changes in non-enzyme (reduced glutathione, GSH) and enzyme antioxidant systems

*Address correspondence to this author at the Department of Health Sciences, Faculty of Health Sciences, Jaén University, Jaén, Spain; Tel: ++0-34-953-212600; Fax: +0-34-953-212943; E-mail: jmmartos@ujaen.es

1568-0096/19 $58.00+.00

(superoxide dismutase, SOD, catalase, CAT, and glutathione peroxidase, GPx) have also been reported [3]. These enzyme and non-enzyme antioxidant defense systems are ready to avoid or decrease brain damages promoted by the excess of free radicals. Furthermore, ROS play an important role in tumor development because the CNS is very sensitive to free-radical damage [4, 5, 13, 14]. However, cancer cells may also be eliminated by ROS, which could block key processes in the cell cycle and induce apoptosis through not completely known mechanisms [14, 15].

Transplacental N-ethyl-N-nitrosourea (ENU)-induced tumors of the nervous system in rodents have been widely used as an experimental brain tumor model [16-20]. This model shows a high rate of tumor induction (normally of 100%) and the appearance of multiple tumors per brain. Also, the profile and time course of tumor progression in this experimental model have been extensively documented [20-22]. However, in this model, potential redox changes and the response of antioxidant systems in the incidence and tumor growth have been little studied, although previous studies have suggested a putative role for oxidative stress in nitrosamine-induced carcinogenesis [20, 23, 24].

In the present work, we analyze, in both tissue and plasma, oxidative stress biomarkers, non-enzyme and enzyme antioxidant defense systems in male and female rats with ENU-induced brain tumors to analyze the participation of redox system components in the brain tumorigenesis. Also, we analyze if plasma levels of the different parameters are related to those on tumor tissue, to establish if they can be used as putative biomarkers of nervous system tumors, due to its greater accessibility. Finally, we analyze several other biochemical parameters in plasma to determine if the administration of the carcinogen affects other physiological functions.

2. MATERIALS AND METHOD

2.1. Animals and Treatments

Female (n=10) and male (n=5) 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 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). Protocols were approved by the Bioethical Committee of the University of Jaen (Reference number CVI09-4957M). 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, a group of pregnant rats was injected i.v. with a single dose of ENU, 75 mg/kg body weight dissolved in saline solution, whereas another group was injected with the vehicle alone. Female and male offspring from the vehicle and ENU-treated rats were used in these experiments. A total of 58 offspring were obtained. The offspring were naturally delivered and weaned at 22 days old. At this time, males and females were housed separated and kept under weekly observation for any sign of neurological or health problems. All rats were weighed once a week for the duration of the study. Rats were sacrificed after 30 weeks.

2.2. Magnetic Resonance Imaging (MRI) Analysis

Magnetic resonance images were acquired at 9.4 Tesla (Bruker Biospec, Ettlingen, Germany) at the Fundación IDI-CHUS (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 on an MR 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 (TR/TE = 2000/80 ms, matrix = 256 x 192 or 128 x 128, two accumulations, Field of View = 30 x 30 mm2). To aid in tumor visualization, animals were injected i.p. with 1.5 ml/kg b.w. 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 (2ml/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 an anticoagulant, allowed to clot, and then centrifuged for 10 minutes at 3000xg to obtain the plasma, which was frozen and stored at -80°C until use. Samples of tumors were quickly removed and prepared for histopathological examination or frozen at –80°C until use. Equivalent brain regions were removed from control animals, according to Paxinos and Watson [25].

2.4. Oxidative Stress Parameters Assays

2.4.1. Bioenergetic Behavior Assay

Mitochondrial activity of brain tissue was assayed by using tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide). This compound 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 [26]. The resulting values were expressed in optical density units/mg of protein.

2.4.2. Lipid Peroxidation Assay

Lipid peroxidation was measured by analyzing the amount of thiobarbituric acid reactive substances (TBARS). Briefly, samples 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. Samples were cooled to room temperature and the absorbance at 532 nm was recorded after subtracting blanks containing TCA and TBA in an equal

volume. The signal was read against a malondialdehyde (MDA) standard curve and the results were expressed as ng MDA/mg of protein [3, 27].

2.4.3. 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 % TCA and centrifuged. Protein precipitates were left to react with 2, 4-dinitrophenylhydrazine 10 mM 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} [3].

2.5. Total Antioxidant Capacity (TAC) Assay

TAC was measured using copper (II)-neocuproine as a chromogenic oxidant (CUPRAC method). Results were compared with a standard curve obtained with trolox. Absorbance was recorded at 570 nm and results were expressed in µmol trolox equivalents/mg of protein [27, 28].

2.6. Nonenzyme Antioxidant Defense Systems

2.6.1. Total Glutathione Assay

Total glutathione was measured using a commercial kit from Biovision, according to manufacturer instructions. Data are presented as nmol of GSH/mg of protein [27].

2.7. Enzyme Antioxidant Defense Systems

2.7.1. Superoxide Dismutase Assay

Samples were mixed with reaction buffer contained 100 mM triethanolamide-diethanolamide buffer (TDB) pH 7.4, 7.5 mM NADH and relation 1:2 EDTA/MnCl2. To start the reaction, 10 mM ß-mercaptoethanol were added. The absorbance was recorded at 340 nm for 2-15 min. Results were 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 [27, 28].

2.7.2. Catalase Activity Assay

Samples were added to 10 mM H2O2 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 [27]. Results were expressed in U/mg of protein. One unit of catalase activity is defined as 1 µmol of H2O2 decomposed per minute under the assay conditions.

2.7.3. 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 carried out in the same reaction volume without the sample. This allows subtracting the non-enzymatic rate of GSH oxidation [27]. 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.

2.8. 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 Bradford's method. Proteins were loaded per lane onto a 15 % SDS-PAGE gel for Cu-Zn superoxide dismutase (23 kDa), or a 10% SDS-PAGE gel for glutathione peroxidase (92 kDa), and 7.5 % SDS-PAGE gel for catalase (64 kDa). After blocking with 5% skim milk in TBS, the membrane was incubated with goat anti-SOD-1 (1:200, Santa Cruz Biotechnology, Inc.), goat anti-GPx-1 (1:200, Santa Cruz Biotechnology, Inc.) and mouse monoclonal anti-catalase 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. Inmunoblots were analyzed by ImageLab TM Software version 2.0.1 (Bio-Rad) to provide quantitative values for relative expression of each protein (all normalized to its own loading control). The optical densities of the bands were measured by ImageLab [29].

2.9. RT-PCR

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

2.10. 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 expressed in g/dL. Total protein level was estimated by the colorimetric method of Bradford. Results are expressed in mg/mL [3].

2.11. Statistical Analysis

All values represent the mean ± standard error of the mean (SEM). Data were analyzed by multiple analysis of variance (MANOVA) plus Newman-Keul´s test, using IBM Pass V.19 software. Values of $P<0.05$ were considered significant.

3. RESULTS

3.1. Parameters of the Carcinogenesis

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. Also, males show a mean of 2.00±0.41 tumors per animal, with a total volume of 350.40±157.76 mm3. Females show a mean of 2.14±0.65 tumors per animal, with a total volume of 206.18±84.88 mm3. No statistically significant differences were found among male and female rats. Fig. (**1**) shows several ENU-induced tumors by MRI and a computerized three-dimensional representation of a tumor as an example of its spatial distribution.

3.2. Oxidative Stress Parameters

3.2.1. Bioenergetic Behavior

Control and tumor tissue bioenergetic behavior showed significantly ($P<0.01$) higher values in both male and female animals with ENU-induced tumors than in their corresponding control groups (Fig. **2**). However, no gender differences were found.

3.2.2. Lipid Peroxidation

The analysis of lipid peroxidation determined through the analysis of thiobarbituric acid reactive substances (TBARS) showed a significant increase ($P<0.01$) in the tissue of male and female animals with ENU-induced tumors when compared with control groups (Fig. **3A**). No statistically significant differences were found among male and female rats. However, no changes were observed in TBARS content in the plasma of these animals (Fig. **3B**).

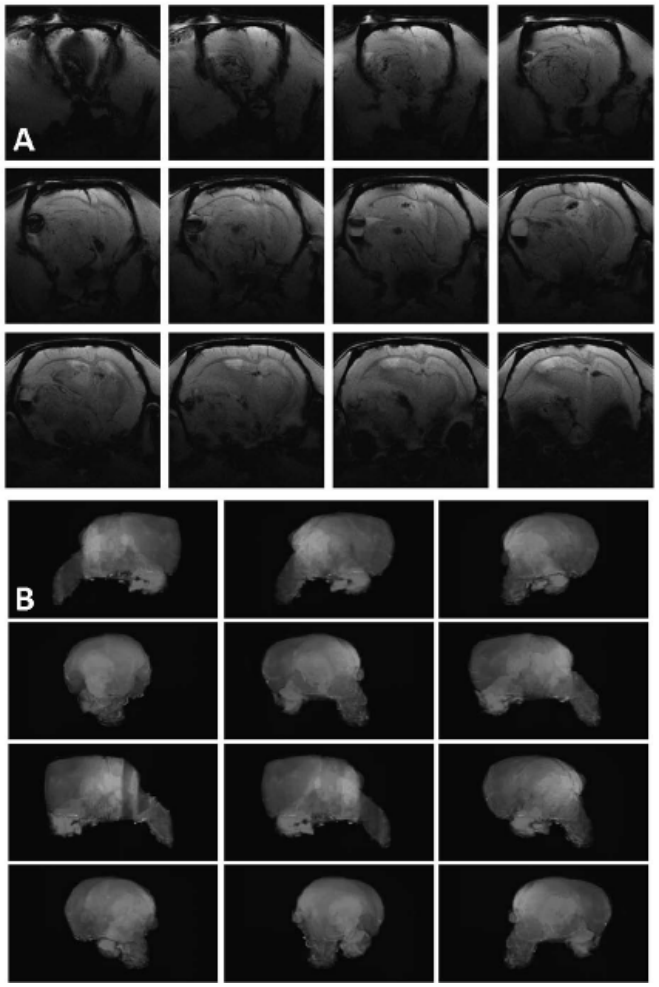

Fig. (1). (**A**) Representative T2-weighted images of ENU-induced tumors in animals by MRI. (**B**) Example of a computerized three-dimensional representation of the tumor.

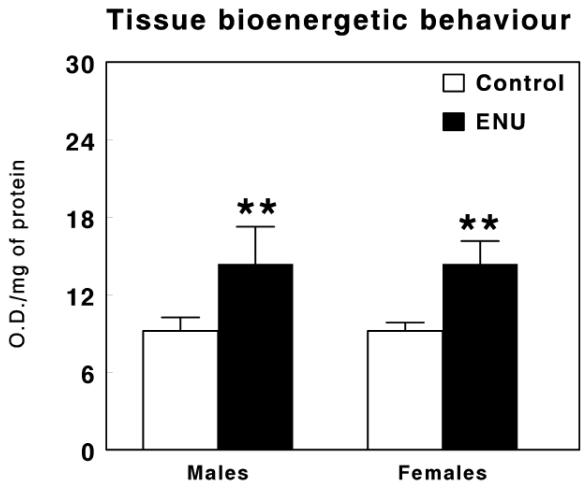

Fig. (2). Tissue bioenergetic behavior of male and female control animals and animals with tumors induced by transplacental N-ethyl-N-nitrosourea (ENU) exposure. Results are expressed in optical density units per mg of protein (Mean ± SEM; **$P<0.01$).

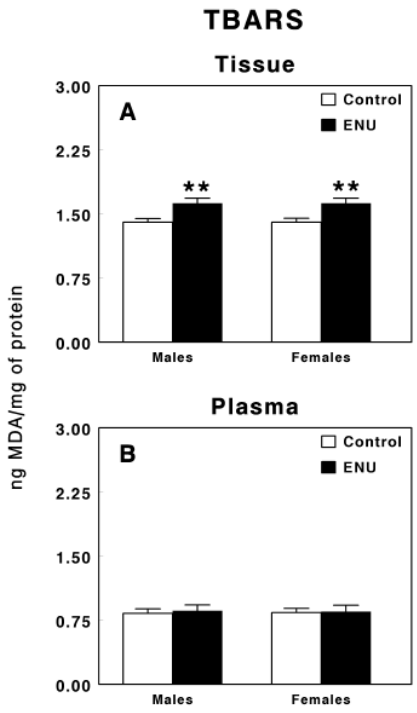

Fig. (3). Thiobarbituric acid reactive substances (TBARS) content in tissue (**A**) and plasma (**B**) of male and female control animals and animals with tumors induced by transplacental N-ethyl-N-nitrosourea (ENU) exposure. Results are expressed in ng of malondialdehyde (MDA) per mg of protein (Mean ± SEM; **P<0.01).

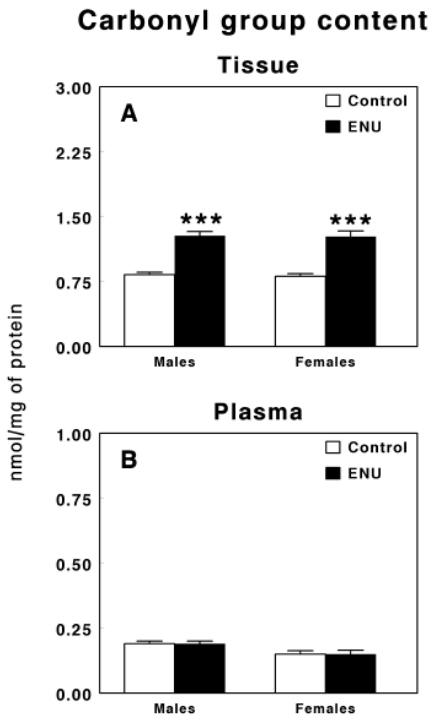

Fig. (4). Carbonyl groups content in tissue (**A**) and plasma (**B**) of male and female control animals and animals with tumors induced by transplacental N-ethyl-N-nitrosourea (ENU) exposure. Results are expressed in nmol per mg of protein (Mean ± SEM; ***P<0.001).

3.2.3. Protein Oxidation

The analysis of protein oxidation (assayed as carbonyl and diene conjugate groups content) also showed a significant increase (P<0.001) in the tissue of male and female animals with ENU-induced tumors (Fig. **4A**), whereas no changes were detected in the plasma between groups (Fig. **4B**). No gender differences were found.

3.3. Antioxidant Defense Systems

3.3.1. Total Antioxidant Capacity

The analysis of total antioxidant capacity (TAC) showed a significant decrease (P<0.05) at tissue level in both male and female animals with ENU-induced tumors when compared with their corresponding controls (Fig. **5A**). No statistically significant differences were found among male and female rats. However, no differences were observed in plasma between groups (Fig. **5B**).

3.3.2. Non-enzymatic Antioxidant Defense System

At tissue level, male and female animals with ENU-induced tumors showed significant (P<0.001) lower levels of total GSH than control groups (Fig. **6A**). Significant decreases in the total glutathione content were also observed in plasma of male (P<0.001) and female (P<0.01) animals with ENU-induced tumors when compared with control groups. However, we found in plasma gender differences in control group, showing female control animals significant (P<0.01) lower levels of total GSH content than male control animals (Fig. **6**).

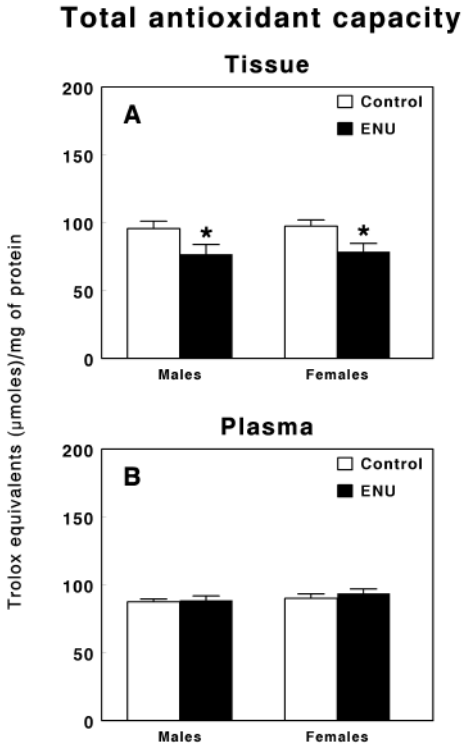

Fig. (5). Total antioxidant capacity (TAC) content in tissue (**A**) and plasma (**B**) of male and female control animals and animals with tumors induced by transplacental N-ethyl-N-nitrosourea (ENU) exposure. Results are expressed in trolox equivalents per mg of protein (Mean ± SEM).

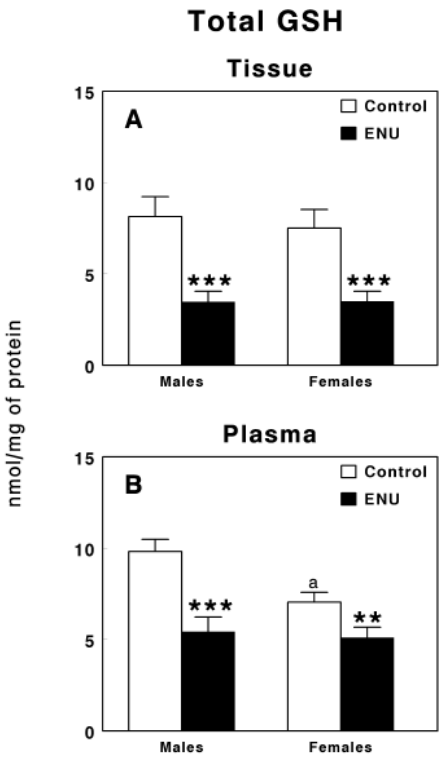

Fig. (6). Total glutathione (GSH) content in tissue (**A**) and plasma (**B**) of male and female control animals and animals with tumors induced by transplacental N-ethyl-N-nitrosourea (ENU) exposure. Results are expressed in nmol per mg of protein (Mean ± SEM; **P<0.01; ***P<0.001; [a]P<0.01).

3.3.3. Enzymatic Antioxidant Defense System

3.3.3.1. Superoxide Dismutase (SOD)

Tissue SOD activity in male and female animals with ENU-induced tumors showed significant (P<0.001) lower levels than control groups (Fig. **7A**). However, no statistically significant differences were found among male and female rats. In the same way, no changes were observed in SOD-1 protein levels (Fig. **7C**) or mRNA expression (Fig. **7D**) between the different groups analyzed. On the contrary, significantly decreased SOD activity was observed in plasma of male (P<0.01) but not female animals with ENU-induced tumors (Fig. **7B**).

3.3.3.2. Catalase

Tissue catalase activity was also significantly (p <0.01) lower in male and female animals treated with ENU, although no significant gender differences were found (Fig. **8A**). However, significant increases (P<0.01) in CAT protein were found in both male and female rats with ENU-induced tumors (Fig. **8C**). Catalase mRNA expression was also significantly higher in both male and female animals with ENU-induced tumors (Fig. **8D**). However, no significant gender differences were found either in tissue activity, protein levels or mRNA expression. On the contrary, plasma catalase activity was significantly decreased in male but not female animals with ENU-induced tumors (Fig. **8B**), although no gender differences were found in plasma between male and female control animals.

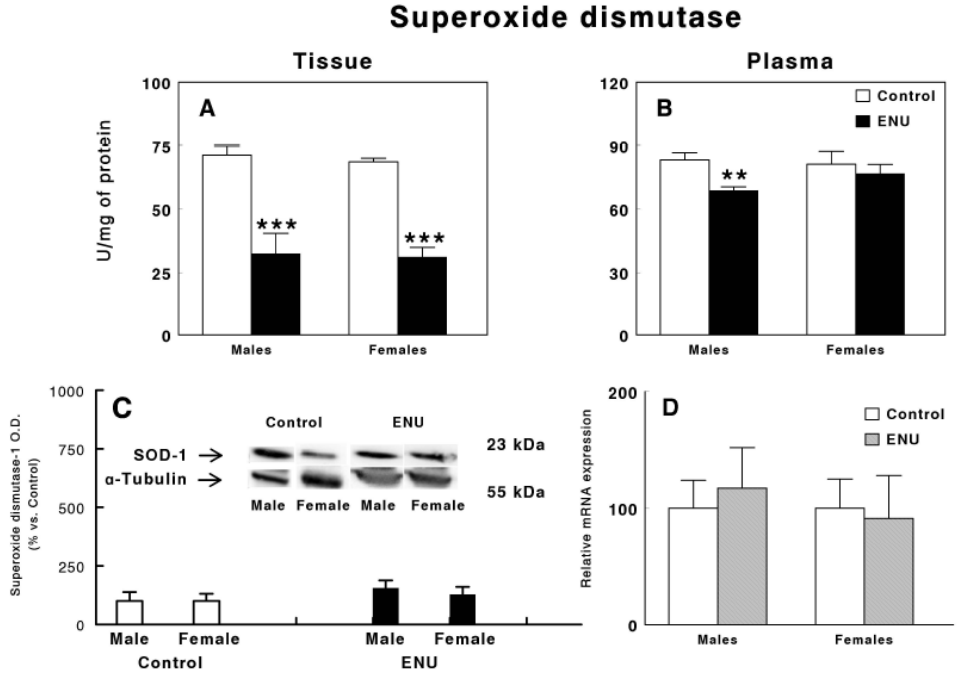

Fig. (7). Superoxide dismutase (SOD) activity in tissue (**A**) and plasma (**B**) of male and female control animals and animals with tumors induced by transplacental N-ethyl-N-nitrosourea (ENU) exposure. Results are expressed in units per mg of protein. Figure (**C**) shows a representative western 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>0.01; ***P<0.001).

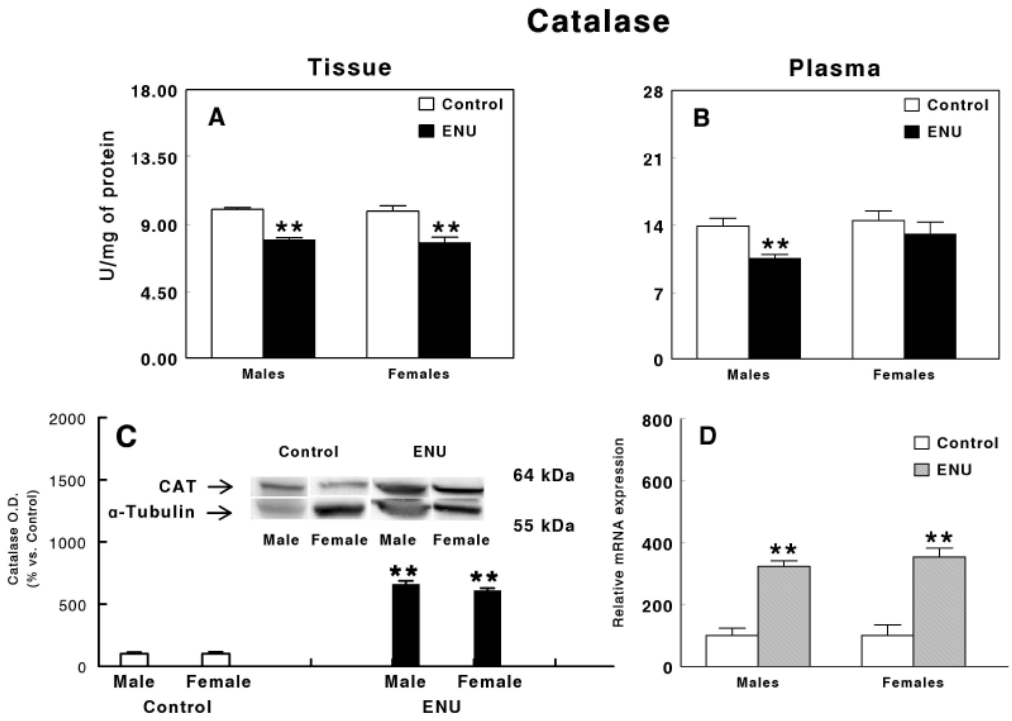

Fig. (8). Catalase (CAT) activity in tissue (**A**) and plasma (**B**) of male and female control animals and animals with tumors induced by trans-placental N-ethyl-N-nitrosourea (ENU) exposure. Results are expressed in units per mg of protein. Figure (**C**) shows a representative western blot showing 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<0.01).

3.3.3.3. Glutathione Peroxidase

Regarding GPx activity, a significant increase (P<0.001) was found at tissue level in both male and female animals with ENU-induced tumors (Fig. **9A**). In the same way, significant increases (P<0.05) in GPx-1 protein were found in both male and female rats with ENU-induced tumors (Fig. **9C**), and GPx-1 mRNA expression was also significantly higher in both male and female animals with ENU-induced tumors (Fig. **9D**). However, no significant gender differences were found either in tissue activity, protein levels or mRNA expression. On the contrary, no changes or gender differences were found in GPx activity in plasma of rats with ENU-induced tumors (Fig. **9B**).

3.4. Blood Chemistry

Table **I** shows the level of several electrolytes (calcium, phosphorus, sodium, potassium and chloride) analyzed in plasma of control animals and animals with ENU-induced tumors. Phosphorus plasma levels were significantly decreased (P<0.01) in females of both control and ENU-induced tumors groups when compared to control males and males with ENU-induced tumors respectively, appearing gender differences not related to ENU exposure. Also, chloride plasma levels were significantly higher (P<0.01) in female animals with ENU-induced tumors than in control females and ENU-treated males. In fact, no gender differences were found in chloride plasma levels in control animals. Finally, no changes were observed in the other electrolytes assayed. All parameters showed values within normal levels.

Table **II** shows plasma levels of glucose and non-protein nitrogenous compounds (urea, creatinine and uric acid) in control animals and animals with tumors induced by trans-placental ENU exposure. No significant differences were observed between groups or between male and female rats. All parameters showed values within normal levels.

Table **III** shows plasma lipid profile (total cholesterol, HDL-cholesterol, LDL-cholesterol, Total cholesterol/HDL-cholesterol ratio and triglycerides) in control rats and rats with ENU-induced tumors. No significant changes in total-cholesterol, HDL-cholesterol and total cholesterol/HDL-cholesterol ratio levels were found when the different experimental groups were compared. By other hand, LDL-cholesterol levels showed gender differences. Thus, male animals showed significant (P<0.01) increased LDL-cholesterol levels in both control and ENU-induced groups than females. Furthermore, a significant increase (P<0.01) of plasma LDL-cholesterol levels were observed in both male and female animals after ENU-exposure. In the same way, plasma triglycerides showed no significant gender differences in the control group. However, in animals with ENU-induced tumors, a significant (P<0.01) decrease was observed in both males and females, being this decrease significantly higher (P<0.01) in males than in females.

Table **IV** shows plasma levels of protein (total protein and albumin) and other enzymes (alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase). No changes were observed in plasma levels of total protein and albumin when the different experimental groups were compared. On the contrary, the analysis of other plasma enzymatic activities showed significant differences. Thus, gender

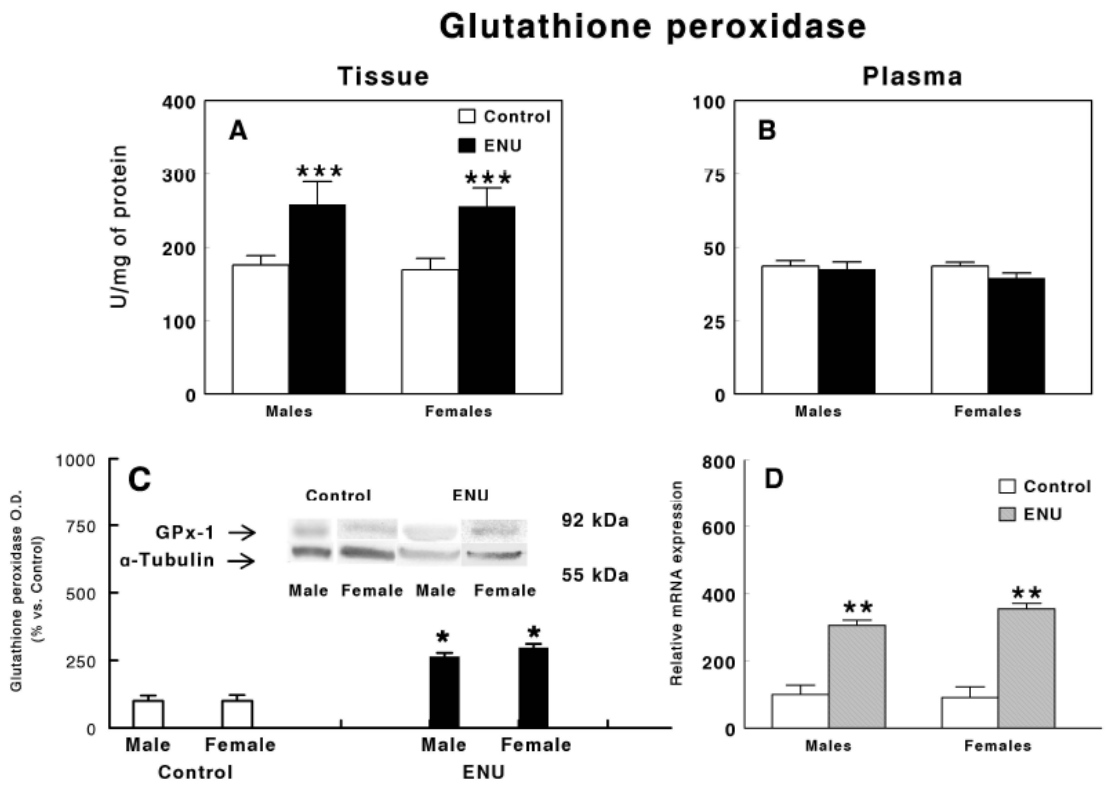

Fig. (9). Glutathione peroxidase (GPx) activity in tissue (**A**) and plasma (**B**) of male and female control animals and animals with tumors induced by transplacental N-ethyl-N-nitrosourea (ENU) exposure. Results are expressed in units per mg of protein. Figure (**C**) shows a representative western 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<0.05; **P<0.01; ***P<0.001).

Table I. Plasma levels of electrolytes in control animals and animals with tumors induced by transplacental N-ethyl-N-nitrosourea (ENU) exposure.

Parameter	Control		ENU		Significance Level
	Male	Female	Male	Female	
Calcium (mg/dL)	10.87±0.06	11.04±0.09	10.33±0.27	10.58±0.16	n.s.
Phosphorus (mg/dL)	6.03±0.44	5.14±0.20[a]	7.13±0.56	5.05±0.35[a]	[a]P<0.01
Sodium (mEq/L)	139.43±0.72	141.00±0.27	142.00±1.73	141.50±0.34	n.s.
Potassium (mEq/L)	6.51±0.46	5.09±0.16	5.30±0.25	4.85±0.15	n.s.
Chloride (mEq/L)	98.00±0.53	98.38±0.18	98.00±2.52	100.50±0.89[b]	[b]P<0.01

Data are expressed in the indicated units as mean ± SEM. [a]Significance level between male and female groups. [b]Significance level between control and ENU groups.

Table II. Plasma levels of glucose and non-protein nitrogenous compounds in control animals and animals with tumors induced by transplacental N-ethyl-N-nitrosourea (ENU) exposure.

Parameter	Control		ENU		Significance Level
	Male	Female	Male	Female	
Glucose (mg/dL)	215.00±20.79	212.13±14.16	171.33±10.84	193.50±8.79	n.s.
Urea (mg/dL)	44.57±1.54	40.75±1.37	49.33±5.04	47.50±7.21	n.s
Creatinine (mg/dL)	0.69±0.04	0.68±0.03	0.67±0.03	0.58±0.02	n.s.
Uric acid ((mg/dL)	1.30±0.18	1.13±0.09	1.43±0.09	1.20±0.06	n.s.

Data are expressed in the indicated units as mean ± EM; n.s., not significant.

Table III. Plasma lipid profile in control animals and animals with tumors induced by transplacental N-ethyl-N-nitrosourea (ENU) exposure.

Parameter	Control		ENU		Significance Level
	Male	Female	Male	Female	
Total cholesterol (mg/dL)	95.29±7.98	84.50±4.04	71.00±8.74	71.67±4.65	n.s.
HDL-cholesterol (mg/dL)	49.43±5.07	47.75±2.50	33.67±5.90	35.33±2.79	n.s
LDL-cholesterol (mg/dL)	12.83±0.39	4.12±1.5[a]	25.33±1.92[b]	15.84±4.00[a]	[a,b]P<0.01
Total cholesterol/HDL-c	1.93±1.57	1.77±1.62	2.11±1.48	2.03±1.67	n.s.
Triglycerides (mg/dL)	165.14±12.19	163.13±15.12	60.00±4.62[b]	102.50±29.29[a]	[a,b]P<0.01

Data are expressed in the indicated units as mean ± SEM. [a]Significance level between male and female groups. [b]Significance level between control and ENU groups.

Table IV. Plasma levels of proteins and enzymes in control animals and animals with tumors induced by transplacental N-ethyl-N-nitrosourea (ENU) exposure.

Parameter	Control		ENU		Significance Level
	Male	Female	Male	Female	
Total protein (mg/mL)	6.50±0.08	6.78±0.14	6.43±0.27	6.77±0.22	n.s.
Albumin (g/dL)	3.11±0.06	3.36±0.11	3.30±0.20	3.75±0.14	n.s
Alanine aminotransferase (UI/L)	69.57±7.04	47.00±7.29[a]	38.67±5.90[b]	38.50±4.43	[a,b]P<0.01
Aspartate aminotransferase (UI/L)	119.29±47.68	118.13±38.34	189.6±25.78[b]	185.83±22.62[ab]41.	[b]P<0.01
Alkaline phosphatase (UI/L)	193.71±16.50	232.00±21.07	54.33±7.69[b]	17±4.51[ab]	[a,b]P<0.01

Data are expressed in the indicated units as mean ± SEM. [a]Significance level between male and female groups. [b]Significance level between control and ENU groups.

differences were observed in alanine-aminotransferase in control group, showing male animals significantly higher levels than female animals (P<0.01). After ENU-exposure, a significant decrease was observed in male (P<0.01) and with borderline significance in female animals in plasma levels of alanine-aminotransferase, but the gender differences did not appear in animals with ENU-induced tumors.

On the other hand, a significant increase (P<0.01) was found in male and female animals with ENU-induced tumors in plasma aspartate aminotransferase activity when compared with their control groups, whereas no gender differences were found in control animals or animals with ENU-induced tumors in this plasma activity.

Finally, plasma levels of alkaline phosphatase activity were significantly lower (P<0.01) in male and female animals with ENU-induced tumors when compared with their control group. Although gender differences were not found in control animals, in animals with ENU-induced tumors male rats showed significantly higher (P<0.01) plasma levels of alkaline phosphatase than female rats. All parameters showed values within normal levels.

4. DISCUSSION

Tumorigenesis is a process which involves multiple cellular and molecular events that promote the transformation of a normal cell into a malignant one. In many of these events, free radicals seem to promote lipid peroxidation of cellular membranes and oxidation of proteins and DNA and induce alterations in chromosome structure, genetic mutations and/or disturbs cell growth [28,30]. This oxidative damage is also promoted by a reduced response of the 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 [6,7,28]. Particularly, it is well known that the CNS is highly sensitive to ROS and brain tumorigenesis has been mainly related to oxidative stress.

On the other hand, ENU-induced tumorigenesis is an experimental brain tumor model extensively described [16, 18, 31] in which rats exposed in utero to a single dose of a mutagen, ENU, preferentially develop brain tumors [19, 32, 33]. 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 [20]. In fact, after ENU exposure, 100 % rats used in our study showed tumors and a mean of two tumors per animal. We also found that tumor volume was slightly higher in males than in females, although no significant differences were observed. Additionally, other studies have indicated a possible role for oxidative stress in nitrosamine induced carcinogenesis [23, 24]. Thus, the ENU-induced tumors model allowed us to test the oxidative processes/redox status at brain tissue level in order to know the putative relationship between oxidative stress and the mechanisms underlying the development of tumors. Furthermore, plasma assays could reflect key steps of oxidative changes that occur in the brain and that may be used as circulating biomarkers.

Bioenergetic behavior analysis showed greater mitochondrial activity in the brain tissue of both male and female animals with ENU-induced tumors when compared to healthy controls and without gender differences. These results indicate an increased oxidative activity in the brain of

male and female animals with ENU-induced tumors, and are in accordance with the levels of oxidative stress parameters analyzed, which also showed increased lipid peroxidation and protein oxidation in brain tissue of male and female animals with ENU-induced tumors. Lipid peroxidation has been reported as a premature marker of oxidative damage because of the augmented propagation of free radicals related to it. Increases of oxidative stress in cells can to modify several cellular targets, promoting cellular damage and, thereafter, the lack of cellular repair mechanisms associated with carcinogenesis [14, 27, 28, 34]. Cancer cells are continuously under oxidative stress due to the high generation of intracellular free radicals, which promotes carcinogenic events through oncogenic stimulation, increased metabolic activity and/or mitochondrial malfunction [11, 14, 28]. Thus, increased levels of oxidative stress induce lipid peroxidation of cell membranes and formation of peroxides that can decompose into several mutagenic aldehyde products, mainly MDA, which is involved in cancer progression [35]. Our results showing increased TBARS levels in the brain of rats with ENU-induced tumors without gender differences are consistent with the findings of other studies [3]. Zengin and col. [36] found increased TBARS levels in tumor tissue samples when compared with peritumoral tissue, which they explained due to high production or unequal clearance of free radicals by the cellular antioxidant defenses. It has been also described in astrocytoma, meningioma, metastatic and other types of tumors that TBARS levels were increased when related to peritumoral adjacent tissue. Also, several differences were found when astrocytoma tumors were compared with other tumor groups. TBARS levels also showed differences when low-grade and high-grade tumors were analyzed. Therefore, it was stated that lipid peroxidation was higher in high-grade tumors. Increased levels of lipid peroxidation support the hypothesis that tumor cells produce high amounts of free radicals, also supporting a relationship between free radicals and carcinogenesis [3].

We have also determined plasma levels of lipid peroxidation in order to determine if a relationship between brain tumor tissue and systemic redox status exists [3]. Cirak and col. [37] studied lipid peroxidation levels in serum and tumor tissue from patients with high and low-grade glial tumors. They found that patients with high-grade tumors had higher MDA levels both in sera and tissue when compared to low grade glial tumor patients and controls. This result implied the possibility that the measurement of TBARS/MDA could be used as a marker of the lipid peroxidation associated with brain tumors. In this way, we had previously described significantly increased levels of TBARS content in serum of animals with C6 glioma subcutaneously implanted [3]. By contrast, the result of present work did not show significant differences between TBARS levels in plasma of rats with ENU-induced levels, and no gender differences were observed. Our results indicate that the systemic lipid peroxidation status does not reflect the oxidative changes observed at the tissue level.

Several products of lipid peroxidation may be also responsible of protein oxidation [38]. So, we have determined the level of protein oxidation (measured as carbonyl and diene conjugate group content) as oxidative stress marker. The results were similar to those previously described for lipid peroxidation; significantly increased concentrations of carbonyl groups were observed in the brain tissue of both male and female rats with ENU-induced tumors but no changes were detected in plasma. These results corroborate that the oxidative changes detected in tumor tissue are not reflected in plasma.

However, the magnitude of the oxidative damage depends not only of free radicals but also on the efficiencies of the antioxidant mechanisms. As already mentioned above, the balance between oxidants and antioxidants systems plays a key role in carcinogenesis [39]. The animals with ENU-induced tumors showed a higher production of free radicals, as reflected in the changes on mitochondrial activity, on lipid peroxidation and on protein oxidation formerly described.

To complete the analysis, we have also studied the total antioxidant capacity. We have found a significant decrease in brain tissue TAC levels in both male and female animals with ENU-induced tumors when compared with healthy control group. However, these changes were not reflected, once again, at plasma level. These decreased TAC levels also imply the failure of the antioxidant systems. Thus, we have found significant differences in brain tissue of male and female animals with ENU-induced tumors in both non-enzyme and enzyme antioxidant defense systems. Thus, the analysis of total GSH content showed a significant decrease at brain tissue level of male and female rats with ENU-induced tumors when compared with their corresponding control groups. We also found decreased levels of total GSH in plasma of both male and female rats. It is well documented that tumor cells have lower-levels of GSH [40] and previous studies have described a significant decrease of GSH in astrocytomas, meningiomas and other types of brain tumor [36, 41]. In the same way, in animals with C6 glioma subcutaneously implanted we also observed a decrease in GSH content in serum [3]. All these results are in accordance with Navarro and col. [42] 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 GSH decrease is related to an increased pro-oxidant milieu that correlates with the increase in lipid peroxidation and protein oxidation observed. Furthermore, the changes found in plasma indicate that GSH could be used as a biomarker in this pathology [4, 5].

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 ($O2\bullet-$) to H_2O_2 and protects cells against $O2\bullet-$-mediated lipid peroxidation; CAT converts H2O2 into H_2O and O_2 and GPx reduces H_2O_2 and other organic peroxides [27, 43]. During prolonged or excessive oxidative stress, changes in SOD, CAT and GPx activities may occur, being or not enough to avoid the oxidative damage and playing a main role in the pathogenesis of several diseases including cancer.

We have found here a significant decrease in SOD activity in brain tissue of both male and female animals with ENU-induced tumors. However, these changes are reflected in plasma only in male animals with ENU-induced tumors. For females, variations in SOD activity detected at the tissue level are not reflected in plasma. Our results are in agree-

ment with a growing body of evidence that demonstrates that SOD has important roles in many aspects of cancer [44]. In fact, different authors have described lower levels of SOD in tumor tissue when compared with normal tissue [45, 46]. Nevertheless, other authors have described higher SOD activity levels in human gliomas when compared with other tumor types. It seems to contradict the observation of low SOD activity in tumor cells [47]. Furthermore, several forms of SOD have been described 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 [44]. Briefly, SOD-1 is located mainly in the cytoplasm [48], SOD-2 in the mitochondrial matrix [49, 50] and SOD-3 is the secreted form of SOD with expression restricted mainly to the lung, kidney and adipose tissue [44, 51]. Most of the studies have been focused on SOD-2 form and it has been suggested that SOD-2 may be a tumor suppressor gene. Lower levels of SOD-2 expression have been described in most types of cancer [52]. However, many evidences show certain heterogeneity in the expression and activity of SOD-2 in different cancer cells, suggesting that SOD-2 expression might be stage-and/or tumor type-dependent [44, 53, 54]. The emerging role of SOD-1 form in cancer biology has been considered by many authors [55]. While the contribution of SOD-1 in the cytoplasm is well known, many authors propose 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 [44, 56]. Huang and col. [57] 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 [56] although, the mechanism by which the inhibition of SOD-1 acts on cancer cells is unclear. In breast cancer, Papa *et al.* [55] have reported an overexpression of SOD-1 and high levels of SOD-1 protein were detected in three mammary tumor models. Furthermore, the analysis of SOD-1 in women primary breast cancers showed that SOD-1 is accumulated in the cytoplasm and the nucleus of cancer cells. These results suggest that the nuclear portion of SOD-1 may also play a role in the survival of cancer cells. However, the malfunction of the anti-oxidant machinery of the mitochondria seems to play a critical role because promotes the production of free radicals in its 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. Finally, the role of SOD-3 in cancer is less understood. Because SOD-3 is extracellular, it is possible that its effect in cancer could be mediated through changes in the tumor microenvironment [44].

Whereas SOD-1 is the major intracellular form of SOD (80% total SOD protein) and given the important role that has been assigned to the SOD-1 in tumor processes, in this study we have determined the levels of SOD-1 protein and mRNA expression in brain samples of animals with ENU-induced tumors and healthy controls. No changes were detected in these parameters. However, lower SOD activity was observed 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 is reflected in the increased oxidative activity observed in rats with ENU-induced tumors.

Catalase is an antioxidant enzyme responsible to the conversion of H2O2 to H2O. Changes in catalase activity have been described in different brain tumors. Although many authors have described significant increases in catalase activity in both glial and meningiomal tumors [45, 58], our results showed lower levels of catalase activity in brain tissue samples of animal with ENU-induced tumors and no gender differences were detected; however, these changes are reflected in plasma only in male animals with ENU-induced tumors. For females, variations in CAT activity detected at the tissue level are not reflected in the plasma, showing similar behavior than SOD. These results may suggest that tumor induction blocks the role of catalase in converting H2O2 to H2O and therefore its antioxidant effect would be compromised; but these results could also indicate that due to the lower SOD activity observed, H2O2 production could be decreased and therefore a lower catalase activity may be necessary to catalyze the reaction from H2O2 to H2O and O2. However, the analysis of protein level and mRNA expression showed significant increases in both male and female animals with ENU-induced tumors. These results may indicate 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. Therefore, it is difficult to establish a causal relationship.

In any case, to complete the study of the redox state, we must take into account the results related to GPx, another enzyme also responsible for the removal of hydrogen peroxide. Although many authors have described lower levels of GPx in different brain tumors [46, 59, 60], we observed a significant increase of GPx activity in brain tissue samples of both male and female animals with ENU-induced tumors, although this change is not reflected in plasma. This increase in GPx activity could compensate the effect of lower CAT activity in the removal of H_2O_2.

Although, several forms of GPx have been cloned [61-63], to study GPx mRNA expression and protein levels we determined GPx-1 isoform because is a crucial antioxidant enzyme, which catalyzes the reduction of H2O2 and a large variety of hydroperoxides (such as DNA hydroperoxides and lipid hydroperoxides) into water and alcohols respectively [64, 65] protecting cells under oxidative stress. In fact, it has been reported that GPx-1 protein levels and mRNA expressions are up-regulated in carcinoma tissues to protect against DNA damage [66], which are also associated with increased risks of cancer [67-69]. Significant increases in GPx-1 protein levels and mRNA expression in males and female rats with ENU-induced tumors were observed in our study.

SOD and CAT do not need cofactors to function, while GPx requires several cofactors and proteins to function at high efficiency. GSH is necessary for GPx develops its function correctly. In this way, we observed lower levels of GSH in animals with ENU-induced tumors; these results may be related to the higher activity of GPx in these animals.

Although CAT and GPx act removing H2O2, their behavior in animals with ENU-induced tumors is different. Antioxidant proteins with similar enzymatic activity may have different effects after modulation due to the different localizations within cells. Both GPx and CAT remove H2O2, but their contribution varies depending on the amount and the localization of H_2O_2 production [70].

The overall effects of the antioxidant system depend on the intracellular equilibrium between the several antioxidant enzymes rather than a single component [39]. The imbalance in the adequate expression and/or activity of antioxidant enzymes can promote the generation of oxidative stress [39,71]. This evidence implied 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.

Finally, we have also measured several blood chemistry parameters (electrolytes, biomarkers of renal and hepatic functions and lipid profile) in order to analyze the potential adverse effects of ENU-induced experimental brain tumors in several physiological processes. Slight modifications occurred when compared animals with ENU-induced tumors to healthy controls, but all of them are considered to be within the normal range; therefore, ENU administration does not seem to cause important changes in other physiological functions.

In any case, the existence of gender differences in brain tumor-related processes -such as the redox status management showed here- indicates that future directions on brain cancer research need to consider sex differences from the molecular and cellular to the organism level, and sex differences must be also considered in preclinical studies, screening and prevention programs, and also in the therapeutic approaches.

5. ANIMAL PROTECTION

All 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 CVI09-4957M).

CONCLUSION

We have analyzed here the redox status and the gender differences in animals with brain tumors induced by ENU administration and their corresponding healthy controls at two levels. Firstly, at tissue level, in order to know the relationship between oxidative stress, the enzyme and non-enzyme antioxidant defense systems and the mechanisms underlying the development of brain tumors; and secondly, at plasma level, in order to know if those key changes of redox balance involved in tumors development are reflected systemically and could be used as biomarkers of this brain tumor. Our results confirm that ENU-induced tumors are related to an increased oxidative stress promoted by several changes in brain enzyme and non-enzyme antioxidant defense systems with gender differences in plasma consisting with a decreased SOD/CAT activities limited to males only. Conversely, plasma GSH levels could be eligible as a circulating biomarker of the disease in this animal model, but its sensitivity, specificity and applicability to humans need to be further evaluated.

LIST OF ABBREVIATIONS

ALP	=	Alkaline phosphatase
ALT	=	Alanine aminotransferase
AST	=	Aspartate aminotransferase
CAT	=	Catalase
cDNA	=	Complementary deoxyribonucleic acid
CNS	=	Central nervous system
DNA	=	Deoxyribonucleic acid
DTNB	=	2-nitrobenzoic acid
EDTA	=	Ethylenediamine tetra-acetic acid
ENU	=	N-ethyl-N-nitrosourea
GAPDH	=	Glyceraldehyde 3-phosphate dehydrogenase
GPx	=	Glutathione peroxidase
GSH	=	Glutathione
HDL	=	High density lipoprotein
LDL	=	Low density lipoprotein
MANOVA	=	Multivariate analysis of variance
MDA	=	Malondialdehyde
MRI	=	Magnetic resonance imaging
mRNA	=	Messenger ribonucleic acid
MTT	=	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NADH	=	β-Nicotinamide adenine dinucleotide
NADPH	=	β-Nicotinamide adenine dinucleotide phosphate
RNA	=	Ribonucleic acid
ROS	=	Reactive oxygen species
RT-PCR	=	Reverse transcription polymerase chain reaction
SDS	=	Sodium dodecyl sulfate
SDS-PAGE	=	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	=	Standard error of mean
SOD	=	Superoxide dismutase
TAC	=	Total antioxidant capacity
TBA	=	thiobarbituric acid
TBARS	=	Thiobarbituric acid-reactive substances
TBS	=	Tris buffered saline
TCA	=	Trichloroacetic acid

TDB = Triethanolamide-diethanolamide buffer.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The study was supported by Junta de Andalucía (PAIDI group BIO296 –currently CTS1039-) and Consejería de Innovación, Ciencia y Empresa through Proyecto de Excelencia Motriz (grant CVI2009-4957M).

ACKNOWLEDGEMENTS

Declared none.

REFERENCES

[1] Davis, F.G.; Malmer, B.S.; Aldape, K.; Barnholtz-Sloan, J.S.; Bondy, M.L.; Brannstrom, T.; Bruner, J.M.; Burger, P.C.; Collins, V.P.; Inskip, P.D.*, et al.* Issues of diagnostic review in brain tumor studies: From the brain tumor epidemiology consortium. *Cancer Epidemiol Biomarkers Prev* **2008**, *17*, 484-489.

[2] Rinaldi, M.; Caffo, M.; Minutoli, L.; Marini, H.; Abbritti, R.V.; Squadrito, F.; Trichilo, V.; Valenti, A.; Barresi, V.; Altavilla, D.*, et al.* Ros and brain gliomas: An overview of potential and innovative therapeutic strategies. *Int J Mol Sci* **2016**, *17*.

[3] Illan-Cabeza, N.A.; Garcia-Garcia, A.R.; Martinez-Martos, J.M.; Ramirez-Exposito, M.J.; Pena-Ruiz, T.; Moreno-Carretero, M.N. A potential antitumor agent, (6-amino-1-methyl-5-nitrosouracilato-n3)-triphenylphosphine-gold(i): Structural studies and *in vivo* biological effects against experimental glioma. *Eur J Med Chem* **2013**, *64*, 260-272.

[4] Martinez-Martos, J.M.; Mayas, M.D.; Carrera, P.; de Saavedra, J.M.A.; Sanchez-Agesta, R.; Arrazola, M.; Ramirez-Exposito, M.J. Phenolic compounds oleuropein and hydroxytyrosol exert differential effects on glioma development via antioxidant defense systems. *Journal of Functional Foods* **2014**, *11*, 221-234.

[5] Ramirez-Exposito, M.J.; Martinez-Martos, J.M. Anti-inflammatory and antitumor effects of hydroxytyrosol but not oleuropein on experimental glioma *in vivo*. A putative role for the renin-angiotensin system. *Biomedicines* **2018**, *6*.

[6] Acharya, A.; Das, I.; Chandhok, D.; Saha, T. Redox regulation in cancer: A double-edged sword with therapeutic potential. *Oxid Med Cell Longev* **2010**, *3*, 23-34.

[7] Herrera, A.C.; Panis, C.; Victorino, V.J.; Campos, F.C.; Colado-Simao, A.N.; Cecchini, A.L.; Cecchini, R. Molecular subtype is determinant on inflammatory status and immunological profile from invasive breast cancer patients. *Cancer Immunol Immunother* **2012**, *61*, 2193-2201.

[8] Papacocea, T.; Papacocea, R.; Badarau, A.; Ion, A.D.; Buraga, I.; Gaman, L.; Papacocea, A. Oxidative stress and antioxidants in brain tumors. *Therapeutics, Pharmacology and Clinical Toxicology* **2011**, *XV*, 234-239.

[9] Rajaraman, P.; Hutchinson, A.; Rothman, N.; Black, P.M.; Fine, H.A.; Loeffler, J.S.; Selker, R.G.; Shapiro, W.R.; Linet, M.S.; Inskip, P.D. Oxidative response gene polymorphisms and risk of adult brain tumors. *Neuro Oncol* **2008**, *10*, 709-715.

[10] Kryston, T.B.; Georgiev, A.B.; Pissis, P.; Georgakilas, A.G. Role of oxidative stress and DNA damage in human carcinogenesis. *Mutat Res* **2011**, *711*, 193-201.

[11] Pelicano, H.; Feng, L.; Zhou, Y.; Carew, J.S.; Hileman, E.O.; Plunkett, W.; Keating, M.J.; Huang, P. Inhibition of mitochondrial respiration: A novel strategy to enhance drug-induced apoptosis in human leukemia cells by a reactive oxygen species-mediated mechanism. *J Biol Chem* **2003**, *278*, 37832-37839.

[12] de Miguel, M.; Cordero, M.D. Oxidative therapy against cancer. In *Oxidative estress and diseases*, Lushchak, V., Ed. In Tech: Croatia, 2012; pp 497-520.

[13] Liang, L.P.; Patel, M. Seizure-induced changes in mitochondrial redox status. *Free Radic Biol Med* **2006**, *40*, 316-322.

[14] Ramirez-Exposito, M.J.; Martinez-Martos, J.M. The delicate equilibrium between oxidants and antioxidants in brain glioma. *Curr Neuropharmacol* **2018**.

[15] Watson, J. Oxidants, antioxidants and the current incurability of metastatic cancers. *Open Biol* **2013**, *3*, 120144.

[16] Druckrey, H.; Ivankovic, S.; Preussmann, R. Teratogenic and carcinogenic effects in the offspring after single injection of ethylnitrosourea to pregnant rats. *Nature* **1966**, *210*, 1378-1379.

[17] Koestner, A.; Swenberg, J.A.; Wechsler, W. Transplacental production with ethylnitrosourea of neoplasms of the nervous system in sprague-dawley rats. *Am J Pathol* **1971**, *63*, 37-56.

[18] Schiffer, D.; Giordana, M.T.; Pezzotta, S.; Lechner, C.; Paoletti, P. Cerebral tumors induced by transplacental enu: Study of the different tumoral stages, particularly of early proliferations. *Acta Neuropathol* **1978**, *41*, 27-31.

[19] Zook, B.C.; Simmens, S.J.; Jones, R.V. Evaluation of enu-induced gliomas in rats: Nomenclature, immunochemistry, and malignancy. *Toxicol Pathol* **2000**, *28*, 193-201.

[20] Mahlke, M.A.; Cortez, L.A.; Ortiz, M.A.; Rodriguez, M.; Uchida, K.; Shigenaga, M.K.; Lee, S.; Zhang, Y.; Tominaga, K.; Hubbard, G.B.*, et al.* The anti-tumor effects of calorie restriction are correlated with reduced oxidative stress in enu-induced gliomas. *Pathobiol Aging Age Relat Dis* **2011**, *1*.

[21] Kokkinakis, D.M.; Rushing, E.J.; Shareef, M.M.; Ahmed, M.M.; Yang, S.; Singha, U.K.; Luo, J. Physiology and gene expression characteristics of carcinogen-initiated and tumor-transformed glial progenitor cells derived from the cns of methylnitrosourea (mnu)-treated sprague-dawley rats. *J Neuropathol Exp Neurol* **2004**, *63*, 1182-1199.

[22] Bulnes, S.; Lafuente, J.V. Vegf immunopositivity related to malignancy degree, proliferative activity and angiogenesis in enu-induced gliomas. *J Mol Neurosci* **2007**, *33*, 163-172.

[23] Bartsch, H.; Hietanen, E.; Malaveille, C. Carcinogenic nitrosamines: Free radical aspects of their action. *Free Radic Biol Med* **1989**, *7*, 637-644.

[24] Hietanen, E.; Bartsch, H. Gastrointestinal cancers: Role of nitrosamines and free radicals. *Eur J Cancer Prev* **1992**, *1 Suppl 3*, 51-54.

[25] Paxinos, G.; Watson, C. *The rat brain in stereotaxic coordinates*. 7th edition. ed.; Elsevier Academic Press: Amsterdam ; Boston, 2013; p 472 pages.

[26] Martinez-Martos, J.M.; Ramirez-Exposito, M.J.; Mayas-Torres, M.D.; Garcia-Lopez, M.J.; Ramirez-Sanchez, M. Utility of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (mtt) assay to measure mitochondrial activity in k+- and atp-stimulated rodent cortex synaptosomes. *Neuroscience Research Communications* **2000**, *27*, 103-107.

[27] Ramirez-Exposito, M.J.; Urbano-Polo, N.; Duenas, B.; Navarro-Cecilia, J.; Ramirez-Tortosa, C.; Martin-Salvago, M.D.; Martinez-Martos, J.M. Redox status in the sentinel lymph node of women with breast cancer. *Ups J Med Sci* **2017**, *122*, 207-216.

[28] Ramirez-Exposito, M.J.; Sanchez-Lopez, E.; Cueto-Urena, C.; Duenas, B.; Carrera-Gonzalez, P.; Navarro-Cecilia, J.; Mayas, M.D.; Arias de Saavedra, J.M.; Sanchez-Agesta, R.; Martinez-Martos, J.M. Circulating oxidative stress parameters in pre- and post-menopausal healthy women and in women suffering from breast cancer treated or not with neoadjuvant chemotherapy. *Exp Gerontol* **2014**, *58*, 34-42.

[29] Puertas, M.C.; Martinez-Martos, J.M.; Cobo, M.P.; Carrera, M.P.; Mayas, M.D.; Ramirez-Exposito, M.J. Plasma oxidative stress parameters in men and women with early stage alzheimer type dementia. *Exp Gerontol* **2012**, *47*, 625-630.

[30] Visioli, F.; Bellomo, G.; Galli, C. Free radical-scavenging properties of olive oil polyphenols. *Biochem Biophys Res Commun* **1998**, *247*, 60-64.

[31] Ikeno, Y.; Shimokawa, I.; Higami, Y.; Ikeda, T. Gfap expression in the subcutaneous tumors of immature glial cell line (hits glioma) derived from enu-induced rat glioma. *J Neurooncol* **1993**, *17*, 191-204.

[32] Kish, P.E.; Blaivas, M.; Strawderman, M.; Muraszko, K.M.; Ross, D.A.; Ross, B.D.; McMahon, G. Magnetic resonance imaging of ethyl-nitrosourea-induced rat gliomas: A model for experimental therapeutics of low-grade gliomas. *J Neurooncol* **2001**, *53*, 243-257.

[33] Slikker, W., 3rd; Mei, N.; Chen, T. N-ethyl-n-nitrosourea (enu) increased brain mutations in prenatal and neonatal mice but not in the adults. *Toxicol Sci* **2004**, *81*, 112-120.

[34] Federico, A.; Morgillo, F.; Tuccillo, C.; Ciardiello, F.; Loguercio, C. Chronic inflammation and oxidative stress in human carcinogenesis. *Int J Cancer* **2007**, *121*, 2381-2386.

[35] Zhang, Y.; Chen, S.Y.; Hsu, T.; Santella, R.M. Immunohistochemical detection of malondialdehyde-DNA adducts in human oral mucosa cells. *Carcinogenesis* **2002**, *23*, 207-211.

[36] Zengin, E.; Atukeren, P.; Kokoglu, E.; Gumustas, M.K.; Zengin, U. Alterations in lipid peroxidation and antioxidant status in different types of intracranial tumors within their relative peritumoral tissues. *Clin Neurol Neurosurg* **2009**, *111*, 345-351.

[37] Cirak, B.; Inci, S.; Palaoglu, S.; Bertan, V. Lipid peroxidation in cerebral tumors. *Clin Chim Acta* **2003**, *327*, 103-107.

[38] Guo, J.; Prokai, L. To tag or not to tag: A comparative evaluation of immunoaffinity-labeling and tandem mass spectrometry for the identification and localization of posttranslational protein carbonylation by 4-hydroxy-2-nonenal, an end-product of lipid peroxidation. *J Proteomics* **2011**, *74*, 2360-2369.

[39] Amstad, P.; Moret, R.; Cerutti, P. Glutathione peroxidase compensates for the hypersensitivity of cu,zn-superoxide dismutase overproducers to oxidant stress. *J Biol Chem* **1994**, *269*, 1606-1609.

[40] Sun, Y.; St Clair, D.K.; Xu, Y.; Crooks, P.A.; St Clair, W.H. A nadph oxidase-dependent redox signaling pathway mediates the selective radiosensitization effect of parthenolide in prostate cancer cells. *Cancer Res* **2010**, *70*, 2880-2890.

[41] Shi, J.; Sun, B.; Shi, W.; Zuo, H.; Cui, D.; Ni, L.; Chen, J. Decreasing gsh and increasing ros in chemosensitivity gliomas with idh1 mutation. *Tumour Biol* **2015**, *36*, 655-662.

[42] Navarro, J.; Obrador, E.; Carretero, J.; Petschen, I.; Avino, J.; Perez, P.; Estrela, J.M. Changes in glutathione status and the antioxidant system in blood and in cancer cells associate with tumour growth *in vivo*. *Free Radic Biol Med* **1999**, *26*, 410-418.

[43] Gilca, M.; Stoian, I.; Lixandru, D.; Gaman, L.; Virgolici, B.; Atanasiu, V. Protection of erythrocyte membrane against oxidative damage by geriforte in healthy human subjects. *Rom J Intern Med* **2009**, *47*, 289-295.

[44] Che, M.; Wang, R.; Li, X.; Wang, H.Y.; Zheng, X.F.S. Expanding roles of superoxide dismutases in cell regulation and cancer. *Drug Discov Today* **2016**, *21*, 143-149.

[45] Popov, B.; Gadjeva, V.; Valkanov, P.; Popova, S.; Tolekova, A. Lipid peroxidation, superoxide dismutase and catalase activities in brain tumor tissues. *Arch Physiol Biochem* **2003**, *111*, 455-459.

[46] Aggarwal, S.; Subberwal, M.; Kumar, S.; Sharma, M. Brain tumor and role of beta-carotene, a-tocopherol, superoxide dismutase and glutathione peroxidase. *J Cancer Res Ther* **2006**, *2*, 24-27.

[47] Del Maestro, R.F.; McDonald, W.; Anderson, R. In *Oxy radicals and their scavenger systems*, Greenwald, R.; Cohen, G., Eds. Elsevier: New York, 1983; pp 28-35.

[48] Crapo, J.D.; Oury, T.; Rabouille, C.; Slot, J.W.; Chang, L.Y. Copper,zinc superoxide dismutase is primarily a cytosolic protein in human cells. *Proc Natl Acad Sci U S A* **1992**, *89*, 10405-10409.

[49] MacMillan-Crow, L.A.; Thompson, J.A. Tyrosine modifications and inactivation of active site manganese superoxide dismutase mutant (y34f) by peroxynitrite. *Arch Biochem Biophys* **1999**, *366*, 82-88.

[50] Kienhofer, J.; Haussler, D.J.; Ruckelshausen, F.; Muessig, E.; Weber, K.; Pimentel, D.; Ullrich, V.; Burkle, A.; Bachschmid, M.M. Association of mitochondrial antioxidant enzymes with mitochondrial DNA as integral nucleoid constituents. *FASEB J* **2009**, *23*, 2034-2044.

[51] Lund, D.D.; Chu, Y.; Miller, J.D.; Heistad, D.D. Protective effect of extracellular superoxide dismutase on endothelial function during aging. *Am J Physiol Heart Circ Physiol* **2009**, *296*, H1920-1925.

[52] Oberley, T.D.; Oberley, L.W. Antioxidant enzyme levels in cancer. *Histol Histopathol* **1997**, *12*, 525-535.

[53] Dhar, S.K.; St Clair, D.K. Manganese superoxide dismutase regulation and cancer. *Free Radic Biol Med* **2012**, *52*, 2209-2222.

[54] Hempel, N.; Carrico, P.M.; Melendez, J.A. Manganese superoxide dismutase (sod2) and redox-control of signaling events that drive metastasis. *Anticancer Agents Med Chem* **2011**, *11*, 191-201.

[55] Papa, L.; Hahn, M.; Marsh, E.L.; Evans, B.S.; Germain, D. Sod2 to sod1 switch in breast cancer. *J Biol Chem* **2014**, *289*, 5412-5416.

[56] Papa, L.; Manfredi, G.; Germain, D. Sod1, an unexpected novel target for cancer therapy. *Genes Cancer* **2014**, *5*, 15-21.

[57] Huang, P.; Feng, L.; Oldham, E.A.; Keating, M.J.; Plunkett, W. Superoxide dismutase as a target for the selective killing of cancer cells. *Nature* **2000**, *407*, 390-395.

[58] Yilmaz, N.; Dulger, H.; Kiymaz, N.; Yilmaz, C.; Bayram, I.; Ragip, B.; Oger, M. Lipid peroxidation in patients with brain tumor. *Int J Neurosci* **2006**, *116*, 937-943.

[59] Gauchez, A.S.; Riondel, J.; Jacrot, M.; Calop, J.; Favier, A. Antioxidant status and lipid peroxidation in athymic mice xenografted with two types of human tumors. *Biol Trace Elem Res* **1995**, *47*, 103-109.

[60] Rao, G.M.; Rao, A.V.; Raja, A.; Rao, S.; Rao, A. Role of antioxidant enzymes in brain tumours. *Clin Chim Acta* **2000**, *296*, 203-212.

[61] Chu, F.F. The human glutathione peroxidase genes gpx2, gpx3, and gpx4 map to chromosomes 14, 5, and 19, respectively. *Cytogenet Cell Genet* **1994**, *66*, 96-98.

[62] Esworthy, R.S.; Doan, K.; Doroshow, J.H.; Chu, F.F. Cloning and sequencing of the cdna encoding a human testis phospholipid hydroperoxide glutathione peroxidase. *Gene* **1994**, *144*, 317-318.

[63] Hall, L.; Williams, K.; Perry, A.C.; Frayne, J.; Jury, J.A. The majority of human glutathione peroxidase type 5 (gpx5) transcripts are incorrectly spliced: Implications for the role of gpx5 in the male reproductive tract. *Biochem J* **1998**, *333 (Pt 1)*, 5-9.

[64] Freeman, B.A.; Crapo, J.D. Biology of disease: Free radicals and tissue injury. *Lab Invest* **1982**, *47*, 412-426.

[65] Meister, A. Glutathione, ascorbate, and cellular protection. *Cancer Res* **1994**, *54*, 1969s-1975s.

[66] Baliga, M.S.; Diwadkar-Navsariwala, V.; Koh, T.; Fayad, R.; Fantuzzi, G.; Diamond, A.M. Selenoprotein deficiency enhances radiation-induced micronuclei formation. *Mol Nutr Food Res* **2008**, *52*, 1300-1304.

[67] Lei, X.G.; Cheng, W.H.; McClung, J.P. Metabolic regulation and function of glutathione peroxidase-1. *Annu Rev Nutr* **2007**, *27*, 41-61.

[68] Zhuo, P.; Diamond, A.M. Molecular mechanisms by which selenoproteins affect cancer risk and progression. *Biochim Biophys Acta* **2009**, *1790*, 1546-1554.

[69] Yagublu, V.; Arthur, J.R.; Babayeva, S.N.; Nicol, F.; Post, S.; Keese, M. Expression of selenium-containing proteins in human colon carcinoma tissue. *Anticancer Res* **2011**, *31*, 2693-2698.

[70] Chandra, J.; Samali, A.; Orrenius, S. Triggering and modulation of apoptosis by oxidative stress. *Free Radic Biol Med* **2000**, *29*, 323-333.

[71] Amstad, P.; Peskin, A.; Shah, G.; Mirault, M.E.; Moret, R.; Zbinden, I.; Cerutti, P. The balance between cu,zn-superoxide dismutase and catalase affects the sensitivity of mouse epidermal cells to oxidative stress. *Biochemistry* **1991**, *30*, 9305-9313.