

Article

Compartment-Specific Remodeling of Superoxide Dismutase Isoforms Reveals Sex-Associated Differences During Experimental Gliomagenesis

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Abstract

Glioma progression is accompanied by alterations in redox homeostasis, yet the coordinated expression of antioxidant enzyme isoforms during tumor development remains incompletely understood. In particular, whether the expression of superoxide dismutase (SOD) isoforms is differentially regulated according to biological sex has received little attention. In this study, we characterized the transcriptional (RT-qPCR) and translational (ELISA) profiles of the three SOD isoforms (SOD1-SOD3) in an N-ethyl-N-nitrosourea (ENU)-induced rat glioma model and evaluated potential sex-associated differences. SOD1 expression remained unchanged at both the mRNA and protein levels in glioma tissue compared with healthy brain, indicating preservation of cytosolic enzyme abundance during tumor development. In contrast, SOD2 was significantly upregulated at both transcriptional and protein levels, with a more pronounced increase in male than in female animals. Conversely, SOD3 showed a significant reduction in both mRNA and protein abundance, although this decrease was less marked in females. These findings demonstrate a compartment-specific pattern of antioxidant remodeling characterized by stable SOD1 expression, increased mitochondrial SOD2, and reduced extracellular SOD3. Because the present study quantified transcript and protein abundance rather than direct reactive oxygen species generation, enzymatic activities, or downstream signaling pathways, the functional implications of these expression changes remain to be established. Nevertheless, the observed sex-associated differences suggest that biological sex influences antioxidant responses during experimental gliomagenesis and support its consideration as an important variable in future mechanistic and translational studies.

Keywords: glioma; oxidative stress; superoxide dismutase; SOD isoforms; biological sex; sexual dimorphism; N-ethyl-N-nitrosourea; RT-qPCR; ELISA; redox homeostasis



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1. Introduction

Gliomas are the most common primary malignant tumors of the central nervous system (CNS) and remain among the most aggressive human cancers. Despite substantial advances in neuroimaging, microsurgical techniques, radiotherapy, and temozolomide-based chemotherapy, the prognosis of patients with high-grade gliomas remains poor because of their diffuse infiltrative growth, pronounced cellular heterogeneity, metabolic

plasticity, and remarkable capacity to develop therapeutic resistance [1]. Increasing evidence indicates that these biological characteristics are closely linked to alterations in cellular redox homeostasis, placing oxidative stress at the center of glioma biology. This imbalance is not merely a byproduct of rapid growth but a driver of metabolic reprogramming, where reactive oxygen species (ROS) act as signaling rheostats that modulate tumor cell plasticity and the acquisition of therapeutic resistance [2,3].

Reactive oxygen species (ROS) are continuously generated during normal aerobic metabolism, primarily through electron leakage from the mitochondrial respiratory chain. Under physiological conditions, ROS function as signaling molecules regulating proliferation, differentiation, and cell survival. However, excessive ROS production or impaired antioxidant defenses disrupt redox homeostasis, promoting oxidative damage to nucleic acids, proteins, and membrane lipids. In cancer cells, this imbalance is exploited to sustain genomic instability, metabolic reprogramming, angiogenesis, and proliferative signaling while maintaining antioxidant defenses sufficient to prevent oxidative stress from reaching cytotoxic levels [4]. Because of its exceptionally high oxygen consumption and lipid-rich composition, the brain is particularly susceptible to oxidative injury, making redox regulation especially relevant during gliomagenesis [3].

Among the endogenous antioxidant systems, the superoxide dismutase (SOD) family constitutes the primary enzymatic defense against superoxide anion. These metalloenzymes catalyze the conversion of superoxide into hydrogen peroxide and molecular oxygen, thereby preventing the accumulation of one of the major reactive oxygen species generated during cellular respiration [5]. Mammals express three SOD isoforms that differ in their cellular localization and physiological roles. Cytosolic Cu/Zn-superoxide dismutase (SOD1) protects the intracellular compartment against cytoplasmic superoxide. Mitochondrial Mn-superoxide dismutase (SOD2) represents the principal antioxidant defense within the mitochondrial matrix, where oxidative phosphorylation continuously generates ROS. Extracellular superoxide dismutase (SOD3) regulates the redox status of the extracellular environment, preserves nitric oxide bioavailability, and contributes to maintaining extracellular matrix homeostasis [5]. Owing to their compartment-specific localization, these enzymes are likely to undergo distinct regulatory adaptations during tumor development.

Evidence accumulated over the last decade indicates that individual SOD isoforms contribute differently to glioma biology. Increased SOD2 expression has been associated with mitochondrial adaptation to oxidative stress and reduced sensitivity to chemotherapy, whereas alterations in SOD3 have been related to changes in the extracellular redox environment that may favor tumor invasion [5]. By contrast, the role of SOD1 appears more context-dependent and remains less clearly defined, with studies reporting stable, increased, or decreased expression depending on tumor type and experimental conditions [5,6]. Nevertheless, most available studies have focused on individual SOD isoforms or cultured glioma cells, whereas comparatively little information is available regarding the coordinated regulation of all three isoforms *in vivo*, particularly when both gene expression and protein expression are analyzed simultaneously.

Another aspect that deserves attention is the influence of biological sex on glioma biology. Glioblastoma is characterized by a striking sexual dimorphism, with males showing higher incidence and significantly poorer outcomes [7]. Recent molecular insights from the 2021 WHO classification emphasize that IDH-wildtype status, TERT promoter mutations, and EGFR amplification define the most aggressive forms, which are often subject to sex-specific metabolic pressures [1,8]. Biological sex dictates redox resilience and glioma pathophysiology. While 17 β -estradiol confers neuroprotection by inducing NRF2-mediated antioxidant responses and maintaining mitochondrial integrity, the up-regulation of androgen receptors in males has been linked to detrimental effects on tumor

growth and poorer clinical outcomes [9,10]. Despite these observations, the sex-dependent remodeling of SOD isoforms, the primary enzymatic defense against superoxide, remains as a gap in understanding how sexual dimorphism influences gliomagenesis [10,11]. Although these observations support the existence of sex-dependent differences in redox regulation, whether the expression of individual SOD isoforms is differentially regulated during gliomagenesis remains largely unknown.

The transplacental N-ethyl-N-nitrosourea (ENU)-induced rat glioma model offers a valuable experimental approach for addressing this question. Unlike transplantation models, ENU administration induces endogenous glial tumors that arise and progress within the native brain microenvironment, reproducing several histopathological and biological characteristics of human gliomas while preserving tumor–host interactions throughout disease development [3].

Therefore, the present study aimed to characterize the transcriptional and translational profiles of the three SOD isoforms in healthy brain tissue and ENU-induced gliomas from male and female rats. Specifically, we quantified *Sod1*, *Sod2*, and *Sod3* mRNA expression by real-time RT-qPCR together with their corresponding protein abundance by ELISA. We therefore investigated whether transcriptional and translational remodeling of the three SOD isoforms occurs during experimental gliomagenesis and whether this remodeling differs according to biological sex.

2. Results

2.1. Relative Gene Expression of SOD Isoforms

The relative mRNA expression of *Sod1*, *Sod2*, and *Sod3* was quantified by real-time RT-qPCR in healthy brain tissue and ENU-induced gliomas from male and female rats. Distinct transcriptional patterns were observed among the three isoforms (Table 1).

Table 1. Relative transcriptional expression of *Sod1*, *Sod2*, and *Sod3* genes in healthy and tumoral brain tissue from male and female Wistar rats at 30 weeks of age.

Experimental Group	<i>Sod1</i> mRNA (Relative Units)	<i>Sod2</i> mRNA (Relative Units)	<i>Sod3</i> mRNA (Relative Units)
Male Healthy Control	1.01 ± 0.08	1.01 ± 0.09	1.01 ± 0.07
Male ENU Glioma	0.96 ± 0.09	2.98 ± 0.18 *	0.38 ± 0.04 *
Female Healthy Control	1.03 ± 0.07	0.98 ± 0.08	1.05 ± 0.08
Female ENU Glioma	0.92 ± 0.08	1.82 ± 0.14 *, †	0.52 ± 0.05 *, †

Values represent mean ± SEM ($n = 10$ – 12 per group: male control $n = 10$, male glioma $n = 12$, female control $n = 10$, female glioma $n = 11$) normalized against the geometric mean of *Actb* and *Gapdh*. * $p < 0.05$ compared to their respective healthy control. † $p < 0.05$ when comparing the female glioma group versus the male glioma.

No significant differences in *Sod1* mRNA expression were detected between healthy brain and glioma tissue in either sex ($p > 0.05$). Likewise, no sex-dependent differences were observed under either physiological or tumor conditions, indicating that transcription of the cytosolic isoform remained stable throughout gliomagenesis.

In contrast, *Sod2* expression increased significantly in glioma tissue compared with the corresponding healthy controls in both males and females ($p < 0.05$). However, the magnitude of this increase differed between sexes. Male gliomas exhibited significantly higher *Sod2* mRNA levels (2.98 ± 0.18 relative units) than female gliomas (1.82 ± 0.14

relative units; $p < 0.05$), indicating a sex-dependent difference in the transcriptional response of the mitochondrial isoform.

Conversely, *Sod3* expression was significantly reduced in glioma tissue in both sexes compared with their respective controls ($p < 0.05$). Nevertheless, this decrease was less pronounced in females (0.52 ± 0.05 relative units) than in males (0.38 ± 0.04 relative units), and the difference between tumor groups reached statistical significance ($p < 0.05$), indicating partial preservation of *Sod3* transcription in female gliomas.

Overall, the transcriptional analysis revealed three distinct expression patterns: stable *Sod1* expression, increased *Sod2* expression, and decreased *Sod3* expression, with the latter two isoforms displaying significant sex-associated differences (Table 1).

2.2. Protein Expression of SOD Isoforms

Protein concentrations of the three SOD isoforms were determined by ELISA in the same experimental groups to evaluate whether the transcriptional changes were reflected at the protein level (Table 2).

Table 2. Absolute protein levels of SOD isoforms in homogenates of healthy brain tissue and ENU-induced glioma in Wistar rats of both sexes.

Experimental Group	SOD1 Protein (pg/mg Total Protein)	SOD2 Protein (pg/mg Total Protein)	SOD3 Protein (pg/mg Total Protein)
Male Healthy Control	310.6 $\pm$ 22.4	42.4 $\pm$ 3.8	450.2 $\pm$ 32.5
Male ENU Glioma	332.8 $\pm$ 38.4	98.6 $\pm$ 8.1 *	112.4 $\pm$ 9.8 *
Female Healthy Control	325.4 $\pm$ 24.1	39.8 $\pm$ 3.2	462.1 $\pm$ 28.9
Female ENU Glioma	345.6 $\pm$ 31.2	74.2 $\pm$ 6.3 *,†	168.3 $\pm$ 13.5 *,†

Data are presented as mean $\pm$ SEM ($n = 10$ – 12 per group: male control $n = 10$, male glioma $n = 12$, female control $n = 10$, female glioma $n = 11$) in pg/mg total protein. * $p < 0.05$ compared to respective control. † $p < 0.05$ comparing female versus male glioma.

Consistent with the gene expression results, SOD1 protein levels did not differ significantly in glioma tissue compared with healthy controls in both sexes ($p > 0.05$). No significant differences were detected between male and female animals, indicating stable abundance of the cytosolic isoform irrespective of tumor development.

In contrast, SOD2 protein concentration increased significantly in glioma tissue from both sexes ($p < 0.05$). Male gliomas exhibited higher SOD2 levels (98.6 ± 8.1 pg/mg total protein) than female gliomas (74.2 ± 6.3 pg/mg total protein), and this difference was statistically significant ($p < 0.05$). Thus, the sex-dependent increase observed at the transcriptional level was also evident at the protein level.

The extracellular isoform SOD3 showed the opposite pattern. Protein levels were significantly reduced in glioma tissue from both males and females compared with the corresponding healthy controls ($p < 0.05$). However, female gliomas retained significantly higher SOD3 concentrations (168.3 ± 13.5 pg/mg total protein) than male gliomas (112.4 ± 9.8 pg/mg total protein; $p < 0.05$), paralleling the differences observed in *Sod3* mRNA expression.

Taken together, the protein analyses closely mirrored the transcriptional findings. SOD1 showed no significant variation, whereas SOD2 increased and SOD3 decreased in glioma tissue. Moreover, the sex-associated differences identified at the mRNA level for

Sod2 and *Sod3* were preserved at the protein level, indicating a high degree of concordance between transcriptional and translational expression (Table 2).

3. Discussion

3.1. Differential Remodeling of the SOD Antioxidant System During Experimental Gliomagenesis

The present study provides a comprehensive evaluation of the transcriptional and translational profiles of the three mammalian SOD isoforms in ENU-induced gliomas from male and female rats. The principal finding is that gliomagenesis is associated with a coordinated but isoform-specific remodeling of the antioxidant system. Whereas *Sod1*/SOD1 expression remained stable, *Sod2*/SOD2 was consistently upregulated and *Sod3*/SOD3 was markedly downregulated. Importantly, these changes were observed at both the mRNA and protein levels, indicating a high degree of concordance between transcriptional regulation and protein abundance.

The second major finding is that this remodeling differs according to biological sex. Male gliomas exhibited a greater increase in SOD2 expression and a more pronounced reduction in SOD3 than female tumors, whereas SOD1 remained unaffected in both sexes. Collectively, these findings indicate that the antioxidant response during gliomagenesis is not uniform but depends on both the cellular compartment in which each isoform operates and the biological sex of the host.

3.2. Stable SOD1 Expression Suggests Preservation of Enzyme Abundance During Glioma Development

Unlike the mitochondrial and extracellular isoforms, neither *Sod1* transcription nor SOD1 protein abundance differed between healthy brain tissue and gliomas. This finding indicates that maintenance of cytosolic SOD1 expression is preserved during tumor development in the ENU model.

Previous studies have reported heterogeneous changes in SOD1 expression in gliomas, ranging from increased expression to reduced abundance in advanced tumors [5,12]. Such discrepancies probably reflect differences in tumor grade, experimental models, analytical methods, and disease stage. Because the ENU model reproduces progressive gliomagenesis rather than rapidly growing xenografts, preservation of SOD1 expression may represent an adaptive response during relatively early or intermediate phases of tumor evolution.

It should be emphasized, however, that the present study evaluated gene and protein expression rather than enzymatic activity. Consequently, unchanged SOD1 abundance cannot be interpreted as evidence of preserved antioxidant function. Oxidative post-translational modifications, including carbonylation or nitration, may impair enzyme activity without altering protein concentration [5]. Previous studies in this experimental model have indeed reported reduced total SOD activity despite relatively stable enzyme expression [3]. The stability of SOD1 expression reflects its role as a metabolic anchor within the glioma microenvironment. Beyond its role as an antioxidant, SOD1 is an essential mediator of cellular adaptation to stress; for instance, its activity can be modulated by GABA signaling through GABA_B receptors in diffuse midline gliomas, mitigating imipridone-induced oxidative stress [13]. Furthermore, SOD1 functions as a downstream effector of the mTORC1 signaling pathway, sustaining tumor cell survival and proliferation under hypoxia and starvation [6]. The preservation of SOD1 levels observed in our study suggests a sustained requirement for this cytosolic defense to maintain genomic stability through mechanisms involving PARP and BRCA1 protection, even as other SOD isoforms undergo dramatic remodeling [14].

3.3. Increased SOD2 Expression Is Consistent with Enhanced Mitochondrial Antioxidant Demand

Among the three isoforms, SOD2 displayed the most pronounced increase during gliomagenesis. Because SOD2 is the principal mitochondrial superoxide scavenger, its induction is consistent with increased oxidative metabolism and elevated mitochondrial ROS production in tumor cells [5,15].

Mitochondria act as metabolic hubs in glioma, where the Warburg effect and altered oxidative phosphorylation generate substantial superoxide levels that must be neutralized to prevent cytotoxicity. The upregulation of SOD2 observed here aligns with the requirement for mitochondrial adaptation to this metabolic reprogramming [15]. Mechanistically, SOD2 expression is often driven by transcriptional activators like Specificity protein 1 (Sp1) and the loss of PINK1, which stabilizes hypoxia-inducible factor-1A via ROS [16,17]. Elevated SOD2 is not merely a marker of progression but a driver of malignancy and temozolomide resistance, often associated with tumor-initiating cell subsets and cross-resistance to irradiation [4,18,19].

An additional observation of our study is that SOD2 induction was significantly greater in male than in female gliomas. Although the underlying mechanisms cannot be established from the present data, this finding is consistent with reports indicating that sex hormones modulate mitochondrial redox homeostasis and antioxidant gene expression [10,11]. Whether the greater SOD2 response observed in males reflects increased mitochondrial oxidative stress, differences in hormonal regulation, or both requires further investigation. Furthermore, the significant enrichment of SOD2 has been identified as a molecular marker of the proneural-to-mesenchymal transition (PMT) in human glioblastoma tissue. In this context, SOD2 upregulation, often occurring in parallel with NADPH oxidase 4 and matrix metalloproteinase 1, facilitates an aggressive migratory phenotype [20]. Our findings of increased SOD2, particularly in the more aggressive male tumors, suggest that biological sex may influence the intensity of this mesenchymal shift mediated by the Nrf2/SOD2 axis [21].

3.4. Reduced SOD3 Expression Indicates Profound Alterations in Extracellular Redox Homeostasis

In contrast to SOD2, the extracellular isoform SOD3 was significantly decreased in glioma tissue at both the transcriptional and protein levels. Because SOD3 constitutes the principal antioxidant enzyme within the extracellular compartment, its reduction suggests substantial remodeling of the extracellular redox environment during tumor development.

Loss of extracellular antioxidant protection has been associated with altered nitric oxide bioavailability, oxidative modification of extracellular matrix components, and enhanced activation of matrix metalloproteinases [5,22]. Downregulation of SOD3 in glioma tissue may foster an immunosuppressive microenvironment by altering tumor macrophage polarization [23]. Although the present study did not evaluate extracellular matrix remodeling or invasive behavior directly, the observed reduction in SOD3 expression is compatible with previous reports describing diminished extracellular antioxidant capacity in aggressive gliomas.

The loss of SOD3, a known tumor suppressor in the extracellular space, represents a step in fostering a permissive microenvironment for glioma progression [22]. Our data showing a depletion of SOD3 in males suggests a higher susceptibility to extracellular redox imbalance. Single-cell RNA sequencing analysis has confirmed that SOD3 downregulation triggers a crosstalk between neoplastic cells and immune infiltrates, specifically promoting the polarization of M2-like pro-tumoral macrophages and increasing neutrophil infiltration [23]. Consequently, the relative preservation of SOD3 expression in female gliomas may provide a comparative immunological advantage, mitigating the development of

an immunosuppressive niche and potentially explaining the better outcomes observed in females [9,23].

3.5. Biological Sex Influences Antioxidant Remodeling During Gliomagenesis

Sex differences represent one of the most consistent findings of the present study. Whereas SOD1 expression remained similar in both sexes, male gliomas exhibited greater SOD2 induction together with a more pronounced reduction in SOD3.

Epidemiological studies have consistently reported a higher incidence and poorer prognosis of gliomas in males than in females [1]. Experimental evidence also indicates that estrogens modulate antioxidant responses through multiple mechanisms, including activation of Nrf2-dependent pathways, regulation of mitochondrial function, and inhibition of NADPH oxidase activity [10,11]. Although our study was not designed to identify the molecular basis of these differences, the observed expression profiles support the concept that biological sex influences antioxidant adaptation during gliomagenesis.

Rather than demonstrating a fundamentally different mechanism of tumor progression between sexes, our findings suggest quantitative differences in the regulation of antioxidant defenses. These differences may contribute to the biological heterogeneity of gliomas and reinforce current recommendations to incorporate sex as a biological variable in experimental cancer research.

3.6. Strengths, Limitations and Perspectives

An important strength of this study is the simultaneous evaluation of the three SOD isoforms at both the transcriptional and protein levels in an established in vivo model of gliomagenesis. This dual analytical approach provides a more comprehensive characterization of antioxidant remodeling than studies focusing on a single molecular level.

First, enzymatic activity and direct reactive oxygen species production were not determined; therefore, functional catalytic changes cannot be directly inferred from expression data alone. Second, downstream redox signaling pathways, post-translational modifications, and cellular localization were not evaluated. Third, because analyses were performed on total tissue homogenates, the present data cannot distinguish whether the observed expression changes originate specifically from neoplastic cells, reactive astrocytes, neurons, vascular endothelial cells, or tumor-infiltrating immune cells [23]. Fourth, although clear sex-associated differences were identified, the precise hormonal and epigenetic mechanisms responsible for these differences remain to be elucidated [10,11]. Our findings highlight SOD isoforms as attractive targets for precision medicine. Future research should explore SOD mimetics, which have shown potential in enhancing the effects of radiotherapy [24], and the role of neuron-glioma synapses in modulating redox homeostasis [25,26]. The sex-dimorphic remodeling of the SOD system emphasizes the need for therapeutic strategies that consider biological sex as a fundamental variable in redox-targeted interventions [27].

Overall, the present findings demonstrate that antioxidant remodeling during experimental gliomagenesis is highly compartment-specific and influenced by biological sex. The coordinated increase in mitochondrial SOD2 together with the reduction of extracellular SOD3 highlights distinct adaptive responses within different cellular compartments and identifies sex-dependent regulation as an important consideration for future studies investigating redox biology in glioma.

4. Materials and Methods

4.1. Animals and Experimental Design

Adult Wistar rats were obtained from Harlan Laboratories (Barcelona, Spain). Ten females and five males were maintained under controlled environmental conditions

(22 ± 2 °C; 12 h light/12 h dark cycle) with free access to standard laboratory chow and water. All experimental procedures were conducted in accordance with the European Community Council Directive 2010/63/EU for the protection of animals used for scientific purposes and were approved by the Bioethical Committee of the University of Jaén (Ref. CVI09-4957M).

Pregnancy was confirmed by the presence of spermatozoa in vaginal smears collected the morning after overnight mating, which was designated as gestational day 1. On gestational day 18, pregnant females received a single intravenous injection of N-ethyl-N-nitrosourea (ENU) (Sigma Aldrich, Madrid, Spain) dissolved in sterile physiological saline. Control animals received an equivalent volume of saline. Offspring were delivered naturally and weaned at postnatal day 22. To eliminate potential litter effects, no more than 1–2 offspring of the same sex per litter were assigned to any single experimental group. A total of 43 offspring derived from 10 ENU-treated and 5 control pregnant dams were stratified into four finalized experimental cohorts: male healthy control ($n = 10$), male ENU glioma ($n = 12$), female healthy control ($n = 10$), and female ENU glioma ($n = 11$). Animals were examined weekly for neurological abnormalities until scheduled sacrifice at 30 weeks of age. The 30-week experimental endpoint was selected based on our established model timeline, as it corresponds to fully formed, high-grade intracranial gliomas with 100% tumor incidence without inducing excessive animal mortality.

4.2. Magnetic Resonance Imaging and Histopathological Confirmation

Tumor development was monitored *in vivo* by high-field magnetic resonance imaging using a 9.4 T Bruker BioSpec system (Bruker BioSpin, Ettlingen, Germany). Animals were anesthetized with equithesin (2 mL/kg, intraperitoneally) and positioned in a dedicated animal holder. T2-weighted spin-echo images were acquired using the following parameters: repetition time (TR), 2000 ms; echo time (TE), 80 ms; acquisition matrix, 256×192 ; slice thickness, 1 mm; and two signal averages. To improve lesion visualization, gadolinium-diethylenetriamine pentaacetic acid (Sigma-Aldrich, Madrid, Spain; Gd-DTPA; 1.5 mL/kg, intraperitoneally) was administered before image acquisition.

As previously characterized and validated by our laboratory in this experimental model, transplacental ENU administration yields a 100% tumor incidence in offspring at 30 weeks, predominantly developing high-grade astrocytic gliomas (WHO grade III–IV equivalents) localized within the cerebral hemispheres (cortex and subcortical white matter) [3,11]. *In vivo* T2-weighted magnetic resonance imaging (MRI at 9.4 T) confirmed hyperintense intracranial expanding lesions with a mean tumor multiplicity of ~ 2.0 tumors per animal and average tumor volumes ranging between 200–350 mm³ depending on biological sex [3,11]. Following euthanasia, brain tissues were collected for histopathological confirmation using established diagnostic criteria (marked cellular pleomorphism, nuclear atypia, and microvascular proliferation) and to exclude necrotic core regions prior to molecular analyses [8]. Anatomically matched cerebral tissue from non-tumor-bearing control animals processed under identical conditions served as healthy controls.

4.3. Tissue Collection and Protein Extraction

Animals were deeply anesthetized with equithesin before brain removal. Brains were rapidly excised, rinsed in ice-cold physiological saline (0.9% NaCl), and dissected immediately. Tumor tissue was carefully isolated, whereas corresponding anatomical regions from control animals were collected using the stereotaxic coordinates described by Paxinos and Watson as anatomical references.

Samples intended for gene expression analyses were immediately frozen in liquid nitrogen and stored at -80 °C until processing.

For protein analyses, tissues were homogenized (10%, *w/v*) in ice-cold 10 mM HCl-Tris buffer (pH 7.4) using a Polytron homogenizer. Homogenates were centrifuged at $100,000 \times g$ for 30 min at 4 °C, and the resulting supernatants were collected as the soluble protein fraction and stored at −80 °C until analysis. Total protein concentration was determined in triplicate using the Bradford assay with bovine serum albumin (BSA) as the calibration standard.

4.4. RNA Extraction and Quantitative Real-Time PCR (RT-qPCR)

Total RNA was extracted from brain tissue using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA concentration and purity were determined spectrophotometrically by measuring the A260/A280 absorbance ratio, and only samples showing adequate purity were included in subsequent analyses.

First-strand complementary DNA (cDNA) was synthesized from 1 µg of total RNA using random hexamer primers and Transcriptor Reverse Transcriptase (Roche Diagnostics, Barcelona, Spain).

Quantitative real-time PCR was performed using SYBR Green chemistry in a MiniOpticon Real-Time PCR Detection System (Bio-Rad, Madrid, Spain). Amplification consisted of an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The specificity of each amplification product was confirmed by melting curve analysis. Standard curves were generated from serial dilutions of pooled cDNA to calculate amplification efficiency, which ranged between 95% and 105% for all primer pairs. Relative expression levels of *Sod1*, *Sod2*, and *Sod3* were calculated using the comparative $2^{-\Delta\Delta C_t}$ method. Gene expression was normalized against the geometric mean of the relative expression quantities of the two endogenous reference genes (*Actb* and *Gapdh*), as implemented in the Bio-Rad software according to the $2^{-\Delta\Delta C_t}$ method, where $\Delta C_t = C_{t_{\text{target}}} - \text{mean}(C_{t_{\text{Actb}}}, C_{t_{\text{Gapdh}}})$, to ensure high normalization stability across healthy and tumoral tissue. All reactions were performed in duplicate. Primer sequences and expected amplicon sizes are listed in Table 3.

Table 3. Oligonucleotide primers used for quantitative gene expression analysis of SOD isoforms and constitutive controls in rat by RT-qPCR. NCBI Gene accession numbers: *Sod1* (NM_017050.1), *Sod2* (NM_017051.2), *Sod3* (NM_012880.2), *Actb* (NM_031144.3), and *Gapdh* (NM_017008.4).

Gene	Direction	Primer Sequence (5' → 3')	Amplicon Size (bp)
<i>Sod1</i>	Forward	TTTGCTCTCCCAGGTTCCG	182
	Reverse	CCCATGCTCGCCTTCAGTTA	
<i>Sod2</i>	Forward	GGACAAACCTGAGCCCTAAG	81
	Reverse	CAAAAGACCCAAAGTCACGC	
<i>Sod3</i>	Forward	TAAGCCCCTAGCATCCCCTT	80
	Reverse	ACATTCGACCTCTGGGGGTA	
<i>Actb</i>	Forward	CCCGCGAGTACAACCTTCT	72
	Reverse	CGTCATCCATGGCGAACT	
<i>Gapdh</i>	Forward	TGAACGGGAAGCTCACTGG	307
	Reverse	TCCACCACCCTGTTGCTGTA	

4.5. Quantification of SOD Proteins by ELISA

Protein abundance of SOD1, SOD2, and SOD3 was quantified in tissue homogenates using commercially available, rat-specific sandwich ELISA kits from Cusabio (Abyntek Biopharma, Vizcaya, Spain; SOD1: cat. CSB-EL022397RA, detection range 15.6–1000 pg/mL;

SOD2: cat. CSB-EL022398RA, detection range 1.87–120 pg/mL; SOD3: cat. CSB-E14981r, detection range 15.6–1000 pg/mL). Tissue homogenates were diluted (1:100 to 1:500) in assay buffer to fit within the linear optical density range. The assays were validated for rat brain homogenates, demonstrating intra-assay coefficients of variation (CV) < 8% and inter-assay CV < 10%. Serial dilutions indicated no appreciable sample matrix interference. Recombinant rat SOD proteins supplied by the manufacturer served as positive controls. Total tissue homogenates containing both intracellular and matrix-associated protein fractions were used to capture the overall tissue-associated SOD3 pool.

Briefly, standards and appropriately diluted samples were incubated in antibody-coated microplates, followed by sequential incubation with the corresponding detection reagents. Color development was achieved using tetramethylbenzidine (TMB), and the reaction was stopped with the supplied acidic stop solution. Absorbance was measured at 450 nm using a microplate reader.

Protein concentrations were calculated from four-parameter logistic (4-PL) standard curves, normalized to total protein concentration, and expressed as pg/mg total protein. All determinations were performed in duplicate.

4.6. Statistical Analysis

Data are presented as mean $\pm$ standard error of the mean (SEM). The normality of the data distribution was assessed using the Shapiro–Wilk test, and homogeneity of variances was verified with Levene’s test. Statistical analyses were performed using GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA, USA).

Differences between experimental groups were evaluated by two-way analysis of variance (ANOVA), considering tissue type (control or glioma) and biological sex (male or female) as independent variables. When appropriate, multiple comparisons were performed using Newman–Keuls post hoc tests. Statistical significance was established at $p < 0.05$.

5. Conclusions

The present study demonstrates that experimental gliomagenesis is associated with a coordinated but isoform-specific remodeling of the superoxide dismutase antioxidant system. While *Sod1*/SOD1 expression remained unchanged, *Sod2*/SOD2 was consistently upregulated and *Sod3*/SOD3 was markedly reduced, with highly concordant changes observed at both the transcriptional and protein levels. These findings indicate that antioxidant adaptation during glioma development is compartment-specific rather than uniform.

A second major finding is the identification of significant sex-associated differences in this remodeling process. Male gliomas exhibited a greater increase in SOD2 together with a more pronounced reduction in SOD3 than female tumors, whereas SOD1 remained unaffected in both sexes. These results support the concept that biological sex influences antioxidant responses during gliomagenesis and should therefore be considered an important biological variable in experimental studies of glioma.

Although the present work was limited to the analysis of gene and protein expression, the observed patterns provide a framework for future investigations addressing enzymatic activity, post-translational regulation, mitochondrial function, and extracellular redox signaling. Further studies integrating these functional approaches will be required to determine how the differential regulation of SOD isoforms contributes to glioma progression and whether these antioxidant pathways may represent targets for personalized therapeutic strategies.

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Abbreviations

The following abbreviations are used in this manuscript:

Actb	Beta-actin gene
BSA	Bovine serum albumin
cDNA	Complementary DNA
Ct	Cycle threshold
ELISA	Enzyme-linked immunosorbent assay
ENU	N-ethyl-N-nitrosourea
GABA	Gamma-aminobutyric acid
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase
IDH	Isocitrate dehydrogenase
LCS-1	SOD1 pharmacological inhibitor
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
mTORC1	Mammalian target of rapamycin complex 1
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
Nrf2	Nuclear factor erythroid 2-related factor 2
PARP	Poly (ADP-ribose) polymerase
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
SOD	Superoxide dismutase
SOD1	Cu/Zn-SOD (Cytosolic superoxide dismutase)
SOD2	Mn-SOD (Mitochondrial superoxide dismutase)
SOD3	Ec-SOD (Extracellular superoxide dismutase)
TMB	Tetramethylbenzidine

References

1. Ostrom, Q.T.; Cote, D.J.; Ascha, M.; Kruchko, C.; Barnholtz-Sloan, J.S. Adult Glioma Incidence and Survival by Race or Ethnicity in the United States from 2000 to 2014. *JAMA Oncol.* **2018**, *4*, 1254–1262. [[CrossRef](#)]
2. Agrawal, K.; Asthana, S.; Kumar, D. Role of Oxidative Stress in Metabolic Reprogramming of Brain Cancer. *Cancers* **2023**, *15*, 4920. [[CrossRef](#)]
3. Ramirez-Exposito, M.J.; Martinez-Martos, J.M. The Delicate Equilibrium between Oxidants and Antioxidants in Brain Glioma. *Curr. Neuropharmacol.* **2019**, *17*, 342–351. [[CrossRef](#)]

4. Reczek, C.R.; Chandel, N.S. The Two Faces of Reactive Oxygen Species in Cancer. *Annu. Rev. Cancer Biol.* **2017**, *1*, 79–98. [\[CrossRef\]](#)
5. Fukai, T.; Ushio-Fukai, M. Superoxide dismutases: Role in redox signaling, vascular function, and diseases. *Antioxid. Redox Signal.* **2011**, *15*, 1583–1606. [\[CrossRef\]](#)
6. König, S.; Strassheimer, F.; Brandner, N.I.; Schroder, J.H.; Urban, H.; Harwart, L.F.; Hehlhans, S.; Steinbach, J.P.; Ronellenfitsch, M.W.; Luger, A.L. Superoxide dismutase 1 mediates adaptation to the tumor microenvironment of glioma cells via mammalian target of rapamycin complex 1. *Cell Death Discov.* **2024**, *10*, 379. [\[CrossRef\]](#)
7. Price, M.; Ballard, C.A.P.; Benedetti, J.R.; Kruchko, C.; Barnholtz-Sloan, J.S.; Ostrom, Q.T. CBTRUS Statistical Report: Primary Brain and Other Central Nervous System Tumors Diagnosed in the United States in 2018–2022. *Neuro Oncol.* **2025**, *27*, iv1–iv66. [\[CrossRef\]](#)
8. Louis, D.N.; Perry, A.; Wesseling, P.; Brat, D.J.; Cree, I.A.; Figarella-Branger, D.; Hawkins, C.; Ng, H.K.; Pfister, S.M.; Reifenberger, G.; et al. The 2021 WHO Classification of Tumors of the Central Nervous System: A summary. *Neuro Oncol.* **2021**, *23*, 1231–1251. [\[CrossRef\]](#)
9. Carrano, A.; Juarez, J.J.; Incontri, D.; Ibarra, A.; Guerrero Cazares, H. Sex-Specific Differences in Glioblastoma. *Cells* **2021**, *10*, 1783. [\[CrossRef\]](#)
10. Lin, H.Y.; Liao, K.H.; Ko, C.Y.; Chen, G.Y.; Hsu, S.P.; Hung, C.Y.; Hsu, T.I. 17 β -estradiol induces temozolomide resistance through NRF2-mediated redox homeostasis in glioblastoma. *Free Radic. Biol. Med.* **2021**, *172*, 430–440. [\[CrossRef\]](#)
11. Ramirez-Exposito, M.J.; Carrera-Gonzalez, M.P.; Mayas, M.D.; Martinez-Martos, J.M. Gender differences in the antioxidant response of oral administration of hydroxytyrosol and oleuropein against N-ethyl-N-nitrosourea (ENU)-induced glioma. *Food Res. Int.* **2021**, *140*, 110023. [\[CrossRef\]](#)
12. Yen, H.C.; Lin, C.L.; Chen, B.S.; Chen, C.W.; Wei, K.C.; Yang, M.L.; Hsu, J.C.; Hsu, Y.H. Alterations of the levels of primary antioxidant enzymes in different grades of human astrocytoma tissues. *Free Radic. Res.* **2018**, *52*, 856–871. [\[CrossRef\]](#)
13. Batsios, G.; Udutha, S.; Taglang, C.; Gillespie, A.M.; Lau, B.; Ji, S.; Phoenix, T.; Mueller, S.; Venneti, S.; Koschmann, C.; et al. GABA production induced by imipridones is a targetable and imageable metabolic alteration in diffuse midline gliomas. *bioRxiv* **2024**. [\[CrossRef\]](#)
14. Ling, M.; Liu, Q.; Wang, Y.; Liu, X.; Jiang, M.; Hu, J. LCS-1 inhibition of superoxide dismutase 1 induces ROS-dependent death of glioma cells and degrades PARP and BRCA1. *Front. Oncol.* **2022**, *12*, 937444. [\[CrossRef\]](#)
15. Agnihotri, S.; Zadeh, G. Metabolic reprogramming in glioblastoma: The influence of cancer metabolism on epigenetics and unanswered questions. *Neuro Oncol.* **2016**, *18*, 160–172. [\[CrossRef\]](#)
16. Agnihotri, S.; Golbourn, B.; Huang, X.; Remke, M.; Younger, S.; Cairns, R.A.; Chalil, A.; Smith, C.A.; Krumholtz, S.L.; Mackenzie, D.; et al. PINK1 Is a Negative Regulator of Growth and the Warburg Effect in Glioblastoma. *Cancer Res.* **2016**, *76*, 4708–4719, Correction in *Cancer Res.* **2022**, *82*, 4695. [\[CrossRef\]](#)
17. Chang, K.Y.; Hsu, T.I.; Hsu, C.C.; Tsai, S.Y.; Liu, J.J.; Chou, S.W.; Liu, M.S.; Liou, J.P.; Ko, C.Y.; Chen, K.Y.; et al. Specificity protein 1-modulated superoxide dismutase 2 enhances temozolomide resistance in glioblastoma, which is independent of O⁶-methylguanine-DNA methyltransferase. *Redox Biol.* **2017**, *13*, 655–664. [\[CrossRef\]](#)
18. Chien, C.H.; Chuang, J.Y.; Yang, S.T.; Yang, W.B.; Chen, P.Y.; Hsu, T.I.; Huang, C.Y.; Lo, W.L.; Yang, K.Y.; Liu, M.S.; et al. Enrichment of superoxide dismutase 2 in glioblastoma confers to acquisition of temozolomide resistance that is associated with tumor-initiating cell subsets. *J. Biomed. Sci.* **2019**, *26*, 77. [\[CrossRef\]](#)
19. Hsueh, W.T.; Chang, K.Y.; Chuang, J.Y.; Liu, M.S.; Chung, P.H.; Chu, J.M.; Chien, C.H. SOD2-mediated TMZ-resistant Glioblastoma Cells Exhibit Cross-resistance to Irradiation. *Anticancer. Res.* **2025**, *45*, 3711–3718. [\[CrossRef\]](#)
20. Tomko, N.; Kluever, M.; Wu, C.; Zhu, J.; Wang, Y.; Salomon, R.G. 4-Hydroxy-7-oxo-5-heptenoic acid lactone is a potent inducer of brain cancer cell invasiveness that may contribute to the failure of anti-angiogenic therapies. *Free Radic. Biol. Med.* **2020**, *146*, 234–256. [\[CrossRef\]](#)
21. Su, I.C.; Su, Y.K.; Setiawan, S.A.; Yadav, V.K.; Fong, I.H.; Yeh, C.T.; Lin, C.M.; Liu, H.W. NADPH Oxidase Subunit CYBB Confers Chemotherapy and Ferroptosis Resistance in Mesenchymal Glioblastoma via Nrf2/SOD2 Modulation. *Int. J. Mol. Sci.* **2023**, *24*, 7706, Correction in *Int. J. Mol. Sci.* **2025**, *26*, 4837. [\[CrossRef\]](#)
22. Griess, B.; Tom, E.; Domann, F.; Teoh-Fitzgerald, M. Extracellular superoxide dismutase and its role in cancer. *Free Radic. Biol. Med.* **2017**, *112*, 464–479. [\[CrossRef\]](#)
23. Liang, X.; Wang, Z.; Dai, Z.; Liu, J.; Zhang, H.; Wen, J.; Zhang, N.; Zhang, J.; Luo, P.; Liu, Z.; et al. Oxidative stress is involved in immunosuppression and macrophage regulation in glioblastoma. *Clin. Immunol.* **2024**, *258*, 109802. [\[CrossRef\]](#)
24. Anwar, S.; Sarwar, T.; Khan, A.A.; Rahmani, A.H. Therapeutic Applications and Mechanisms of Superoxide Dismutase (SOD) in Different Pathogenesis. *Biomolecules* **2025**, *15*, 1130. [\[CrossRef\]](#)
25. Cueto-Urena, C.; Ramirez-Exposito, M.J.; Martinez-Martos, J.M. Neuron-Glioma Synapses in Tumor Progression. *Biomedicines* **2025**, *14*, 72. [\[CrossRef\]](#)

26. Guerreiro, J.F.; Gomes, M.; Pagliari, F.; Jansen, J.; Marafioti, M.G.; Nistico, C.; Hanley, R.; Costa, R.O.; Ferreira, S.S.; Mendes, F.; et al. Iron and copper complexes with antioxidant activity as inhibitors of the metastatic potential of glioma cells. *RSC Adv.* **2020**, *10*, 12699–12710. [[CrossRef](#)]
27. Cueto-Urena, C.; Ramirez-Exposito, M.J.; Carrera-Gonzalez, M.P.; Martinez-Martos, J.M. Hydroxytyrosol and Brain Tumors: Mechanisms of Action and Therapeutic Potential. *Curr. Issues Mol. Biol.* **2025**, *47*, 667. [[CrossRef](#)]

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