

Clonidine Attenuates Oxidative Stress Induced by H₂O₂ in C6 Glial Cells

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ABSTRACT


Oxidative stress plays a critical role in the pathogenesis of neurodegenerative diseases and glioma progression, with glial cells being central to maintaining redox homeostasis. Clonidine, an α_2 -adrenergic and imidazoline receptor agonist, has demonstrated neuroprotective and antioxidant effects in various experimental models. This study aimed to evaluate the protective effects of clonidine against hydrogen peroxide (H₂O₂)-induced oxidative damage in C6 glial cells. C6 glioma cells were pretreated with clonidine (0.3–4.8 μ M) for 1 hour, followed by exposure to 0.5 mM H₂O₂ for 24 hours, and cell viability was assessed using the XTT assay, while total antioxidant status (TAS), total oxidant status (TOS), and superoxide dismutase (SOD) levels were measured using commercial kits and ELISA. H₂O₂ exposure significantly reduced cell viability and antioxidant parameters while increasing oxidative markers; however, clonidine treatment significantly improved cell viability, elevated TAS and SOD levels, and decreased TOS levels compared to the H₂O₂ group, demonstrating its dose-dependent protective effect against oxidative stress. These findings suggest that clonidine mitigates oxidative damage in C6 glial cells, potentially through antioxidant, anti-inflammatory, and imidazoline receptor-mediated mechanisms, and may have therapeutic relevance in conditions characterized by glial oxidative stress, including neurodegenerative diseases and glioma.


Keywords: Clonidine, Glial cells, Hydrogen peroxide, Neuroprotection, Oxidative stress.



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1. Introduction

Glial cells are distributed across the entire nervous system and play critical roles in maintaining homeostasis, supporting neuronal function, and mediating immune responses. Among these cells, astrocytes are particularly important for preserving neuronal stability and protecting the brain from oxidative damage [1–3]. Because of their central role in regulating redox balance, astrocytes have become important targets in studies investigating neurodegeneration and oxidative stress.

Recent advances have redefined glial cells, particularly astrocytes and microglia, as central contributors to the development of neurodegenerative disorders. Rather than being passive responders to neuronal injury, these cells actively contribute to disease through impaired redox homeostasis, neuroinflammation, and disruption of metabolic support. Genetic studies have further supported this view, identifying glia-enriched genes such as *APOE* and *TREM2* as key risk factors in Alzheimer's disease. Accordingly, therapeutic strategies that restore or preserve glial antioxidant capacity may hold promise for modulating disease progression [4].

Under normal physiological circumstances, a dynamic equilibrium exists between the production of reactive oxygen species (ROS) and the antioxidant defense systems

that neutralize them. Disruption of this balance in favor of ROS—either due to excessive production or impaired antioxidant response—leads to oxidative stress [5,6]. Elevated ROS levels damage cellular macromolecules such as proteins, lipids and DNA, ultimately disturbing key intracellular signaling pathways [7]. Although ROS—including hydrogen peroxide (H₂O₂)—are essential signaling molecules at low levels, their accumulation leads to genotoxicity, mitochondrial dysfunction, and cell death [8,9].

Oxidative stress not only plays a major role in neurodegeneration but is also implicated in tumor initiation and progression. Elevated ROS levels can activate multiple transcription factors and inflammatory pathways, promoting tumor cell proliferation, invasion, and resistance to chemotherapy and radiotherapy. Additionally, ROS produced by immune cells like macrophages contribute to various stages of carcinogenesis [10].

Numerous biochemical parameters have been proposed to evaluate oxidative stress and antioxidant defenses. However, assessing all individual oxidant and antioxidant molecules simultaneously is impractical. Therefore, integrated indicators that reflect the overall

redox balance have been developed. Among these, total oxidant status (TOS) represents the cumulative oxidant burden in a biological sample, whereas total antioxidant status (TAS) reflects the combined activity of endogenous and exogenous antioxidants. The reliability and validity of these measurements have been demonstrated in previous studies, and standardized commercial assay kits are now widely available for their determination [11,12].

Clonidine is an agonist of alpha-2 adrenergic receptors and primarily acts on the central nervous system (CNS) by stimulating presynaptic α_2 -adrenoceptors to regulate blood pressure. [13]. Since the 1970s, α_2 -adrenergic receptor agonists have been widely used to manage hypertension and reduce cardiac afterload. Beyond cardiovascular effects, they also produce analgesic, sedative, anxiolytic, and sympatholytic effects. Notably, studies have shown that these agents can improve both structural and functional outcomes when administered during ischemic injury to the nervous system [14]. Clonidine also activates imidazoline I_1 receptors, which is linked to both the antihypertensive action [15] and the modulation of oxidative stress and nitric oxide pathways, which influence cognitive function and neuroprotection. By interacting with these systems clonidine may help counteract neuroinflammation and oxidative damage, supporting its potential protective role in neurodegenerative conditions [16].

This study aims to evaluate the possible protective role of clonidine against oxidative damage induced by hydrogen peroxide (H_2O_2) in the C6 glial cell line, a commonly used in vitro model for astrocyte-like glial responses.

2. Materials and Methods

2.1. Cell Culture and Chemicals

C6 glioma cells were purchased from the American Type Culture Collection (ATCC, USA) and cultured in 25 cm^2 flasks using DMEM (ThermoFisher Scientific, USA) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, and 1% penicillin-streptomycin. The cultures were incubated at 37°C in a humidified environment containing 5% CO_2 . Subculturing was carried out once the cultures reached approximately 80% confluency, and experiments were initiated after the third passage. FBS, L-glutamine, penicillin/streptomycin, clonidine hydrochloride and H_2O_2 were supplied by Sigma-Aldrich (USA).

2.2. XTT Cell Viability Assay

Cell viability was evaluated using the XTT assay (Roche Diagnostics, MA, USA) as previously described [17]. C6 glioma cells were plated in 96-well plates at a concentration of 1×10^4 cells per well using 100 μL of complete DMEM and incubated overnight at 37 °C in a humidified environment with 5% CO_2 to allow attachment.

Cells were divided into the following experimental groups: control, H_2O_2 -only, clonidine-only, and clonidine + H_2O_2 . Clonidine was applied at concentrations of 0.3, 0.6, 1.2, 2.4, and 4.8 μM . The selected concentration range was based on previously reported in vitro studies demonstrating protective and antioxidant effects of clonidine in cultured cells [18]. After pre-treatment with clonidine for 1 hour, 0.5 mM H_2O_2 was introduced to the appropriate wells to induce oxidative stress, as previously described in C6 glial cells [19], and the cells were further incubated for 24 hours. Following incubation, the culture medium was discarded, and wells were gently washed twice with phosphate-buffered saline (PBS). Subsequently, 100 μL of phenol-red free DMEM and 50 μL of XTT labeling solution were added to each well, and the plates were incubated at 37 °C for 4 hours.

The absorbance was then recorded at 450 nm using a microplate reader (Thermo Fisher Scientific, Altrincham, UK). All experiments were conducted in triplicate, and cell viability was calculated as a percentage compared to the control group without treatment, which was defined as 100% viability [20]. The experimental workflow is summarized in Figure 1.

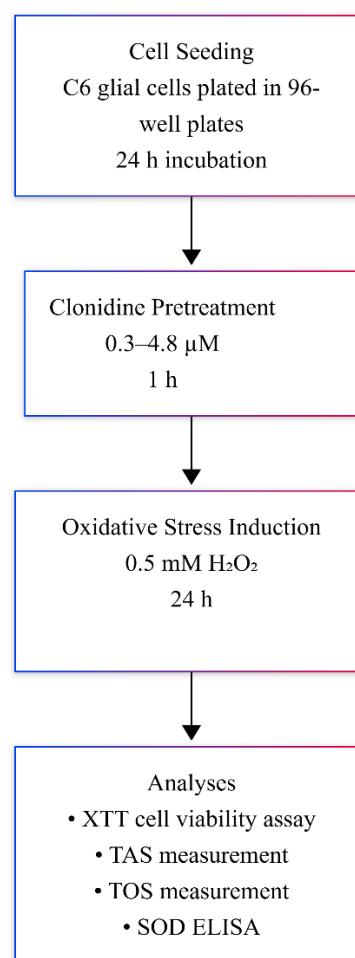


Figure 1. schematic representation of the experimental workflow used to evaluate the protective effects of clonidine against H_2O_2 -induced oxidative stress in C6 glial cells

2.3. Preparation of Cell Homogenates

Cell homogenates were prepared following a previously described method [17]. Briefly, cells from each experimental group were harvested into sterile tubes and centrifuged at 2000 rpm for 10 minutes. The supernatants were discarded, and the resulting cell pellets were resuspended in phosphate-buffered saline (PBS, pH 7.4) to obtain a final concentration of approximately 1×10^6 cells/mL. Cell lysis was performed through subjecting the suspensions to repeated freeze–thaw cycles, allowing the release of intracellular contents. The lysates were centrifuged at 4000 rpm for 10 minutes at 4 °C, after which the supernatant fractions were carefully separated for subsequent biochemical assays. Total protein concentrations in the samples were determined using the Bradford assay kit (Merck Millipore, Darmstadt, Germany) [21,22].

2.4. Assessment of Total Antioxidant, Oxidant, and Superoxide Dismutase (SOD) Levels

To evaluate the antioxidant and oxidant status after H₂O₂-induced oxidative stress, the 4.8 μM clonidine concentration, which demonstrated the highest protective effect in cell viability assays, was selected for TAS, TOS, and SOD analyses.

TAS and TOS levels in the collected cell supernatants were quantified using commercially available assay kits (Rel Assay Diagnostics, Gaziantep, Turkey) according to the manufacturer's protocols originally established by Erel [11,12].

The TAS assay evaluates the total antioxidant capacity of the sample based on the ability of antioxidants to reduce the blue-green colored ABTS radical cation (ABTS•⁺) to a colorless form. Reagent 1 (buffer solution) and Reagent 2 (containing ABTS•⁺) were sequentially added to the wells containing standards and cell supernatants. After a 5-minute incubation at 37 °C, absorbance was recorded at 660 nm. The degree of decolorization reflects the antioxidant capacity, and results were expressed as micromoles of Trolox equivalents per milligram of protein (μmol Trolox Eq/mg protein), using Trolox as the standard calibrator.

The TOS assay determines total oxidant status by measuring the oxidation of a ferrous ion–chelator complex to ferric ion in an acidic medium. Reagent 1 (buffer) and Reagent 2 (prochromogen containing xylenol orange) were added to the samples. Enhancer molecules present in the medium promote the oxidation reaction. Ferric ions generated in the process react with xylenol orange to produce a colored complex, and absorbance

was measured at 530 nm after a 5-minute incubation at 37 °C. Results were calculated based on a hydrogen peroxide calibration curve and reported as micromoles of hydrogen peroxide equivalents per milligram of protein (μmol H₂O₂ Eq/mg protein).

Superoxide dismutase (SOD) levels in cell supernatants were measured using a commercial sandwich ELISA kit (SunLong Biotech Co., Ltd., Hangzhou, China; Cat. No. SL0664Ra), following the manufacturer's protocol. The assay is based on the antigen–antibody binding principle using a 96-well microplate pre-coated with an anti-SOD antibody. Standards and samples were added to the wells and incubated to allow binding of SOD. After washing, an HRP-conjugated detection antibody was added, followed by chromogenic substrate (TMB).

The enzymatic reaction was terminated with stop solution, and the resulting color change was quantified at 450 nm using a microplate reader (Thermo Fisher Scientific, Altrincham, UK). SOD concentrations in the samples were calculated by interpolation from a standard curve generated using known concentrations of rat SOD. Final results were normalized to total protein content and expressed as nanograms per gram of protein (ng/g protein).

2.5. Statistical Analysis

Data were analyzed using IBM SPSS Statistics 29.0 for Windows (IBM Corp., Armonk, NY, USA). Results were expressed as the mean ± standard error of the mean (SEM). Statistical comparisons among groups were performed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test to identify group differences. A p-value of less than 0.05 was regarded as statistically significant.

3. Results and Discussion

3.1. Effect of Clonidine on Cell Viability Following H₂O₂-Induced Oxidative Stress

The XTT assay was used to evaluate the protective effects of clonidine against hydrogen peroxide (H₂O₂)-induced oxidative stress in C6 glial cells. As shown in Figure 2, Exposure to 0.5 mM H₂O₂ for 24 hours led to a significant decline in cell viability compared to the control group without treatment (p < 0.05). However, co-treatment with clonidine at all tested concentrations (0.3, 0.6, 1.2, 2.4, and 4.8 μM) significantly increased cell viability compared to the H₂O₂-only group (p < 0.05), indicating a dose-dependent protective effect.

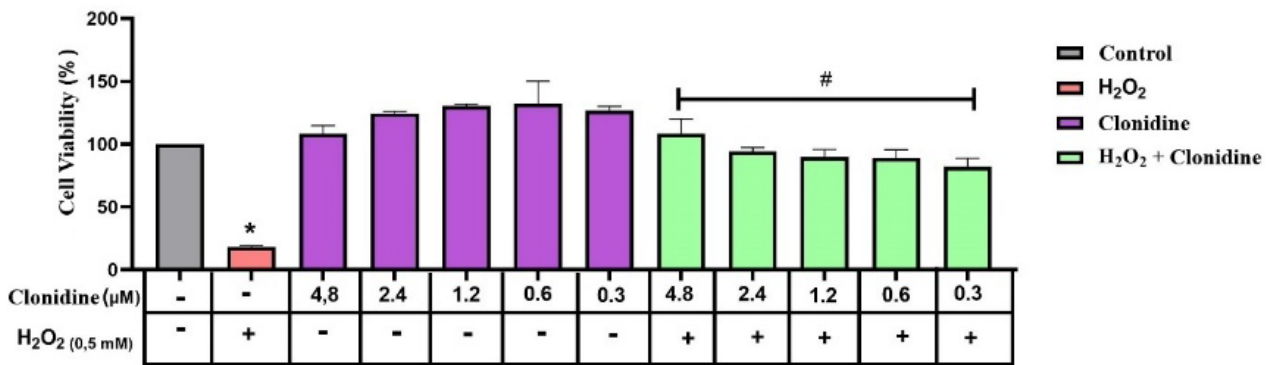


Figure 2. Influence of clonidine on the viability of C6 cells exposed to oxidative stress induced by H₂O₂. Data are expressed as mean ± SEM. *p < 0.001 vs. Control group; #p < 0.001 vs. H₂O₂ group.

Oxidative stress plays a crucial role in promoting cellular dysfunction in the CNS and is strongly implicated in both neurodegenerative disease progression and tumor biology. In the present work, we investigated the possible antioxidative properties of clonidine in the C6 glial cell line under conditions of oxidative stress induced by hydrogen peroxide. Our results demonstrate that clonidine significantly enhanced cell viability and restored redox balance, as evidenced by increased TAS and SOD levels and decreased TOS levels. These findings support the notion that clonidine can mitigate oxidative damage in glial-like cells.

Consistent with our findings, previous studies have demonstrated that clonidine can exhibit antioxidative activity in various in vivo models. For example, clonidine improved total antioxidant capacity and reduced lipid peroxidation and protein oxidation indicators in the hearts of spontaneously hypertensive rats [23] and reduced oxidative damage in multiple organs following hemorrhagic shock in rats [24]. Similarly, its neuroprotective properties in models of neonatal hypoxic-ischemic brain damage have been linked to its capacity to reduce oxidative stress and neuroinflammation, primarily through stimulation of the Nrf2/HO-1 signaling pathway and NF-κB suppression [25]. These mechanisms may underlie the observed improvements in antioxidant status in our C6 glial cell model.

Our findings also align with previous work demonstrating that clonidine attenuates oxidative damage in kidney tissues subjected to ethanol or cisplatin toxicity, likely via modulation of the p38 MAPK pathway and activation of imidazoline-1 receptors [26, 27]. Beyond these in vivo observations, clonidine has also shown antioxidative effects in vitro. Previous work in rat C6 glioma cells demonstrated that clonidine can modulate nitric oxide synthase activity and influence nitric oxide production, indicating a potential interaction between clonidine and oxidative signaling pathways [28].

Dysregulation of nitric oxide signaling is known to contribute to oxidative and nitrosative stress as well as neuroinflammatory processes implicated in neurodegeneration [29]. In human brain microvascular endothelial cells exposed to angiotensin II, clonidine pretreatment (0.1–10 μM) significantly attenuated intracellular ROS generation and preserved cell viability in a concentration-dependent manner, supporting a direct cellular antioxidant/cytoprotective action [18]. The shared antioxidant effect across organ systems suggests that clonidine’s redox-modulatory action may be generalizable and mechanistically conserved, possibly involving transcriptional regulation of antioxidant enzymes, including superoxide dismutase.

3.2. Effect of Clonidine on TAS, TOS, and SOD Levels Following H₂O₂-Induced Oxidative Damage

As shown in Figure 3, exposure to 0.5 mM H₂O₂ for 24 hours significantly decreased total antioxidant status (TAS) in C6 cells compared to the control group (p < 0.001). Treatment with clonidine at 4.8 μM alone significantly increased TAS levels relative to control (p < 0.05), and both clonidine alone and clonidine + H₂O₂ groups showed significantly higher TAS levels compared to the H₂O₂-only group (p < 0.001) (Figure 3A).

Total oxidant status (TOS) was significantly elevated in the H₂O₂ group compared to control (p < 0.001). Treatment with 4.8 μM clonidine, either alone or in combination with H₂O₂, significantly reduced TOS levels relative to the H₂O₂ group (p < 0.001), with no statistically significant difference compared to the control group (p > 0.05) (Figure 3B).

H₂O₂ exposure also significantly decreased SOD levels compared to control (p < 0.001). Although the clonidine + H₂O₂ group continued to show lower SOD levels than control (p < 0.001), both the clonidine-alone and clonidine + H₂O₂ groups exhibited significantly higher SOD levels compared to the H₂O₂-only group (p < 0.05) (Figure 3C).

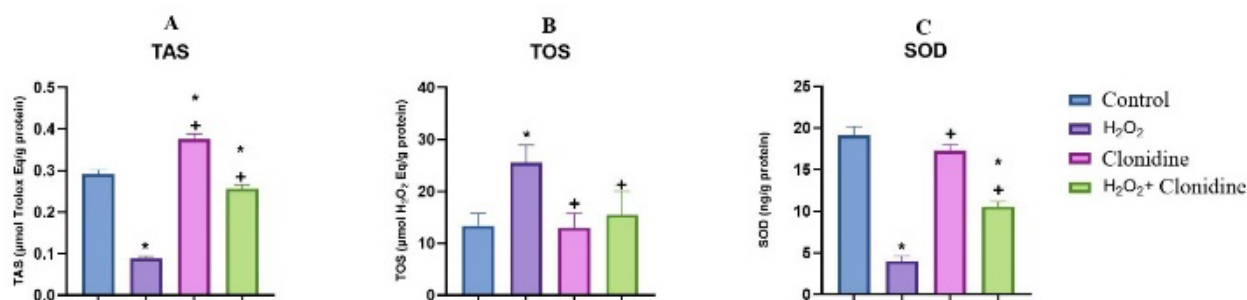


Figure 3. Influence of clonidine on TAS (A), TOS (B), and SOD (C) levels in C6 cells following oxidative stress induced by H₂O₂. Data are presented as mean ± SEM. *p < 0.05 vs. Control group; +p < 0.05 vs. H₂O₂ group.

Our results are particularly important given the emerging recognition of glial oxidative stress as a primary contributor to the pathological processes underlying neurodegeneration. Recent evidence indicates that glial dysfunction precedes neuronal degeneration in disorders like amyotrophic lateral sclerosis (ALS) and Alzheimer's disease. This dysfunction is often characterized by excessive ROS production, excitotoxicity, and impaired protein clearance [9]. By reducing oxidative stress in glial cells, clonidine may help preserve their homeostatic and neuroprotective roles, potentially interrupting feedback loops that drive disease progression.

The protective effects of clonidine may also be partly attributed to its agonistic action at imidazoline receptors (IRs), which play a significant neuroprotective role by modulating cellular defenses against oxidative stress—an underlying mechanism implicated in the development of neurodegenerative disorders like Alzheimer's disease, Huntington's disease, Parkinson's disease, and ischemic stroke [30,31]. Their antioxidant effects are mediated through multiple pathways that collectively preserve neuronal viability and cellular homeostasis. One critical mechanism involves stabilization of lysosomal membranes, thereby protecting cells from oxidative stress-induced lysosomal rupture and subsequent cytotoxicity [32]. Additionally, IRs have been shown to enhance autophagy—a fundamental cellular process that clears damaged organelles and maintains proteostasis—by promoting the transformation of LC3-I to LC3-II, a key autophagosomal marker, and supporting the lysosomal localization of cathepsin D [33,34]. Furthermore, IR activation reduces the accumulation of p62/SQSTM1, a stress-related protein whose buildup is known to impair autophagic flux under oxidative conditions [35]. Beyond autophagy, IR signaling boosts the function of intrinsic antioxidant enzymes like catalase and superoxide dismutase, thereby aiding in the neutralization of reactive oxygen species and the mitigation of oxidative damage within neuronal systems [36]. These multifaceted effects highlight the potential of IRs as key modulators of cellular resilience in oxidative environments and may partially explain the antioxidant and cytoprotective effects observed with clonidine in our study.

Taken together, these findings suggest that clonidine exerts its protective effects through a multi-faceted mechanism involving direct antioxidant action,

enhancement of endogenous defense pathways (e.g., Nrf2, IR signaling), and possibly suppression of inflammation. These properties make clonidine a promising candidate for further investigation in models of neurodegenerative and neuroinflammatory disorders, where glial oxidative stress is a central feature. Although the present findings provide evidence for the antioxidant potential of clonidine in C6 glial cells, this study represents an initial experimental investigation, and further studies involving additional cellular models, in vivo experiments, and detailed molecular analyses are required to better clarify the underlying mechanisms.

Despite these findings, several limitations should be acknowledged. The present study was conducted in an in vitro glial cell model and primarily focused on biochemical and cell viability assays to evaluate oxidative stress and antioxidant responses. Morphological evaluation of the cells was not performed, which may provide additional insight into the cellular effects of clonidine. Future studies incorporating microscopic analysis and additional molecular markers would further clarify the protective mechanisms observed.

Conflict of Interest

There are no conflicts of interest in this work.

Acknowledgments

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Declaration of Generative AI

The authors did not use any generative AI or AI-assisted technologies in the preparation of this manuscript, including the data analysis and writing stages.

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