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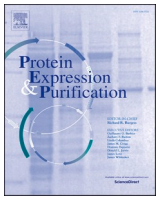

Research Article

Expression and purification of recombinant glutaredoxin 1 and protection against oxidative stress injury during cerebral ischemia-reperfusion injury

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ABSTRACT

Glutaredoxin (Grx) is a small molecular protein widely found in both prokaryotes and eukaryotes, serving various biological functions, including participation in redox reactions and exerting anti-apoptotic effects[1]. To evaluate the protective effect of recombinant Grx1 against oxidative stress, we constructed the pET-30a (+)/Grx1 recombinant plasmid and performed soluble expression and purification of the recombinant Grx1. In vitro experiments, including ABTS and DPPH radical scavenging assays, showed that recombinant Grx1 has significant antioxidant activity. Reactive oxygen species detection revealed that the levels of reactive oxygen species in the Grx1 treatment group decreased by 33.01 % compared to the H_2O_2 group. Flow cytometry analyses indicated that the number of apoptotic cells in the Grx1 treatment group decreased by 23.51 % relative to the H_2O_2 group. Additionally, qRT-PCR analysis showed that Grx1 significantly reduced the expression levels of genes such as IL-1β, TNF-α, IL-6, and caspase-3 in PC12 cells. *In vivo*, recombinant Grx1 was utilized to treat cerebral ischemia-reperfusion injury (CIRI). Histological staining revealed that recombinant Grx1 significantly mitigated hippocampal tissue damage. Western blotting analysis demonstrated that Grx1 can reduce neuronal apoptosis following CIRI by decreasing Bax expression while increasing Bcl-2 expression. Furthermore, Grx1 was shown to modulate the HO-1/Nrf2 signaling pathway by elevating the expression of Nrf2 and HO-1. In summary, this study successfully overexpressed biologically active Grx1 in *E. coli,* and confirms that recombinant Grx1 exhibits remarkable antioxidant activity in both *in vitro* and *in vivo* experiments, effectively alleviating oxidative stress damage associated with ischemic stroke.

1. Introduction

Oxidative stress represents an imbalance between oxidants and antioxidants in living organisms, resulting in increased oxidation and disruption of redox signaling [1]. The study of oxidative stress encompasses various fields, including biology, physiology, pathology, medicine, health, and disease [2,3]. It is widely acknowledged as a significant contributor to toxicity and disease. Numerous studies have established links between oxidative stress and aging, as well as various diseases such as atherosclerosis, ischemic stroke, and Alzheimer's disease, with many underlying biochemical mechanisms elucidated [4,5]. Given its pivotal role in clinical pathogenesis, the development of effective antioxidant drugs are essential. Glutaredoxin (Grx), which are small proteins containing active cysteine residues, have recently been found in various subtypes across different organisms, and their functions have been thoroughly investigated [1,6]. Chai et al. demonstrated that glutaredoxins specifically catalyze reversible protein S-glutathionylation, thereby regulating redox signaling and preventing the irreversible oxidation of protein thiols [7]. Haseena et al. showed that the glutaredoxin system safeguards the brain during oxidative stress by reducing oxidized glutathione levels, consequently decreasing the risk of neurodegenerative diseases [8]. The glutaredoxin family plays a crucial role in regulating glutathionylation and deglutathionylation, which are vital for controlling glutathione-dependent redox reactions, thus achieving antioxidant effects by maintaining normal GSH levels in the body [1].

Glutaredoxin 1(Grx1), a member of the glutaredoxin family, plays a crucial role in regulating cellular functions and maintaining redox status under varying environmental conditions [9,10]. It catalyzes the reduction of disulfide bonds that occur between proteins, as well as mixed disulfide bonds formed between protein thiols and small molecule

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thiols, such as the common mixed disulfide bond between proteins and glutathione (GSH Pr-S-S-G) [11]. The regulation of glutathionylation by glutaredoxin 1 is linked to the pathogenesis of various diseases, including neurodegenerative disorders, fatty liver, and cancer Increasing evidence supports anti-inflammatory, anti-apoptotic, and antioxidant functions [12–15]. Mannix et al. demonstrated that Grx1 significantly influences cardiovascular and cerebrovascular diseases, including myocardial ischemia-reperfusion injury, cardiac hypertrophy, and atherosclerosis [16]. Ischemia-reperfusion injury (IRI) is a common complication of stroke, involving complex mechanisms such as calcium overload, mitochondrial autophagy, cell apoptosis, and oxidative stress [17–20]. The development of effective drugs to prevent IRI remains a significant challenge. Studies indicate that recombinant Grx1 may be a potential therapeutic agent for brain ischemia-reperfusion injury. However, its efficacy and underlying mechanisms are not yet fully understood. In this study, we expressed and purified recombinant Grx1 using *Escherichia coli (E. coli)* and demonstrated its antioxidant activity both *in vivo* and *in vitro*. Cellular experiments confirmed its effectiveness in alleviating oxidative stress damage. Additionally, animal experiments showed that recombinant Grx1 effectively treats rat brain ischemia-reperfusion injury. This study established a method for the efficient expression and purification of recombinant Grx1 in *E. coli* and demonstrated the antioxidant activity of Grx1 both *in vitro* and *in vivo*. Furthermore, we have shown that Grx1 is capable of activating the Nrf2/HO-1 pathway, which downregulates inflammatory and pro-apoptotic factors. This research lays a foundation for further exploration of the role of recombinant Grx1 in oxidative stress damage and provides new insights into the treatment of diseases associated with oxidative stress.

2. Materials and methods

2.1. Materials and reagents

According to the Grx1 protein sequence (NCBI Reference Sequence: NP_001112362.1),the company optimized the codons in the gene sequences and synthesized the generated gene sequences. Gene sequencing and primer synthesis were performed by Zhejiang Sunya Biotechnology Company. The pET30a (+) plasmid and *Escherichia coli (E. coli)* strains *BL21*(DE3) and DH5α are preserved in our laboratory. Plasmid extraction kit was purchased from Axygen Biotechnology Company. The anti-Grx1 tag rabbit monoclonal antibody was obtained from Beijing Bio-Tech Bio-Technology Co., Ltd. (Beijing, China). Additionally, the anti-His tag mouse monoclonal antibody, horseradish peroxidase-conjugated goat anti-mouse IgG, horseradish peroxidase-conjugated goat anti-rabbit IgG, and protein marker were purchased from Shanghai Beyotime Biotechnology Co., Ltd. (Shanghai, China). Monoclonal antibodies were procured from Proteintech Group, Inc., China. SPF-grade male SD rats were provided by the animal experimental research center of Zhejiang Chinese Medical University.

2.2. Construction of recombinant plasmid pET-30a (+)/Grx1

To enhance the expression level, we first optimized the codon usage of the Grx1 gene based on the Grx1 amino acid sequence. After synthesis, the Grx1 gene fragment was inserted into the *Nde* I and *Xho* I restriction sites of the pET30a (+) vector. The pET-30a (+)/Grx1 recombinant plasmid was then transformed into *E. coli* BL21(DE3) competent cells, and screening was performed on Luria-Bertani (LB) agar plates containing kanamycin at a concentration of 50 μg/ml.

2.3. Expression of recombinant Grx1 in E. coli

A single colony from an agar plate was used to inoculate 10 mL of LB media supplemented with 100 μg/mL kanamycin, and the culture was shaken overnight at 37 °C. For small-scale expression cultures, 50 μL of the overnight culture was used to inoculate 5 mL of LB media in a conical flask. The pre-cultures were grown to an absorbance of 0.6–0.8 at 600 nm. At this point, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the subculture to induce Grx1 expression. The recombinant proteins were induced at 20 °C for 12 h. Cells of *E.coli* were harvested and sonicated on ice using an ultrasonic disintegrator, after which the cell suspensions were centrifuged at 5000 g for 20 min at 4 °C. The supernatant and precipitate were analyzed using 12 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Subsequently, the recombinant Grx1 expression conditions were optimized by varying IPTG concentrations (0 mM, 0.25 mM, 0.5 mM, and 1 mM), incubation times (8 h, 10 h, 12 h, and 14 h), and temperatures (16 °C, 20 °C, 24 °C, and 28 °C). The expression levels of soluble Grx1 were also analyzed by SDS-PAGE.

2.4. Purification of recombinant Grx1

Recombinant Grx1 was purified using Ni-NTA affinity chromatography. In brief, the fermentation broth was collected and centrifuged at 4 °C at 12,000 rpm for 20 min. The resulting pellet was sonicated on ice for 20 min and then subjected to a second centrifugation at 4 °C at 12,000 rpm for 20 min. The supernatant was filtered through a 0.45 μm membrane and subsequently purified with a Ni-NTA affinity column (Biodragon, Suzhou, China). The eluates were collected, concentrated using a 3 kDa ultrafiltration tube at 4 °C, and analyzed by SDS-PAGE.

2.5. Identification of the recombinant Grx1

The recombinant Grx1 was identified using Western blotting. Recombinant proteins were transferred to a nitrocellulose (NC) membrane, which was subsequently blocked with 5 % non-fat milk at room temperature (RT) for 2 h. The membrane was then incubated overnight at 4 °C with 1:1000 dilutions of anti-His rabbit polyclonal antibody and anti-GRX1 mouse polyclonal antibody. Following this, the membrane was washed three times with 1 × TBST buffer (10 min per wash) to remove unbound primary antibodies. It was then incubated with horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse IgG (H&L) antibodies for 2 h. After another washing step with TBST to eliminate unbound secondary antibodies, the membrane was then exposed to a Super Signal chemiluminescent substrate kit (Beyotime Biotechnology, Shanghai, China) and incubated. Protein bands were visualized using a Tanon-6200 imaging system and photographed for documentation.

2.6. In vitro antioxidant capacity evaluation

To assess the antioxidant activity of recombinant Grx1 *in vitro*, we employed the ABTS and DPPH radical scavenging assays. The recombinant Grx1 protein was diluted to various concentrations (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mg/mL). The diluted solutions were added to the micro-plate according to the provided instructions. Following a reaction shielded from light, absorbance was using with a microplate reader to calculate the scavenging rate.

2.7. Inhibition of oxidative stress damage

PC12 cells in the logarithmic growth phase were seeded in a 96-well plate at a density of 100,00 cells per well and cultured in a CO_2 incubator for 20 h. The cells were then subjected to oxidative stress by incubating in serum-free DMEM medium containing 200 μM H_2O_2 for 10 h. Following this treatment, the old culture medium was removed from the 96-well plate, and the cells were gently washed three times with PBS. Various concentrations of Grx1 (0, 1.5, 3.0, 6.0, 12.0, 24.0, 48.0 μM) were subsequently added to complete DMEM medium. The Petri dish was then returned to the incubator for an additional 12 h. Cell morphology was observed, and cell viability was assessed using the cell

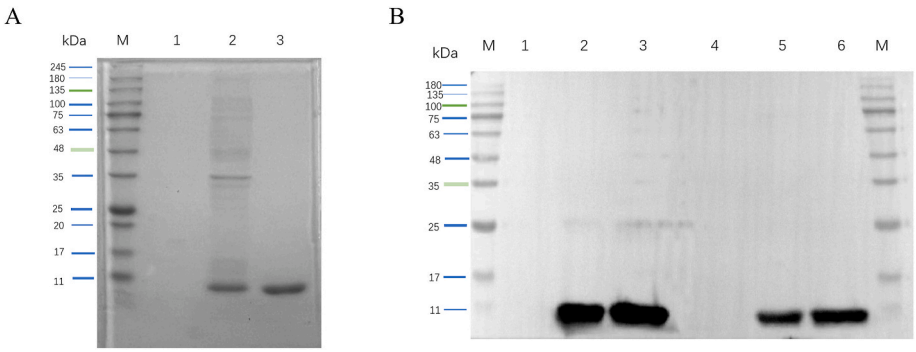

Fig. 1. Optimization of protein expression and identification by Western blotting. **A**: Expression and purification of Grx1 protein; M: Marker; Lane 1: Supernatant of non-induced sample; Lane 2: Supernatant of induced sample; Lane 3: Purification with Ni-NTA chromatography.
B: Western blotting analysis of Grx1 protein; Lane 1: Supernatant of non-induced sample (anti-his antibody); Lane 2: Supernatant of induced sample (anti-his antibody); Lane 3: Purification with Ni-NTA chromatography (anti-his antibody); Lane 4: Supernatant of non-induced sample (anti-Grx1 antibody); Lane 5: Supernatant of induced sample (anti-Grx1 antibody); Lane 6: Purification with Ni-NTA chromatography (anti-Grx1 antibody).

counting kit-8 (CCK-8) method.

2.8. Cell ROS staining

PC12 cells in the logarithmic growth phase were seeded into a 24-well plate and divided into four groups: normal, H_2O_2, positive control, and Grx1 treatment. The plate was then incubated in a CO_2 incubator. Grx1 (12 μM) was added to the Grx1 group, followed by an incubation period of 12 h. The positive control group received treatment with appropriate drugs and was incubated at 37 °C for 30 min. After washing with PBS, each well was treated with 100 μL of a 10 μM DCFH-DA probe and incubated at 37 °C for an additional 30 min. Following another wash with serum-free medium, the cells were observed under a fluorescence microscope in the dark for analysis.

2.9. Flow cytometry

PC12 cells were seeded into a 6-well plate at a concentration of 1×10^5 cells/mL and divided into Control, H_2O_2, and Grx1 groups. Following the attainment of the logarithmic growth phase, oxidative stress modeling was performed as previously described (Section 2.7). The cells were gently resuspended in 195 μL of Annexin V-FITC binding solution, to which 5 μL of Annexin V-FITC and 10 μL of propidium iodide staining solution were added. The mixture was gently mixed and incubated in the dark at room temperature (20–25 °C) for 10–20 min, after which it was placed on ice and analyzed using a flow cytometer. The results were processed and analyzed using CytExpert software.

2.10. qRT-PCR

Total RNA was extracted from the cells using the Trizol method and dissolved in 25 μL of RNase-free water. After measuring the concentration, 2 μg of total RNA was reverse-transcribed using the RevertAid First Strand cDNA Synthesis Kit. (Thermo Scientific™ K1621) according to the manufacturer's instructions, and the resulting cDNA was stored at −80 °C. PCR amplification was performed using the qTOWER 3G fluorescence quantitative PCR instrument, following the instructions provided for the TB® Green qPCR Kit (Table S1). After obtaining the sample CT value, the relative expression of the target gene is calculated using formula $2^{-\Delta\Delta Ct}$.

2.11. Focal cerebral ischemia model

The rat model of middle cerebral artery occlusion (MCAO) was established using the modified suture-occluded method [8]. All rats were housed in an accredited animal husbandry facility approved by the Animal Center of Zhejiang Chinese Medical University. The rats were divided into three groups (n = 8 each): the sham group, the MCAO group, and the Grx1 group. All experimental procedures were conducted in accordance with national and international guidelines and regulations, and were approved by the Laboratory Animal Management and Ethics Committee of Zhejiang Chinese Medical University (Approval No. ACUC-20211018-12).

Following a 12-h fasting period, 2 % sodium pentobarbital (0.2 mL/100 g, provided by Zhejiang Experimental Animal Center) was administered intraperitoneally to induce anesthesia in the rats. A monofilament nylon suture was then inserted through the common carotid artery into the internal carotid artery. One hour after MCAO, reperfusion was achieved by removing the nylon sutures. After the rats recovered from anesthesia, recombinant GRX1 protein (600 μg/kg) was injected into the tail vein once daily for a duration of 7 days.

2.12. Western-blotting

The hippocampal tissue from the modeling side was isolated on ice. A portion of this tissue was then added to RIPA lysis buffer at a 20-fold volume ratio. Following grinding, the samples were lysed on ice for 30 min, with oscillation occurring every 10 min. After centrifugation at 4 °C for 15 min at 12,000 rpm, the supernatant was transferred to a new tube, and each sample was subsequently diluted 10-fold. The protein concentration was determined using the Bicinchoninic Acid Assay (BCA). Samples were prepared based on their protein concentrations, subjected to SDS-PAGE electrophoresis, and transferred to PVDF membranes (0.2 μm). The membranes were blocked with TBST solution containing 5 % non-fat milk and then incubated with primary antibodies overnight at 4 °C, followed by a 2-h incubation with corresponding secondary antibodies. The antibodies were diluted at a ratio of 1:1000 for use.

2.13. Statistical analysis

The experimental data were analyzed using SPSS version 25.0 statistical software. Descriptive statistics for categorical data were presented as mean ± standard deviation (mean ± SD). Group comparisons of multiple sample means were conducted using one-way analysis of variance (ANOVA) followed by the LSD post-hoc test. Significance testing was performed using a two-tailed test, with significance levels represented by P values: $P < 0.05$ indicating a significant difference, and $P < 0.01$ indicating a highly significant difference.

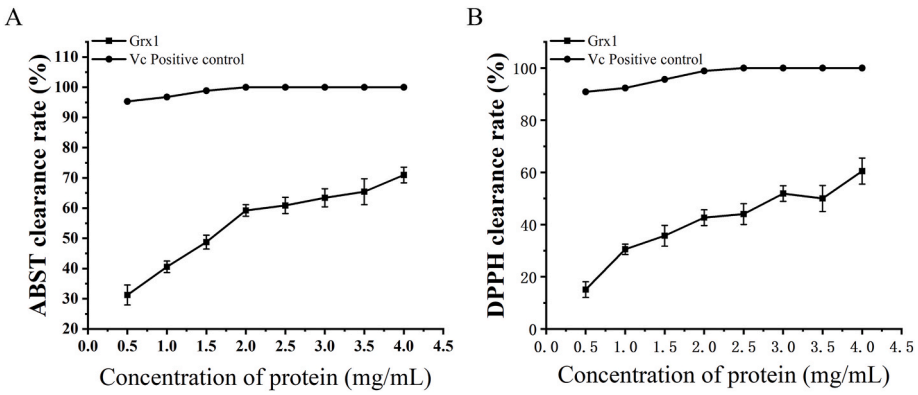

Fig. 2. Antioxidant activity *in vitro*. **A**: Scavenging rate of Grx1 on ABTS radicals. "Vc" corresponds to vitamin C. The scavenging rate of Grx1 on ABTS radicals demonstrates that as the concentration of Grx1 protein increases, its capacity to scavenge ABTS free radicals also increases. This finding confirms the antioxidant activity of Grx1 protein *in vitro*.
B: Scavenging rate of Grx1 on DPPH radicals. "Vc" corresponds to vitamin C. The scavenging rate of Grx1 on DPPH radicals was assessed using a DPPH free radical scavenging experiment to validate the findings from part A. The results indicated that the Grx1 protein exhibits scavenging activity against DPPH free radicals, thereby confirming its antioxidant biological activity *in vitro*.

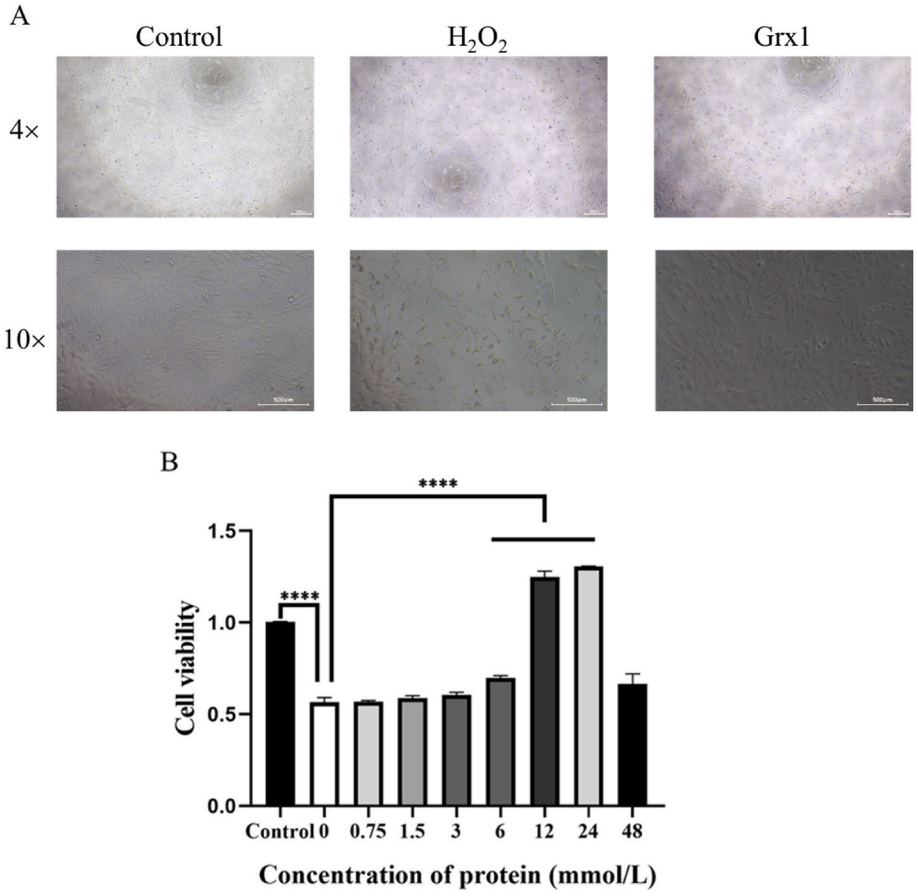

Fig. 3. Treatment of cellular oxidative stress damage.A: Quantity and morphology of cells under low and high magnification. In the H_2O_2 treatment group, the cells lose their original morphological structure, with the characteristic spindle shape transforming into a rounded form. The cells aggregate into clumps, and cell death is clearly observable. In contrast, the Grx1 treatment group (At a concentration of 12.0 μM) exhibits normal cellular morphology, with a significantly reduced number of cells condensing into clumps compared to the H_2O_2 treatment group. This observation indicates that recombinant Grx1 can effectively mitigate oxidative stress damage caused by H_2O_2.
B: Cell viability at different drug concentrations. The cell viability was measured at 56.50 % when treated solely with H_2O2. As the concentration of the drug increased, cell viability correspondingly improved, achieving a maximum value of 130.69 % at a Grx1 concentration of 24 mM *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. All experiments were conducted using three different batches (N = 3); columns represent mean values, while bars indicate standard deviation.

3. Results

3.1. Recombinant plasmid construction

Following codon optimization, the Grx1 gene was synthesized by General Biotechnology Company (China) and subsequently inserted into the *Nde* I and *Xho* I restriction sites of the pET-30a (+) plasmid (Fig. S1). Recombinant pET-30a(+)/Grx1 was then transfected into competent E. coli BL21 (DE3) cells. Plasmid transformants were screened on LB agar plates containing 50 μg/ml kanamycin and were subsequently sent to the company for sequencing. The sequencing results confirmed that the constructs were 100 % accurate (Fig. S2).

3.2. Expression and purification of recombinant Grx1

We successfully expressed the GRX1 protein, with a molecular weight of approximately 11 kDa, which is consistent with the expected value of 11,776 Da. To optimize the yield of soluble recombinant Grx1 protein, different IPTG concentrations, durations, and temperatures were used to induce recombinant protein expression. Fig. S4A demonstrates that recombinant protein expression was significantly higher at 0.25 mM IPTG. Fig. S4B indicates that the protein expression level was maximized after 14 h of induction. From Fig. S4C, it is evident that the optimal expression temperature was 20 °C. Therefore, the optimal conditions for expressing Grx1 are 0.25 mM IPTG, 14 h of induction, and 20 °C. Following the purification and elution of the supernatant from cell lysis (Fig. S4D), a gradient wash with 20,40,60, and 80 mM imidazole was performed, followed by elution with 300 mM imidazole in PBS, resulting in the target protein being primarily found in the elution fraction. The target protein was analyzed by SDS-PAGE (Fig. 1A) and identified by western blotting (Fig. 1 B). The results indicated that the recombinant Grx1 was successfully expressed and purified (Fig. S3).

3.3. Antioxidant activity evaluation

To determine the *in vitro* antioxidant activity of recombinant Grx1, we employed ABTS and DPPH free radical scavenging assays. The recombinant Grx1 protein was diluted to various concentrations. The diluted solutions were then added to the microplate in accordance with the provided instructions. Following the photoprotection reaction, scavenging rate was assessed by measuring absorbance using a microplate reader. The results indicate that Grx1 can effectively inhibit the production of $ABTS^{+}$ and DPPH free radicals *in vitro*, demonstrating significant antioxidant function against free radicals. As shown in Fig. 2, the increase in Grx1 concentration is correlated with the enhancement of antioxidant activity, providing preliminary evidence that the recombinant protein Grx1 has antioxidant biological activity.

3.4. Recombinant Grx1 alleviates cellular oxidative damage

To evaluate the effect of recombinant Grx1 on the survival rate of PC12 cells exposed to hydrogen peroxide, PC12 cells were treated with Grx1 at concentrations of 0, 1.5, 3.0, 6.0, 12.0, 24.0, and 48.0 μM and subsequently cultured for 12 h. The results indicated that, compared to the control group, cell viability in the negative control group decreased to 56.50 %. Morphological observations revealed a significant number of cells exhibiting clear contraction, which is indicative of cell death. Compared to the H_2O_2 group, the cell viability in the Grx1 treatment group increased to 130.69 %. In contrast, the Grx1 treatment groups exhibited a significant increase in cell number compared to the H_2O_2 treatment group and showed no notable morphological changes (Fig. 3A). The survival rate was higher in the Grx1 groups compared to the control, with more pronounced effects observed at concentrations of 12 μM and 24 μM (Fig. 3B). These findings suggest that hydrogen peroxide induced significant cell death, while Grx1 effectively alleviated oxidative damage.

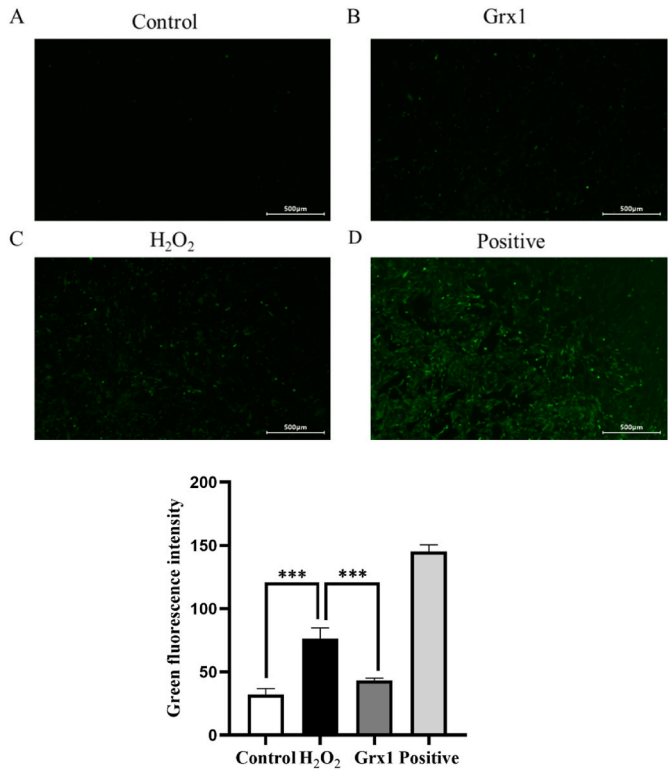

Fig. 4. Cell ROS staining. A: Normal group. **B:** Grx1 group. **C:** H_2O_2 group. **D:** Positive control group. *P < 0.05, **P < 0.01, ***P < 0.001. All experiments were performed with three different batches (N = 3); In the Normal group, cells were cultured under standard conditions, in the Positive control group, cells were treated with Rosup to significantly enhance reactive oxygen species production; Columns represent mean values; bars represent standard deviation.

3.5. Recombinant Grx1 reduces the ROS level

Reactive oxygen species (ROS), a byproduct of cellular metabolism, can lead to oxidative damage when accumulated excessively. To investigate whether recombinant Grx1 inhibits ROS production, fluorescence microscopy was employed to observe the generation of reactive oxygen species in various cell groups. The results demonstrated that, following hydrogen peroxide treatment, the H2O2 group exhibited increased green fluorescence compared to the control group, indicating elevated ROS levels. Conversely, the Grx1 treatment groups displayed significantly reduced green fluorescence, suggesting lower ROS levels (Fig. 4). These findings indicate that recombinant Grx1 significantly inhibits ROS production in PC12 cells.

3.6. Recombinant Grx1 inhibits cell apoptosis

To investigate whether recombinant Grx1 can inhibit cell apoptosis, we employed flow cytometry to quantify the apoptotic cells. The number of apoptotic cells was significantly higher in the H_2O_2 treatment group compared to the control group ($P < 0.01$). Conversely, the Grx1 treatment group exhibited a significant reduction in apoptotic cells relative to the H_2O_2 group ($P < 0.01$). In summary, these results suggest that Grx1 can inhibit cell apoptosis under conditions of oxidative stress (Fig. 5).

3.7. Recombinant Grx1 decrease gene expression levels of IL-1β, TNF-α, IL-6, and Caspase-3

We elucidated the role of Grx1 in the inflammatory response of PC12 cells induced by H2O2 and found that H2O2 significantly increased the transcription levels of pro-inflammatory factors and pro-apoptotic

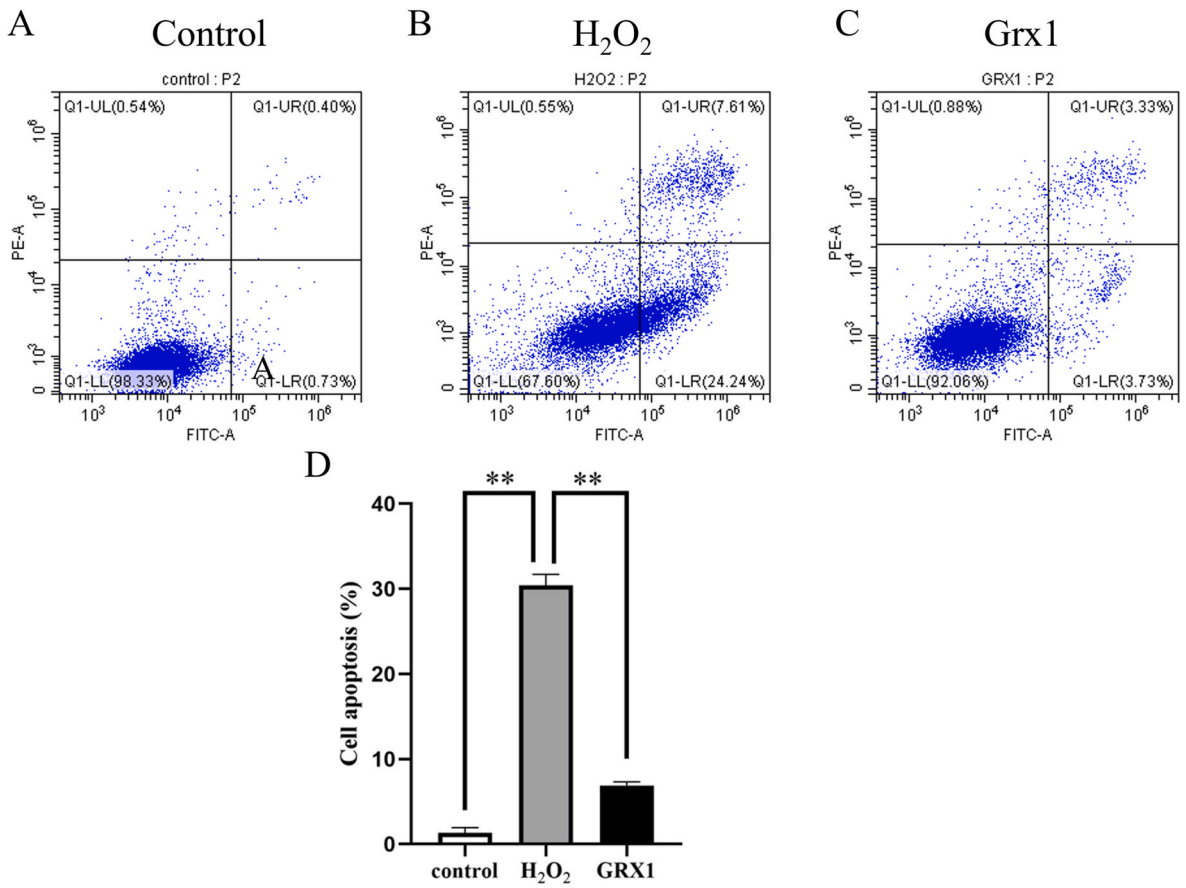

Fig. 5. Flow cytometry for cell apoptosis identification. A: Normal group. **B:** H_2O_2 group. **C:** Grx1 group. *P < 0.05, **P < 0.01, ***P < 0.001. All experiments were performed with three different batches (N = 3); Columns represent mean values; bars represent standard deviation.

factors, including IL-1β (Fig. 6A), TNF-α (Fig. 6B), IL-6 (Fig. 6C), and Caspase-3 (Fig. 6D). Furthermore, Grx1 was shown to inhibit the H_2O_2-induced release of these pro-inflammatory and pro-apoptotic factors (Fig. 6). These results indicate that Grx1 has the potential to mitigate the cellular inflammatory response and apoptosis triggered by H_2O_2.

3.8. Inhibit the expression of apoptosis-related proteins

Following cerebral ischemia-reperfusion injury (CIRI), the expression of apoptosis-related proteins was evaluated using Western blotting. As illustrated in Fig. 7A, the MCAO model in SD rats exhibited a decrease in Bcl-2 protein expression and a significant increase in Bax protein levels when compared to the Sham group. Notably, after Grx1 treatment, Bax protein levels were reduced, while Bcl-2 expression increased significantly in comparison to the CIRI group.

3.9. Regulating the HO-1/NrF2 signaling pathway

We investigate the expression of Nrf2 and HO-1 proteins in rat brain cells following cerebral ischemia-reperfusion injury (CIRI). As illustrated in Fig. 7B, middle cerebral artery occlusion (MCAO) modeling in Sprague-Dawley (SD) rats significantly decreased the levels of Nrf2 and HO-1 proteins compared to the sham group. Following Grx1 treatment, the levels of Nrf2 and HO-1 proteins increased significantly relative to the CIRI group (Fig. 7B). These findings suggest that Grx1 can enhance the expression of Nrf2, promote the overexpression of HO-1, and elevate the levels of various antioxidant enzymes, such as superoxide dismutase (SOD) and catalase (CAT), thereby exerting antioxidant effects.

4. Discussion

Oxidative stress generates a significant amount of reactive oxygen species, which can directly or indirectly damage DNA and proteins, leading to lipid peroxidation and the development of various diseases. Recent advances in the understanding of oxidative stress-induced diseases have valuable new insights. For instance, oxidants can damage DNA and exacerbate inflammatory pathways that promote various stages of tumorigenesis, including cell transformation, tumor growth, proliferation, invasion, angiogenesis, and metastasis. The observed increase in H_2O_2 and reactive oxygen species (ROS) in patients with hypertension underlines the potential for developing antioxidant drugs. In the present study, we verified the antioxidant function of Grx1 through ABTS and DPPH free radical scavenging assays, demonstrating that Grx1 can reduce ROS production in PC12 cells and effectively mitigate cell death induced by H_2O_2. This evidence underscores the significant role of Grx1 in oxidative signal transduction [21] and redox homeostasis. Previous studies have indicated that Grx1 protects against H_2O_2-induced neuronal apoptosis and oxidative stress by regulating the GSK-3β/Nrf2 signaling pathway [22]. Our study corroborates these findings, confirming that Grx1 alleviates apoptosis and oxidative stress.

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a critical transcription factor involved in the cellular antioxidant response. It is present in various cell types and is responsive to redox changes. Research has demonstrated that Nrf2 plays a significant role in redox processes and offers endogenous neuroprotection [23,24]. During cerebral ischemia, Nrf2 interacts with the Keap1, PI3K/AKT, MAPK, NF-κB, and HO-1 pathways to reduce oxidative stress and inflammation, maintain mitochondrial homeostasis, protect the blood-brain barrier, and inhibit ferroptosis, thereby alleviating cerebral ischemia-reperfusion injury

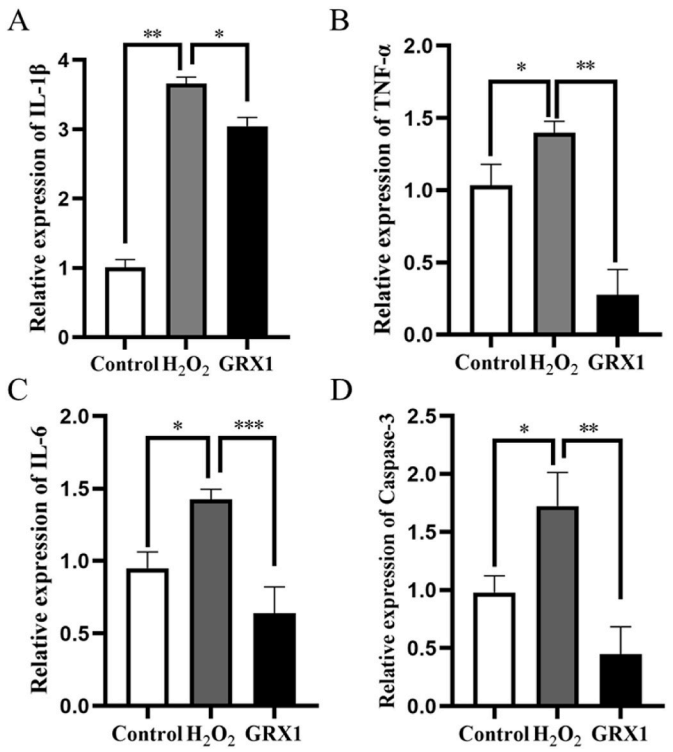

Fig. 6. qRT-PCR detection of relative expression of IL-1β, TNF-α, IL-6, and Caspase-3 in cells. A: IL-1β. **B:** TNF-α. **C:** IL-6. **D:** Caspase-3.
*P < 0.05, **P < 0.01, ***P < 0.001. All experiments were performed with three different batches (N = 3); Columns represent mean values; bars represent standard deviation.

(CIRI) [25–27]. Studies have demonstrated that upregulating HO-1 via the SIRT1/Nrf2 pathway and inhibiting ferroptosis can mitigate doxorubicin-induced myocardial damage [28]. Furthermore, activating the AMPK/GSK-3β/Nrf2 pathway enhances the expression of HO-1, which in turn inhibits the accumulation of iron in cardiomyocytes and reduces myocardial damage resulting from ischemia-reperfusion [29]. Studies have demonstrated that scopolamine mitigates neuronal damage in rats suffering from cerebral ischemia-reperfusion injury by modulating the ERK1/2 and Nrf2/HO-1 pathways, consequently inhibiting cell apoptosis, inflammation, and oxidative stress [30]. Our research indicates that Grx1 can activate the Nrf2 pathway and enhance the expression of HO-1. On one hand, the degradation of the heme group is advantageous in preventing its pro-oxidative effects. On the other hand, the by-products biliverdin and its reduced form, bilirubin, exhibit effective reactive oxygen species (ROS) scavenging activity, which protects against peroxides, peroxynitrite, hydroxyl radicals, and superoxide free radicals. This mechanism contributes to the reduction of oxidative stress and cellular apoptosis, aligning with the findings of Han et al. [30].

Grx1 is a member of the glutaredoxin family and is widely found in viruses, plants, and humans [1]. The relationship between glutaredoxin 1 (Grx1) and human diseases has been demonstrated, particularly in connection with oxidative stress damage. In this experiment, we utilized the pET30a (+) plasmid and *E. coli* BL21 to construct a recombinant Grx1 expression system, successfully expressing the soluble recombinant protein. The Grx1 protein, purified via Ni-NTA chromatography, exhibited a molecular weight of approximately 12 kDa. The production process was optimized by screening various expression conditions, the optimal expression condition were induced with 0.25 mM IPTG at 20 °C for 14 h. In free radical scavenging experiments, the highest scavenging rates for both free radicals exceeded 60 %. Additionally, reactive oxygen species (ROS) level detection indicated a 33.02 % decrease in ROS levels

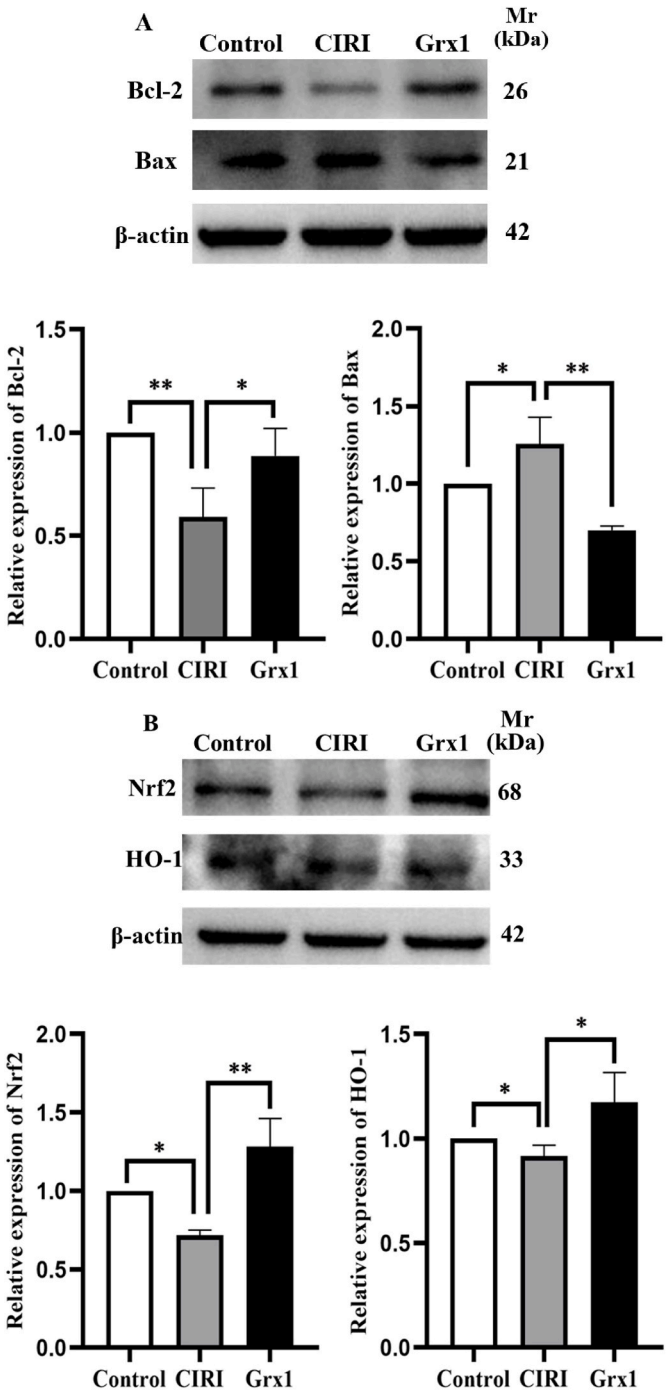

Fig. 7. Western blot analysis of apoptosis and Nrf2/HO-1 signaling pathway relative protein Data are expressed as mean ± SD (n = 3 holes in each group). *P < 0.05, **P < 0.01, ***P < 0.001. Columns represent mean values; bars represent standard deviation.
A: Bcl-2 and Bax protein, with β-actin as the reference control.
B: Nrf2 and HO-1 protein, with β-actin as the reference control.

following Grx1 treatment group, which is consistent with findings reported by Kommaddi et al. [31], both indicating that the presence of Grx1 can affect ROS levels. Grx1 was shown to reduce cell apoptosis, decreasing the percentage of apoptotic cells from 30.39 % to 6.89 %, and also reduced the expression levels of related inflammatory factors, aligning with the conclusions drawn by Liu et al. [32]. Additionally, this study examined the relationship between Grx1 and the HO-1/Nrf2 signaling pathway. The Nrf2 system serves as a crucial defense

mechanism for cells and organisms in response to oxidative stress. Nrf2 is activated by reactive oxygen species and exerts its transcriptional activity to induce the expression of various downstream antioxidant enzymes [33]. Western blotting results indicated that Grx1 mitigated cerebral ischemia-reperfusion injury in rats by enhancing the expression of Nrf2 and HO-1.

5. Conclusion

In the current research, we have developed an *E. coli* expression system to effectively express and purify recombinant Grx1 protein. Our research results indicate that the recombinant Grx1 protein has antioxidant biological activity *in vitro*. In addition, *in vivo*, we have demonstrated that it can reduce the production of ROS within cells and alleviate severe oxidative stress damage. Grx1 also activates the Nrf2/HO-1 pathway, downregulates inflammatory and pro apoptotic factors. Based on these findings, we propose that Grx1 has antioxidant and anti-apoptotic properties in the treatment of oxidative stress and cerebral ischemia-reperfusion injury.

CRediT authorship contribution statement

Zi-teng Li: Writing – original draft, Data curation. **Tong Lin:** Software, Formal analysis. **Yu Sun:** Methodology. **Xin-yi Wang:** Investigation, Formal analysis. **Yi-xuan Yang:** Validation, Formal analysis. **Li Gan:** Methodology, Investigation. **Jia-ming Xu:** Investigation. **Xu-ting Wei:** Validation, Methodology. **Huang-qing Zhu:** Visualization, Resources. **Wei-chun Zhao:** Supervision. **Zhen-hong Zhu:** Writing – review & editing, Project administration.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviations

Grx, Glutaredoxin; IPTG, isopropyl-β-D-thiogalactopyranoside; qRT-PCR, quantitative real-time polymerase chain reaction; Kan, kanamycin; SDS, sodium dodecyl sulfate; DPPH, 2,2-Diphenyl-1-picrylhydrazyl; ABTS, (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)); IRI, Ischemia-reperfusion injury; CIRI, Cerebral ischemia-reperfusion injury.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pep.2025.106689.

Data availability

Data will be made available on request.

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