

ORIGINAL RESEARCH

Wedelolactone attenuates cerebral ischemia-reperfusion injury by blocking GPX4-mediated ferroptosis

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Abstract

Cerebral ischemia is currently the third leading cause of death worldwide. Among its complications, cerebral ischemia-reperfusion (I/R) is considered the most inevitable problem. To address this condition, there is a need for the development of more drugs. Wedelolactone, the main active chemical component extracted from dried lotus leaves, exhibits a wide range of pharmacological effects. Here we constructed an oxygen-glucose deprivation/reperfusion (OGD/R) model. Cell counting kit-8 (CCK-8) assays evaluated the impact of Wedelolactone on cell growth. Real-time quantitative polymerase chain reaction (RT-qPCR) assessed its effects on inflammation. We employed 2',7'-Dichlorofluorescein (DCF) staining and an iron detection kit to measure ferroptosis. Flow cytometry (FCM) and immunoblot assays were utilized to investigate cell apoptosis. The results showed that Wedelolactone promoted OGD-induced HT-22 cell viability and alleviated OGD/R-induced cellular inflammation, OGD/R-stimulated ferroptosis and OGD/R-induced apoptosis. Mechanically, Wedelolactone inhibited OGD/R-stimulated ferroptosis *via* mediating glutathione peroxidase 4 (GPX4) expression. In conclusion, Wedelolactone may attenuate cerebral ischemia-reperfusion (CIR)/I by blocking GPX4-mediated ferroptosis.

Keywords

Cerebral ischemia-reperfusion (I/R); Wedelolactone; Apoptosis; Ferroptosis; GPX4

1. Introduction

Cerebral ischemia currently ranks as the third leading cause of both mortality as well as severe disability [1]. There has been a concerning increase in the incidence, prevalence and absolute number of disabilities and fatalities caused by stroke. Ischemic stroke, a major subtype of stroke, primarily results in the loss of brain function [2], which accounts for 70–80% of all strokes [3]. Presently, the primary treatment approach for ischemic stroke focuses on restoring blood flow to brain tissues, with achieving successful revascularization of cerebral vessels through drug or mechanical thrombolysis being the critical objective following a stroke [4]. However, complications frequently arise after revascularization. The pathological mechanism of cerebral ischemia-reperfusion (I/R) injury is complex and not yet fully understood. Whether during the ischemic phase or upon reperfusion, a series of events unfolds in brain tissues, including oxidative stress, intracellular calcium overload, neurotoxicity, inflammatory responses and apoptosis [5]. When the blood supply to the brain is restored, surviving neurons are also challenged.

Wedelolactone, derived from the leaves of *Eclipta chinensis*, is a prominent chemical compound widely used in traditional medicine across the Americas, Asia and Africa [6]. Recent

studies have unveiled its versatile pharmacological properties, including liver protection, antioxidant capabilities, anti-tumor effects and anti-inflammatory effects [7]. For instance, Wedelolactone has been shown to activate protein kinase A (PKA) signaling to inhibit inflammasome activation and pyroptosis by promoting NOD-like receptor thermal protein domain associated protein 3 (NLRP3) phosphorylation [8]. It may also alleviate acute pancreatitis by suppressing ferroptosis through GPX4 [9]. Additionally, Wedelolactone protects against doxorubicin-induced podocyte inflammation and oxidative damage by modulating the I κ K/I κ B/nuclear factor kappa-B (NF- κ B) axis and exhibits neuroprotective potential by preventing aluminum-induced neurodegeneration. However, despite these promising properties, the role of Wedelolactone in cerebral I/R injury remains unclear.

In this study, we investigated the effects of Wedelolactone on cerebral I/R and found that it can attenuate such injuries by suppressing GPX4-mediated ferroptosis.

2. Materials and methods

2.1 Establishment of OGD/R model and treatment

HT-22 cells (American Type Culture Collection) were maintained in glucose-free Dulbecco's Modification of Eagle's Medium (DMEM) in a hypoxic chamber with a gas mixture comprising 94% nitrogen (N₂), 5% carbon dioxide (CO₂) and 1% oxygen (O₂) at 37 °C for oxygen and glucose deprivation. Control cells were not subjected to OGD/R. Wedelactone (Sigma, USA) was added at 0 μM, 5 μM, 10 μM, 20 μM and 40 μM concentration at the initiation of OGD and continued throughout the hypoxic period. The model was constructed as previously described [3].

2.2 Cell viability

The CCK-8 reagent (C0038, Beyotime, Beijing, China) was added to each well to assess cell viability and incubated for 4 hours. Then, cell absorbance was measured at 450 nm using a microplate reader.

2.3 Real-time qPCR

Total RNA was reverse transcribed into cDNA using reverse transcriptase (Promega, USA). cDNA was amplified using the following primers: tumor necrosis factor-α (TNF-α): Forward: 5'-GGTGCCTATGTCTCAGCCTCTT-3', Reverse: 5'-GCCATAGAAGTATGAGAGGGAG-3'; interleukin-1β (IL-1β): Forward: 5'-ACAAGGAGAAGAAAGTAATGAC-3', Reverse: 5'-GCTGTAGAGTGGGCTTAT-3'; IL-6: Forward: 5'-AGACAGCCACTCACC-3', Reverse: 5'-TTCTGCCAGTGCCTCTT-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH): Forward: 5'-AGAAGGCTGGGGCTCATTTG-3', Reverse: 5'-AGGGCCATCCACAGTCTTC-3'.

2.4 DCF staining

The HT-22 cells were fixed and blocked with goat serum for one hour. Subsequently, they were incubated with the DCF/reactive oxygen species (ROS) detection kit (ab238535, Abcam, Cambridge, UK) following the manufacturer's instructions. After washing with phosphate buffered saline (PBS), images were captured using a confocal fluorescence microscope (LSM880, Carl Zeiss, Oberkochen, Germany).

2.5 Iron level detection

Iron levels in HT-22 cells were assessed using the iron detection kit (ab83366, Abcam, Cambridge, UK) following the manufacturer's instructions.

2.6 Cell apoptosis

HT-22 cells were trypsinized and suspended in a binding buffer, followed by the addition of Annexin V and propidium iodide (PI) for a 5-minute incubation. Cell apoptosis was assessed using a flow cytometer (BD Biosciences, New Jersey, USA).

2.7 Immunoblot

Samples were collected and subjected to electrophoresis on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, followed by transfer onto polyvinylidene difluoride (PVDF) membranes. The membranes were subsequently blocked with 5% fat-free milk, then incubated with primary antibodies, including Bax (Rabbit, 1:1000, ab32503, Abcam), Bcl-2 (Rabbit, 1:1000, ab182858, Abcam), cleaved caspase-3 (Rabbit, 1:1000, ab32042, Abcam), GPX4 (Rabbit, 1:1000, ab125066, Abcam), and β-actin (Mouse, 1:3000, ab8226, Abcam), for 1 hour, followed by incubation with secondary antibodies.

2.8 Statistics

Data analysis was conducted using GraphPad 8.0 software (GraphPad Software, Inc., La Jolla, CA, USA). Each experiment was repeated for 3 times. Error bars are used to represent the mean ± standard deviation (SD). Statistical comparisons were performed *via* one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test, with significance set at $p < 0.05$.

3. Results

3.1 Wedelactone promotes OGD/R-induced HT-22 cell viability

To evaluate the impact of Wedelactone on cerebral I/R, we utilized an OGD/R model with HT-22 cells. The chemical structure formula of Wedelactone is shown in Fig. 1A. Our CCK-8 assays revealed that lower concentrations of Wedelactone (5, 10 and 20 μM) had mild effects on HT-22 cell growth, whereas a higher concentration (40 μM) inhibited their proliferation (Fig. 1B). Consequently, we selected the lower concentrations of Wedelactone (5, 10 and 20 μM) for subsequent experiments with HT-22 cells. Further CCK-8 assays demonstrated that OGD/R treatment impaired HT-22 cell growth, but Wedelactone treatment enhanced the viability of OGD/R-induced HT-22 cells (Fig. 1C). Therefore, these results suggest that Wedelactone can promote the viability of HT-22 cells subjected to OGD/R.

3.2 Wedelactone alleviates OGD/R-stimulated cellular inflammation

Subsequently, we assessed the impact of Wedelactone on the inflammation of HT-22 cells subjected to OGD/R. Our RT-qPCR assays revealed that OGD/R treatment induced inflammation in HT-22 cells, as evidenced by increased mRNA levels of IL-1β, IL-6 and TNF-α (Fig. 2). However, Wedelactone treatment resulted in reduced mRNA levels of these factors in response to OGD/R treatment (Fig. 2). Thus, Wedelactone may mitigate OGD/R-induced cellular inflammation.

3.3 Wedelactone alleviates OGD/R-stimulated ferroptosis

We investigated the impact of Wedelactone on ferroptosis in HT-22 cells subjected to OGD/R. Our DCF staining re-

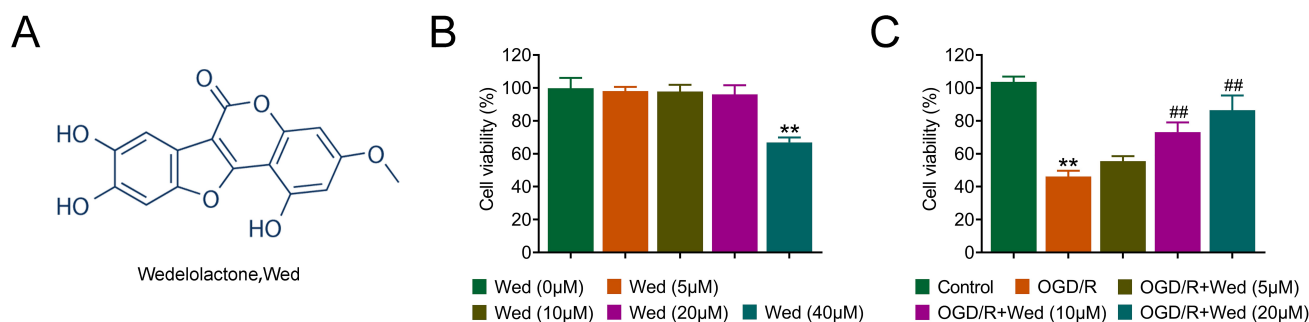


FIGURE 1. Wedelactone promotes HT-22 cell viability. (A) Chemical structure formula of Wedelactone. (B) CCK-8 assays assessing HT-22 cell growth with Wedelactone (5, 10, 20 and 40 μM). The corresponding 450 values are also shown. Error bars represent SD. ** $p < 0.01$, Wedelactone vs. control. (C) CCK-8 assays evaluating HT-22 cell growth under various treatments, with OD450 values measured. Error bars represent SD. ** $p < 0.01$, OGD/R vs. control, ## $p < 0.01$, OGD/R + Wedelactone vs. OGD/R. OGD/R: oxygen-glucose deprivation/reperfusion.

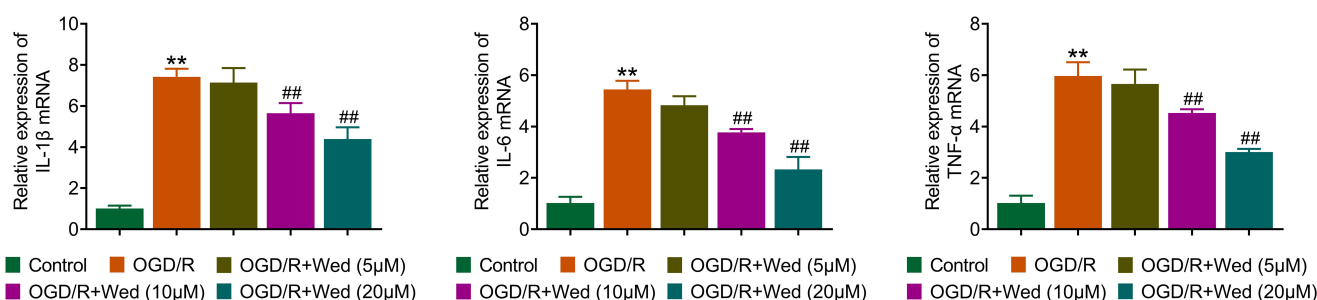


FIGURE 2. Wedelactone alleviates OGD/R-induced cellular inflammation. RT-qPCR assays showing the mRNA levels of inflammation factors in HT-22 cells under OGD/R and Wedelactone treatment (5, 10 and 20 μM). Error bars represent SD. ** $p < 0.01$, OGD/R vs. control, ## $p < 0.01$, OGD/R + Wedelactone vs. OGD/R. OGD/R: oxygen-glucose deprivation/reperfusion, IL: interleukin, TNF- α : Tumor necrosis factor- α .

sults revealed that OGD/R induced ROS production in HT-22 cells, as evidenced by the elevated DCF staining levels (Fig. 3A). However, Wedelactone treatment suppressed ROS production in HT-22 cells following OGD/R (Fig. 3A). Additionally, we assessed the levels of released iron in HT-22 cells and observed that OGD/R treatment led to increased iron levels, whereas Wedelactone treatment decreased these iron levels (Fig. 3B). Furthermore, we examined the expression of GPX4 and found that Wedelactone treatment reduced GPX4 expression in OGD/R-induced HT-22 cells (Fig. 3C). Thus, Wedelactone mitigates OGD/R-stimulated ferroptosis.

3.4 Wedelactone alleviates OGD/R-stimulated apoptosis

Flow cytometry (FCM) assays showed that OGD/R induction led to HT-22 cell apoptosis (Fig. 4A). Additionally, our findings demonstrated that Wedelactone treatment suppressed apoptosis in OGD/R-induced HT-22 cells, as indicated by a reduction in the number of apoptotic cells (Fig. 4A). Furthermore, immunoblot assays showed that OGD/R increased the expression levels of Bax and cleaved caspase-3 while decreasing Bcl-2 expression (Fig. 4B). Notably, treatment with Wedelactone suppressed the expression of Bax,

cleaved caspase-3 and enhanced Bcl-2 expression (Fig. 4B). Overall, these results suggest that Wedelactone can alleviate OGD/R-induced apoptosis.

3.5 Wedelactone inhibits OGD/R-stimulated ferroptosis by regulating GPX4 expression

Lastly, we investigated the mechanism through which Wedelactone suppresses OGD/R-induced ferroptosis in HT-22 cells. Immunoblot analysis revealed that OGD/R treatment led to a decrease in GPX4 expression in HT-22 cells, while Wedelactone treatment (20 μM) increased GPX4 expression (Fig. 5A). Moreover, siGPX4 transfection further reduced GPX4 expression in Wedelactone-treated cells exposed to OGD/R (Fig. 5A). In addition, CCK-8 assays indicated that Wedelactone treatment enhanced the growth of OGD/R-induced cells and that this effect could be reversed when GPX4 was depleted (Fig. 5B). DCF staining also showed that GPX4 depletion counteracted ROS level reduction caused by Wedelactone in OGD/R-induced cells (Fig. 5C). Similarly, our analysis of iron levels in cells demonstrated that Wedelactone decreased iron levels in OGD/R-induced cells, while GPX4 ablation further increased iron levels induced by Wedelactone treatment (Fig. 5D). Taken together, these findings suggest

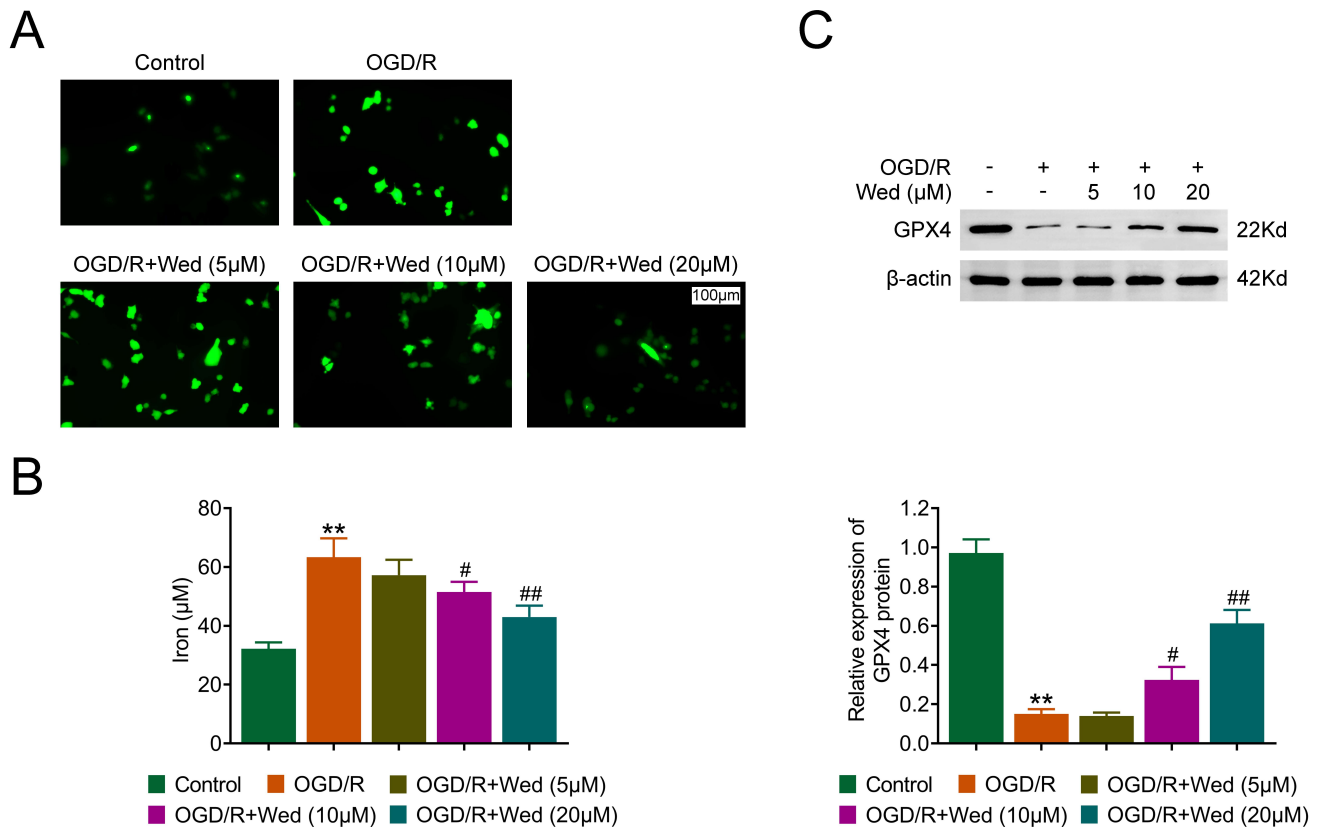


FIGURE 3. Wedelactone alleviates OGD/R-induced ferroptosis. (A) DCF staining illustrating ROS levels in HT-22 cells under OGD/R treatment and Wedelactone treatment (5, 10 and 20 μM). (B) Iron detection kit measurements of iron release in HT-22 cells under OGD/R treatment and Wedelactone treatment (5, 10 and 20 μM). (C) Immunoblot showing GPX4 expression in HT-22 cells under OGD/R treatment and Wedelactone treatment (5, 10 and 20 μM). Error bars represent SD. ** $p < 0.01$, OGD/R vs. control, # $p < 0.05$, ## $p < 0.01$, OGD/R + Wedelactone vs. OGD/R. OGD/R: oxygen-glucose deprivation/reperfusion; GPX4: Glutathione peroxidase 4.

that Wedelactone inhibits OGD/R-induced ferroptosis by regulating GPX4 expression.

4. Discussion

Cerebrovascular disease is a prevalent and frequently occurring health issue, ranking as the leading cause of disability worldwide, with ischemic disease accounting for approximately 75% of all cerebrovascular diseases [10]. Reperfusion following ischemia can exacerbate cellular injury, a phenomenon referred to as I/R injury [11]. Acute cerebral ischemic injury is intricately linked to inflammation, and the inflammatory response is a key contributor to reperfusion injury [12]. Inflammation represents a crucial pathological event in the cascade of cell damage and can lead to secondary neuronal damage [13]. The primary approach to treating cerebral I/R injury involves the use of neuroprotective drugs like edaravone and butylphthalide to improve brain damage [14]. Patients may also require medications aimed at neutralizing oxygen free radicals, which can be managed with neuroprotectants designed to ameliorate cerebral I/R injury.

In various regions, such as Asia and South America, the traditional use of Wedelia has been well-established for addressing a range of health issues, including septic shock, liver

disease, viral infections and snakebites. Wedelolactone, a prominent compound found in Wedelia, is believed to contribute significantly to its liver-protective properties, alongside its application in Mohwa [15]. Recent pharmacological research has revealed the diverse potential of Wedelolactone (WEL). It acts as an inhibitor of $\text{Na}^+\text{-K}^+\text{-ATPase}$ and isomerase type II, which makes it promising for several applications, including liver protection, managing postmenopausal osteoporosis, immunosuppression, and potential anticancer properties [16]. WEL has demonstrated the ability to suppress caspase-11 expression induced by lipopolysaccharide (LPS) and induce apoptosis in prostate cancer cells by targeting protein kinase c epsilon (PKC ϵ) [17]. Through a series of *in vitro* experiments, our research demonstrates that Wedelactone can alleviate cellular inflammation, ferroptosis, and apoptosis triggered by OGD/R.

The data confirmed that Wedelactone effectively inhibits ferroptosis *via* GPX4, a pivotal component in the regulation of iron-induced cell death that has an important role in cerebral I/R injury. Upon OGD/R exposure, we observed a significant reduction in GPX4 protein levels, GSH content, and GPX4 activity in HT-22 cells, which led to impaired ability of cells to eliminate lipid peroxides, resulting in the accumulation of

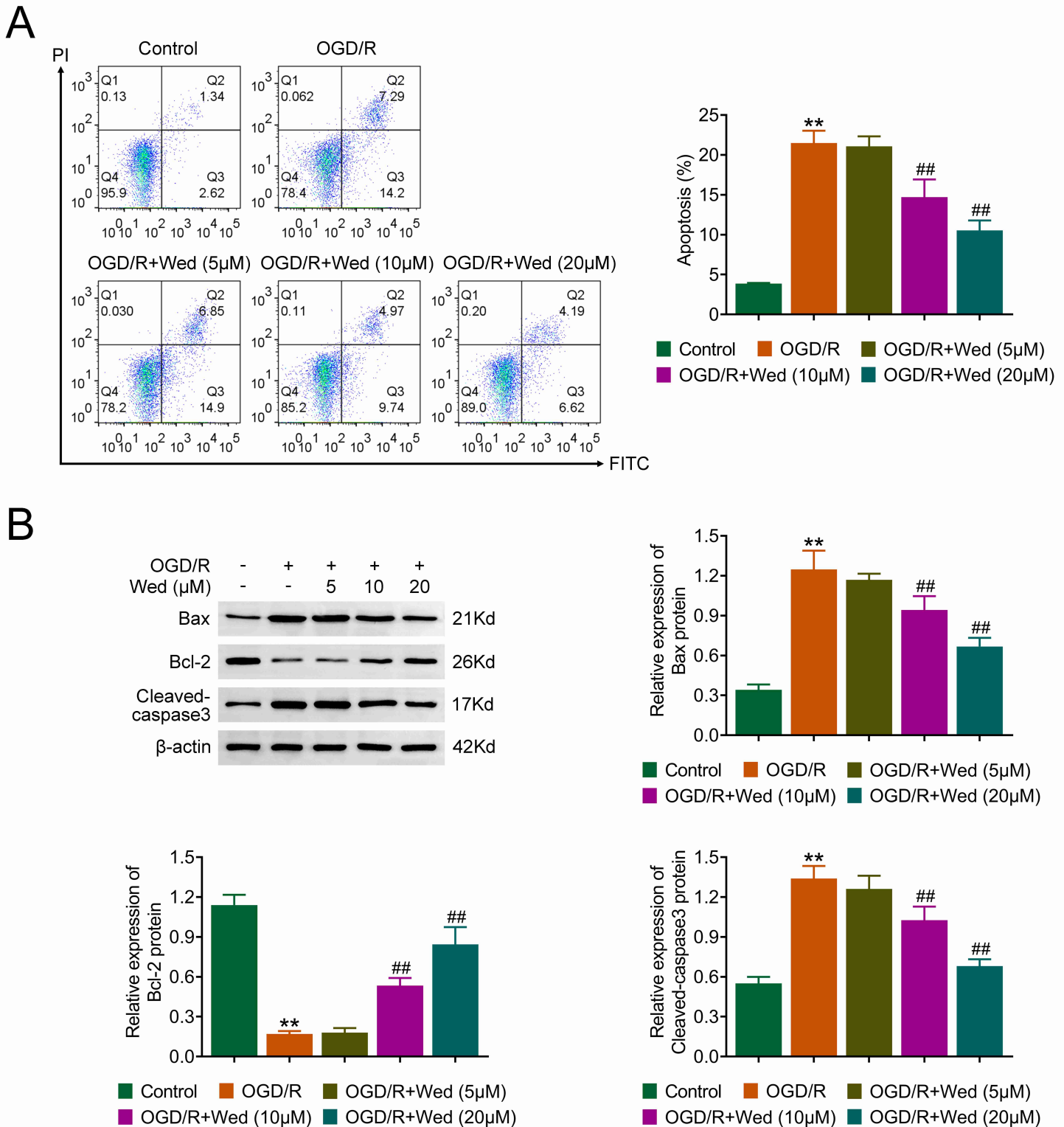


FIGURE 4. Wedelactone alleviates OGD/R-induced apoptosis. (A) FCM assays showing the apoptosis levels in HT-22 cells under OGD/R treatment and Wedelactone treatment (5, 10 and 20 μM). (B) Immunoblot assays displaying Bax, Bcl-2 and cleaved caspase-3 expression in HT-22 cells under OGD/R treatment and Wedelactone treatment (5, 10 and 20 μM). Error bars represent SD. ** $p < 0.01$, OGD/R vs. control, ## $p < 0.01$, OGD/R + Wedelactone vs. OGD/R. OGD/R: oxygen-glucose deprivation/reperfusion.

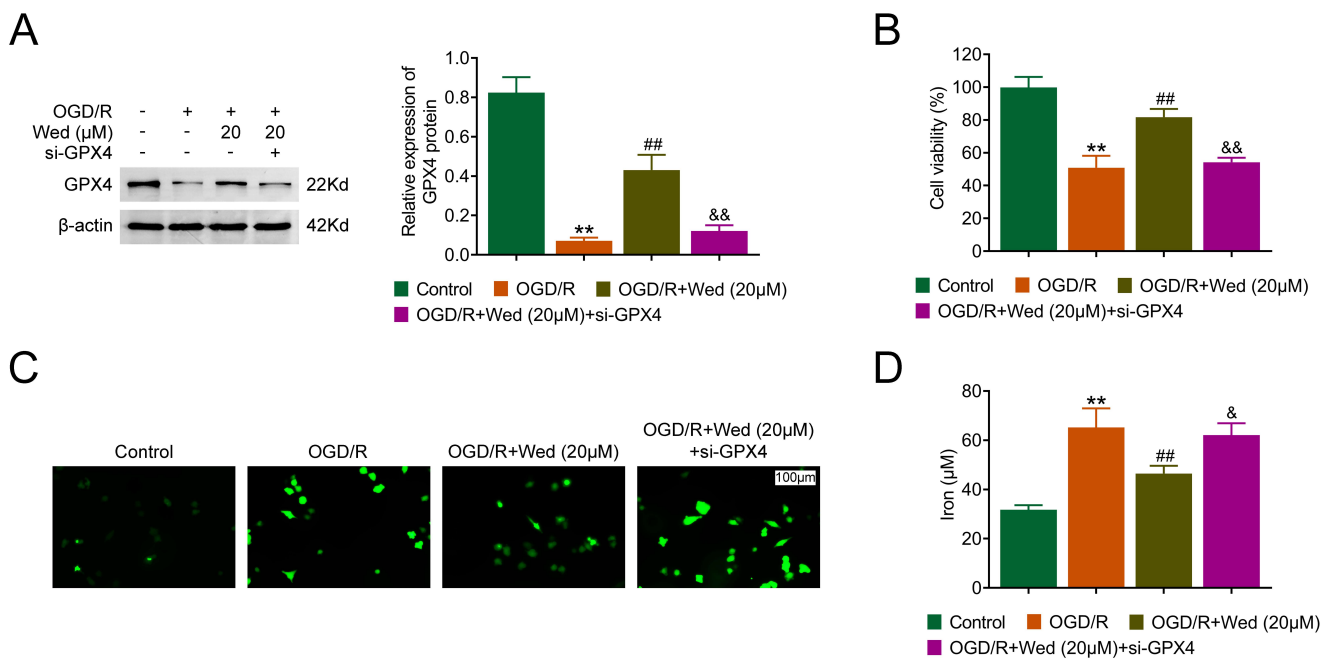


FIGURE 5. Wedelactone inhibits OGD/R-stimulated ferroptosis by regulating GPX4 expression. (A) Immunoblot assays showing GPX4 expression in HT-22 cells under the indicated treatment. (B) CCK-8 assays assessing HT-22 cell viability under the indicated treatment, with OD450 values measured. (C) DCF staining indicating ROS levels in HT-22 cells under the indicated treatment. (D) DCF staining displaying ROS levels in HT-22 cells under the indicated treatment. Error bars represent SD. $**p < 0.01$, OGD/R vs. control, $###p < 0.01$, OGD/R + Wedelactone vs. OGD/R, $\&p < 0.05$, $\&\&p < 0.01$, OGD/R + Wedelactone + siGPX4 vs. OGD/R + Wedelactone + siNC. NC: negative control; OGD/R: oxygen-glucose deprivation/reperfusion; GPX4: Glutathione peroxidase 4.

numerous oxidative byproducts, including malondialdehyde (MDA). Ferroptosis, characterized by a decrease in GPX4 activity and glutathione depletion, is a newly recognized form of cell death [7]. Our data further confirmed that Wedelactone inhibits OGD/R-induced ferroptosis by mediating GPX4.

Ferroptosis has been increasingly recognized for its involvement in various diseases, including cancers, cardiovascular disorders, and ischemia-related conditions, with a particularly strong association observed in cerebral I/R injury [18]. Studies have found that ferroptosis is vital in promoting cerebral I/R injury, and intervention against ferroptosis has a protective effect on the nervous system [19, 20]. Our data further confirm the role of ferroptosis in cerebral I/R injury. However, it should be noted that one of the main limitations of this present study is the lack of *in vivo* validation, thereby requiring the need for further deeper investigations to confirm these findings.

5. Conclusions

In summary, our findings demonstrate that Wedelolactone can attenuate cerebral I/R injury by inhibiting GPX4-mediated ferroptosis.

AVAILABILITY OF DATA AND MATERIALS

The data are contained within this article.

AUTHOR CONTRIBUTIONS

HYY—performed material preparation and the experiments. DL—performed data collection and analysis. YPW—written the first draft of the manuscript. All authors commented on previous versions of the manuscript. All authors contributed to the study conception and design. All authors read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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