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Melatonin alleviates heme-induced ferroptosis *via* activating the *Nrf2/HO-1* pathway in neurons

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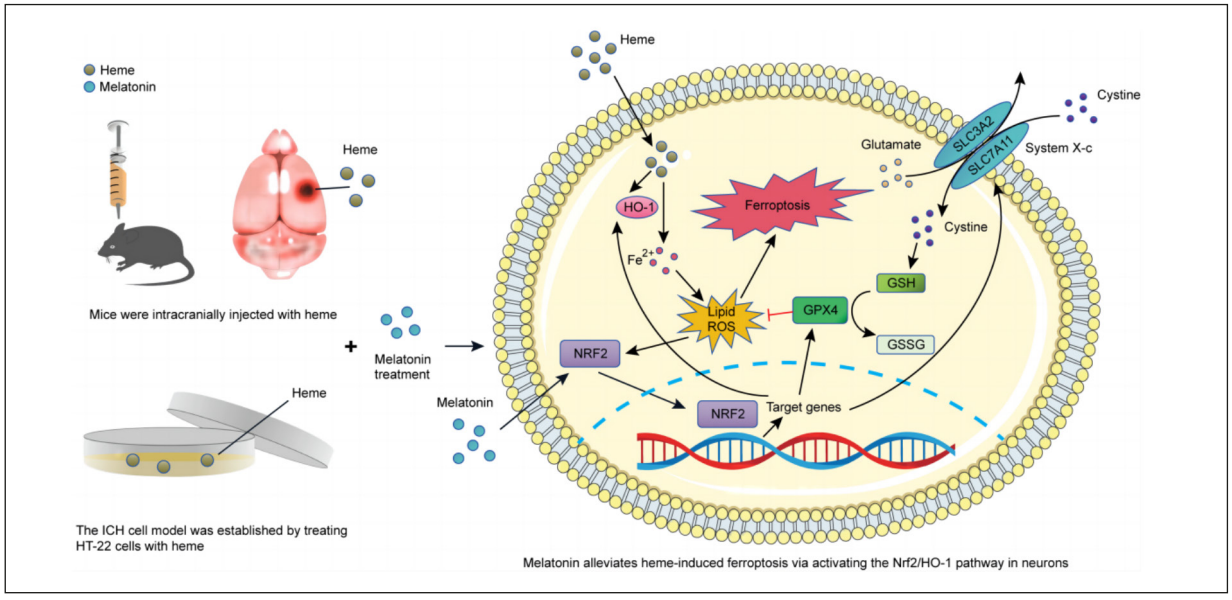
Abstract. – OBJECTIVE: Ferroptosis of neurons is a significant cause of brain injury following intracerebral hemorrhage (ICH). As an iron-containing compound in hemoglobin, heme contributes to nerve injury post-ICH. Melatonin has been shown to mitigate the effects of ICH, yet its specific functions remain largely elusive. In this study, we aimed to explore the roles and mechanisms of melatonin in heme-induced ferroptosis subsequent to ICH.



Graphical Abstract. This graphical abstract illustrates the protective effects of melatonin on heme-induced ferroptosis in neurons following ICH. *In vivo*, heme was injected into the brains of mice, and *in vitro*, heme stimulation was used to model ICH. Heme, released from hemoglobin during ICH, induces ferroptosis by increasing iron levels and promoting lipid peroxidation, which is marked by a significant decrease in the key ferroptosis regulators *GPX4* and *SLC7A11*. Melatonin treatment not only reduced neuronal death and alleviated brain function impairment, but also restored the expression of these critical ferroptosis markers by activating the *Nrf2/HO-1* pathway, indicating its protective role against ferroptosis.

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MATERIALS AND METHODS: C57BL/6 mice were intracranially injected with heme and then treated with melatonin. Behavior tests [modified neurological severity score (mNSS), forelimb placing, and corner turn tests], H&E staining, Nissl staining, and Prussian blue staining were used to evaluate mouse brain tissue injury. *In vitro*, HT-22 cells were stimulated with heme and cell viability was determined by crystal violet staining. The iron contents were determined in heme-treated brains and cells, and the levels of 4-hydroxynonenal (4-HNE) and malonaldehyde (MDA) were assessed by ELISA. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used to investigate the mRNA levels of nuclear factor erythroid 2-related factor 2 (*Nrf2*) and heme oxygenase-1 (*HO-1*). Immunoblotting was used to analyze the protein expression of glutathione peroxidase 4 (*GPX4*), solute carrier family 7 member 11 (*SLC7A11*), *Nrf2*, and *HO-1*. Finally, small interfering RNA (siRNA) was used to knock down *Nrf2* in HT-22 cells.

RESULTS: Melatonin treatment alleviated heme-induced injuries to neural function, as indicated by improved behavior in the mice. Moreover, melatonin decreased cell death and iron concentrations, increased MDA and 4-HNE levels, and reversed the decreases in *GPX4*, *SLC7A11*, *Nrf2*, and *HO-1* induced by heme *in vitro* and *in vivo*. These results indicated that melatonin could improve the ferroptosis induced by heme. In addition, we found that *Nrf2* knockdown attenuated the therapeutic effect of melatonin on neuronal ferroptosis induced by heme.

CONCLUSIONS: In general, melatonin alleviates heme-induced ferroptosis by activating the *Nrf2/HO-1* pathway, which implies that melatonin is a promising treatment for ferroptosis in ICH.

Key Words:
Melatonin, Heme, Ferroptosis, *Nrf2/HO-1* pathway, Intracerebral hemorrhage.

Abbreviations

ICH: intracerebral hemorrhage; 4-HNE: 4-hydroxynonenal; MDA: malonaldehyde; *Nrf2*: nuclear factor erythroid 2-related factor 2; *HO-1*: heme oxygenase-1; *GPX4*: glutathione peroxidase 4; *SLC7A11*: solute carrier family 7 member 11; siRNA: small interfering RNA; ROS: reactive oxygen species; PBST: phosphate-buffered saline with tween-20; RT-qPCR: reverse transcription quantitative polymerase chain reaction; *GAPDH*: glyceraldehyde-3-phosphate dehydrogenase; ELISA: enzyme-linked immunosorbent assay; H&E: hematoxylin-eosin staining; mNSS: modified neurological severity score.

Introduction

Intracerebral hemorrhage (ICH) is one of the most life-threatening causes of stroke, accounting for 15% of all stroke cases, and is characterized by high morbidity, disability, and mortality[1]. Although the pathophysiological mechanisms of ICH have been extensively studied, effective treatments for this disease have not been identified and need to be further explored. In ICH hematoma, hemoglobin is broken down, and heme, an iron-containing compound, is released. Hemolysis is catalyzed by heme oxygenase to produce biliverin, carbon monoxide, and iron ions and is involved in lipid peroxidation, neuroinflammation, and cytotoxic processes in ICH[2,3].

Iron is released from degraded heme and accumulates in brain tissues around hematomas[4], predicting a poor prognosis and an increased risk of death[5]. Ferroptosis is an iron-dependent form of nonapoptotic cell death that is manifested by the overproduction of reactive oxygen species (ROS), iron deposition, lipid peroxidation, and cytokine changes[6]. Ferroptosis is involved in neuronal death and neurological impairment after ICH[7]. Recently, ferroptosis inhibitors, such as ferrostatin-1, have been reported to have neuroprotective effects on ICH-induced secondary brain injury[8]. However, the roles and mechanisms of ferroptosis in heme-induced brain injury are still poorly understood.

Melatonin (N-acetyl-5-methoxytryptamine) is a neuroendocrine hormone produced by the pineal gland and is secreted during the circadian cycle[9]. It is released into the blood and cerebrospinal fluid to reach the central nervous system and peripheral organs[10]. It acts as an effective antioxidant[11] that can scavenge ROS, protect mitochondrial oxidoreductase, indirectly stimulate antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GPx) and reduce oxidative damage[12]. Melatonin has been reported to improve ICH, cerebral ischemia, and subarachnoid hemorrhage (SAH) as a promising neuroprotective agent[13-16]. Recent studies[17] have shown that melatonin plays a protective role in neuronal injury by activating the *Nrf2*/*HO-1* pathway. The *Nrf2* pathway regulates the transcription of antioxidant-related genes and maintains the cellular redox balance by binding to antioxidant response elements (AREs) in antioxidant and detoxification genes, such as *HO-1*[18]. Thus, we speculate that melatonin attenuates neuronal ferroptosis in ICH.

In this study, we aimed to investigate whether melatonin could protect against heme-induced ferroptosis in ICH and further explore the role of the *Nrf2*/*HO-1* pathway in this protective effect.

This will help to elucidate the functions and mechanisms of melatonin in ICH treatment.

Materials and Methods

Animals

To eliminate any potential influence of the animals' sex on the results, equal numbers of female and male C57BL/6 mice (from the State Key Laboratory of Cancer Biology and Department of Pathology, the Fourth Military Medical University, Xi'an, Shannxi, China) (8-12 weeks, 20-24 g) were housed in a temperature-controlled and humidity-controlled facility and maintained under a 12 h/12 h light-dark cycle. The experiment was approved by the Biomedical Ethics Committee of the Health Science Center of Xi'an Jiaotong University (No. XJTUAE 2023-2166), and the animals were randomly assigned to each experimental group.

Mouse ICH Model and Treatment

The mouse ICH model was constructed according to previous studies[19]. Specifically, the mice were anesthetized with 50 mg/kg pentobarbital sodium, and their body temperature was maintained at 37.0 ± 0.5°C *via* a heating blanket. They were then placed in a stereotaxic apparatus (Render Biotech Co., Ltd., Shenzhen, Guangdong, China). A hole with a diameter of 1 mm was drilled 0.5 mm posterior to the bregma, 2.3 mm lateral to the left (striatal part), and 3.7 mm to the ventral part. A microinfusion pump was connected to a 50 μL syringe (50 μL, Shanghai Gaoge Industry and Trade Co., Ltd., Shanghai, China), and a 30 μL saline solution containing 15 μg of hematin (Nanjing BioDuly Co., Ltd., Nanjing, Jiangsu, China) was injected into the striatum at a rate of 2 μL/min. After the injection was completed, the syringe was left in place for 5 min and was slowly withdrawn to prevent backflow after the injection. The drill hole was then sealed with bone wax. Melatonin was dissolved in anhydrous ethanol and subsequently diluted with normal saline to achieve a final ethanol concentration of 2%. The mice were intraperitoneally injected with 10 mg/kg melatonin 1 h, 24 h, and 48 h after surgery.

Cell Culture and Treatment

The mouse hippocampal neuron cell line HT-22 (Cell Bank of the Chinese Academy of Sciences, Shanghai, China) was cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; ExCell Bio, Suzhou, Jiangsu, China) and 100 U/ml penicillin or streptomycin at 37°C with 5% CO_2. Heme and melatonin were chosen as described previously[14,20]. The ICH cell model was established by treating HT-22 cells with 50 μM heme for 24 h. At the same time, the HT-22 cells were treated with 60 μM melatonin.

In addition, HT-22 cells at 60-70% confluence were transfected with siRNA targeting *Nrf2* (si-*Nrf2*) (GenePharma, Shanghai, China) or negative control siRNA (si-NC) (GenePharma, Shanghai, China) *via* Lipofectamine 3,000 (Invitrogen, Carlsbad, CA, USA).

Behavior Tests

The neurological impairment test was performed *via* the 18 score-modified neurological severity score (mNSS), forelimb placing test, and corner turn tests 3 days after heme administration, as described previously[21]. Neurological deficits are scored to indicate motor, sensory, reflexes, and balance functions. The mNSS included the following steps:

(1) Exercise test: the tail lift test was used to observe the flexion of the anterior and posterior limbs and the deviation angle of the head within 30 s. Mice were placed on the floor to determine whether they could walk normally.
(2) Sensory test: placement test (visual and tactile test) and proprioception test (deep sensation, paw pressing on the edge of the table to stimulate limb muscles).
(3) Balance beam test.
(4) Loss of reflex and abnormal movement: auricle reflex (shaking head when touching the external auditory canal), corneal reflexes (blinking while touching the cornea with cotton wire), panic reflex (motion reflex to the noise of fast fingering cardboard), epilepsy, myoclonus, and dystonia. The test is scored on a scale of 0 to 18, with higher scores indicating more severe nerve injury. For the forelimb placing test, the success rate was assessed by suspending the mouse forelimb in the air and stimulating the mouse tentacles. In the corner turn test, the mice were allowed to progress into 30° angled corners, and the numbers of left and right turns were recorded. The turning direction frequency equals the percentage of the number of right turns to the total number of turns. The tests were conducted in a double-blind manner.

Hematoxylin-Eosin (H&E) Staining

The brain tissue sections of the injured areas were stained with hematoxylin-eosin (HE) and then photographed by cellSens entry (Olympus Corporation, Tokyo, Japan).

Nissl Staining

After dewaxing and rehydration, the paraffin-embedded sections were stained with toluidine blue solution at 50°C for 40 min. Next, the sections were rinsed with distilled water, dehydrated in graded concentrations of alcohol (70%, 80%, 95%, and 100%) for 3 min, cleared in xylene for 5 min, and covered with neutral balm. ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used to count the number of surviving neurons.

Prussian Blue Staining

Prussian blue staining was used to reveal iron-labeled cells in the brain tissue. Briefly, after three washes with Phosphate-buffered saline with tween-20 (PBST), the brain sections were covered with a PBS-Triton X solution at room temperature for 5 min. Subsequently, the sections were stained with Perl's Prussian blue stain for 30 min and then washed with PBST 3 times. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 15 min, after which the sections were washed with PBST 3 times. The sections were subsequently incubated with 3,3'-diaminobenzidine (DAB) and counterstained with hematoxylin.

Crystal Violet Staining

Cell viability was determined *via* crystal violet staining. HT-22 cells were incubated with 11% glutaraldehyde for 20 min and washed with deionized water. Next, the sections were stained with 0.1% crystal violet solution (Sangon Biotech Co., Ltd., Shanghai, China) for 30 min and washed with distilled water. The crystal violet solution was extracted with 10% acetic acid. After 1 h, the optical density (OD) at 570 nm was measured *via* a microplate reader (Multiskan Go, Thermo Fisher, Shanghai, China). The experiments in each group were performed in triplicate.

Iron Content Measurement

Mouse brain homogenates and HT-22 cells were washed twice with sterile PBS. Then, according to the instructions of the iron content assay kit (Solarbio, Beijing, China), the absorbances at 520 nm and 510 nm were determined for the brain homogenate and HT-22 cells, respectively. Each experiment was repeated three times.

Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted from brain tissue and HT-22 cells *via* TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed into cDNA *via* the Primer Script RT Reagent Kit (TaKaRa, Kusatsu City, Shiga Prefecture, Japan). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an endogenous control. The primers used are listed in Table I. The specific procedure was as follows: denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. Relative gene expression was measured *via* the $2^{-\Delta\Delta Ct}$ method. Each experiment was repeated three times.

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was performed according to the manufacturer's instructions (Elabscience Biotechnology Co., Ltd., Wuhan, Hubei, China) to assess the concentrations of 4-HNE and MDA. The optical density of each well at 450 nm was determined *via* a microplate reader. The experiments in each group were performed in triplicate.

Western Blot

Total protein was extracted from brain tissue and HT-22 cells *via* radio-immunoprecipitation assay (RIPA) buffer and quantified *via* the Bradford method. Equal amounts of protein were separated *via* SDS-PAGE and transferred

Table I. qPCR primers.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Nrf2	CTCCAGCGAGCAGGCTATCT	ACGCACAGCAATCCACCAA
HO-1	AC-CGCCTTCCTGCTCAACATTG	CTCTGACGAAGTGAC-GCCATCTG
GAPDH	GGTGAAGGTCGGTGTGAACG	CTCGCTCCTGGAAGATGGTG

to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were blocked with PBST (5% skim milk) for 1 h and incubated with antibodies specific for *GPX4* (A1933, 1:1000; ABclonal, Shanghai, China), *SLC7A11* (26864-1-AP, 1:1000; Proteintech, Wuhan, Hubei, China), β-actin (AC026, 1: 5000; ABclonal, Shanghai, China), *Nrf2* (A0674, 1:1000; ABclonal, Shanghai, China) and *HO-1* (ADI-SPA-896-F, 1:1000; Enzo, Farmingdale, New York, NY, USA) at 4°C overnight. After washing with PBST, the membranes were incubated with secondary antibodies conjugated to peroxidase at room temperature for 1 h. Visualization was conducted *via* an enhanced chemiluminescence system and electrophoretic image analyzer, with β-actin serving as the internal reference.

Statistical Analysis

The statistical analysis was performed using SPSS 21.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA). The measurement data are expressed as the mean ± standard deviation (SD) and were normally distributed with homogeneous variance. The significance of differences between the two groups was analyzed using Student's *t*-test, and for more than two groups, it was determined using two-way analysis of variance (ANOVA), followed by data checking with Tukey's multiple comparison test. A *p*-value lower than 0.05 was considered to indicate statistical significance.

Results

Melatonin Improves Nerve Injury in Intracerebral Heme-Injected Mice

It was reported that intracerebral heme injection induces lipid peroxidation and inflammation[19]. Here, melatonin was used to treat a mouse model of ICH *via* intracerebral heme injection. At 72 h after intracerebral heme injection, the mice exhibited severe neurobehavioral disturbances, which were attenuated by melatonin (Figure 1A). Furthermore, coronary sections of heme-injected brain tissue also exhibited a significantly black heme deposition cavity and a pale color in response to melatonin (Figure 1B). H&E staining revealed significant edema, neuronal necrosis, and intracellular vacuoles after heme treatment (Figure 1C). We used Nissl staining to further assess the protective effect of melatonin on neurons in the injured area. The number of surviving neurons was significantly reduced by heme but increased in the melatonin-treated tissues (Figure 1D-E). In addition, the number of Prussian blue-positive cells was increased in the injured area of brain tissue after heme injection, while melatonin treatment relieved iron deposition (Figure 1F). In summary, intracerebral heme injection induced nerve function damage, neuronal injury and iron deposition in mice, which were alleviated by melatonin.

Melatonin Alleviates Neuronal Ferroptosis in Intracerebral Heme-Injected Mice

Ferroptosis is considered an important process involved in heme-induced neuronal injury after ICH. Here, a significant increase in Fe^{2+} deposition and lipid peroxidation products (4-HNE and MDA) was observed after heme administration, and these changes were reduced by melatonin (Figure 2A-C). In addition, the expression of the ferroptosis inhibitors *GPX4* and *SLC7A11* in the homogenate of injured brain tissue was inhibited by heme but increased by melatonin (Figure 2D). These experiments revealed the protective role of melatonin in neuronal ferroptosis, lipid oxidation, and brain injury in intracerebral heme-injected mice.

Melatonin Protects Against Heme-Induced Ferroptosis in HT-22 Cells

To confirm the regulatory role of melatonin in mediating neuronal ferroptosis *in vitro*, HT-22 cells were treated with heme. Crystal violet staining revealed decreased cell viability following heme treatment. After melatonin treatment, cell viability increased (Figure 3A). Additionally, the Fe^{2+} concentration and 4-HNE and MDA concentrations were notably increased in heme-treated cells but were inhibited by melatonin (Figure 3B-D). Furthermore, the protein expression of *GPX4* and *SLC7A11* was suppressed by heme, while melatonin attenuated these changes in expression (Figure 3E). In conclusion, melatonin attenuated heme-induced ferroptosis in HT-22 cells.

Melatonin Promotes the Expression of Nrf2 and HO-1 in Neurons

Activation of the *Nrf2*/*HO-1* pathway inhibits neural cell apoptosis, oxidative stress, and neuroinflammation after ICH[22-24]. Moreover, melatonin is believed to activate the *Nrf2*/*HO-1* pathway and enable astrocytes to resist heme toxicity,

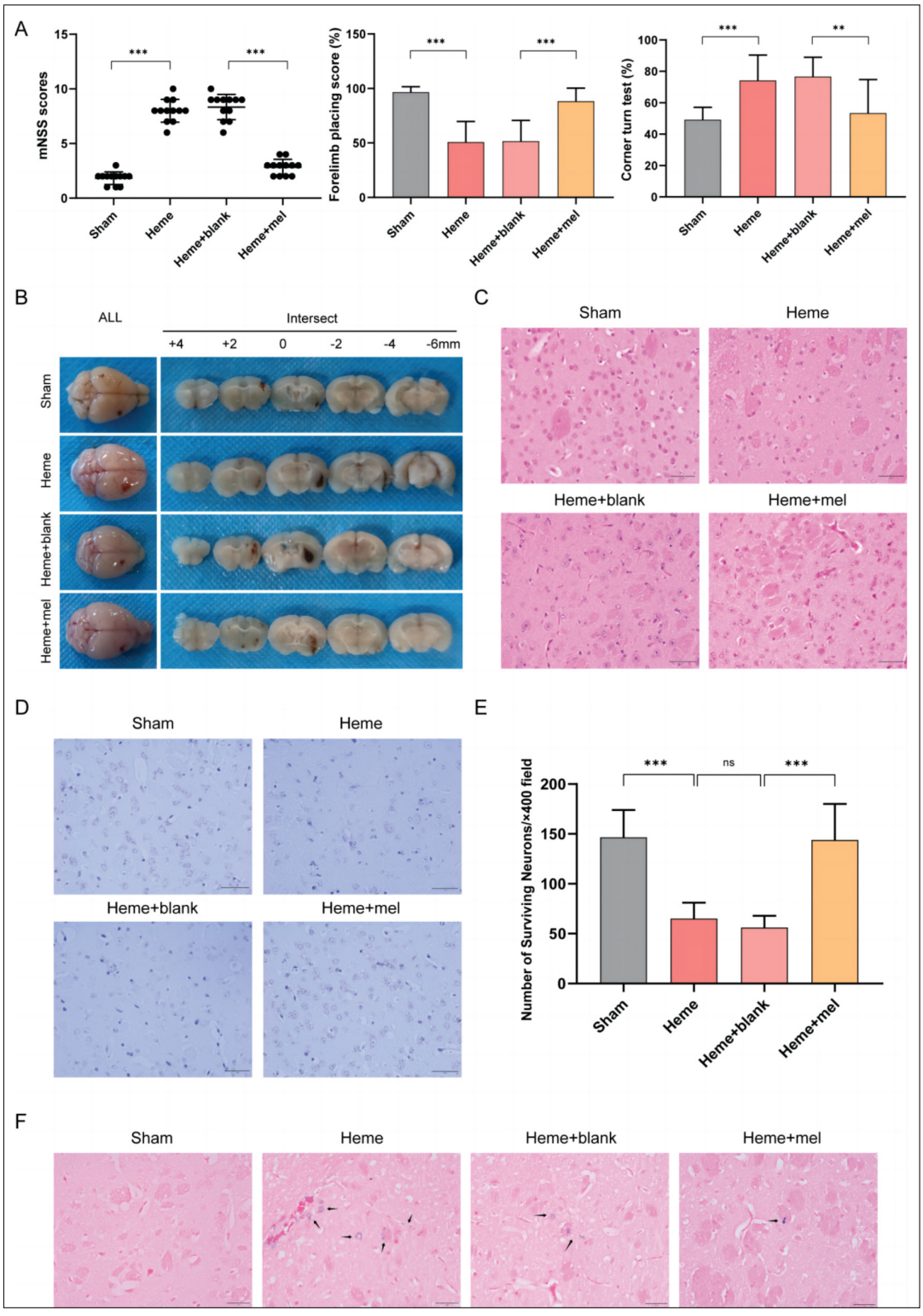

Figure 1. Melatonin improves nerve injury in intracerebral heme-injected mice. After the intracerebral heme injection model was established, melatonin was administered intraperitoneally. **A**, 18-mNSS score, forelimb placing test and corner turn test (n = 12). **B**, Coronary facial sections of brain tissue 72 h after intracerebral heme injection. **C**, Histopathological changes in the brain tissue were visualized *via* H&E staining (40× magnification). **D**, Representative Nissl-stained sections of mouse peristriatal brain injury sites, with arrows indicating surviving neurons (40× magnification). **E**, The number of surviving neurons at the injury site was analyzed. **F**, Iron deposition at sites of brain injury was visualized *via* Prussian blue staining (40× magnification). N = 6, the data are presented as the means ± SDs and were analyzed *via* Student's *t*-test. After the analysis, the data were tested *via* Tukey's multiple comparison test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

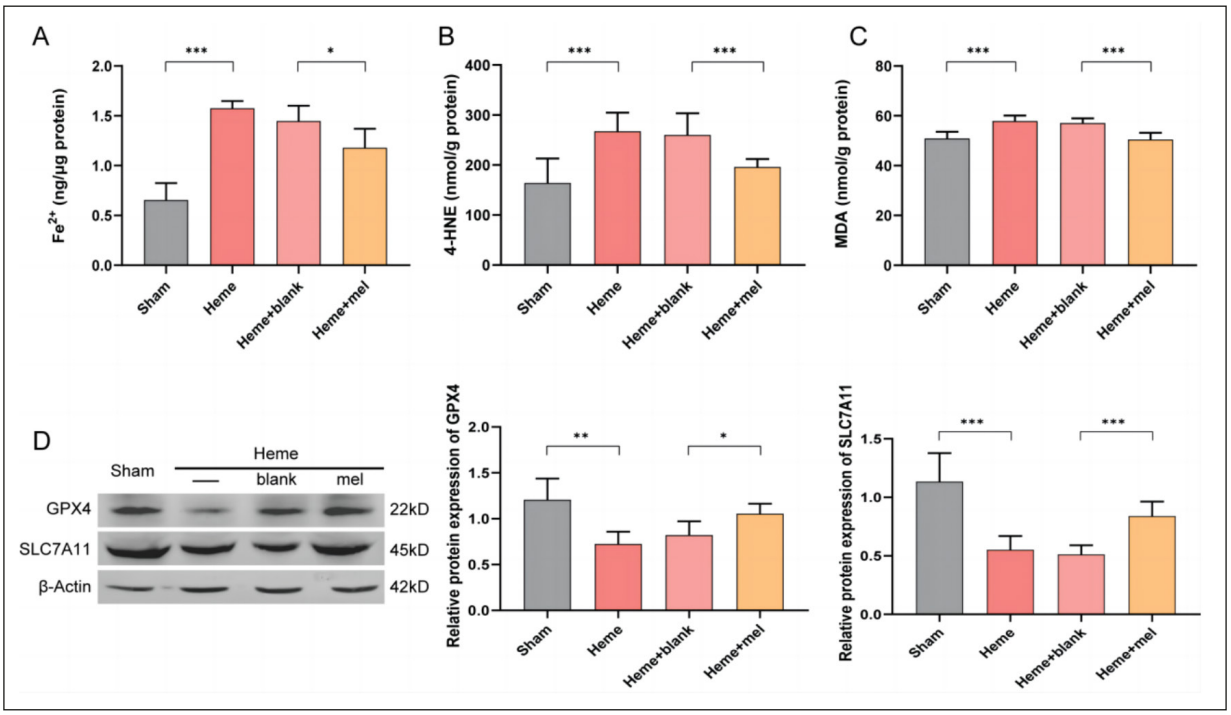

Figure 2. Melatonin alleviates neuronal ferroptosis induced by heme in the mouse brain. **A**, The relative concentration of Fe^{2+} in brain tissue homogenates was determined *via* an iron assay kit. **B**, The content of 4-HNE in the brain tissue was determined by ELISA. **C**, MDA levels in brain tissues were measured *via* ELISA. **D**, The expression levels of *GPX4* and *SLC7A11* were determined *via* western blotting. N = 6, the data are presented as the means ± SDs and were analyzed *via* Student's *t*-test. After the analysis, the data were tested *via* Tukey's multiple comparison test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

oxidative stress and apoptosis[14]. However, whether the *Nrf2*/*HO-1* pathway is involved in the protective effect of melatonin on heme-induced neuronal ferroptosis is still unknown. Therefore, we tested the mRNA and protein levels of *Nrf2* and *HO-1* in heme-treated brains. As expected, *Nrf2* expression was significantly reduced by heme but increased by melatonin (Figure 4A). In addition, *HO-1* was significantly increased by melatonin (Figure 4B). Similar results were observed in HT-22 cells *in vitro* (Figure 4C-D). Taken together, these data highlight that melatonin is capable of promoting the expression of the *Nrf2* and *HO-1* genes in neurons in heme-induced ICH models both *in vitro* and *in vivo*.

Silencing of Nrf2 Exacerbates Heme-Induced Ferroptosis in HT-22 Cells

To further investigate the regulatory role of the *Nrf2*/*HO-1* pathway in neuronal ferroptosis *in vitro* ICH models, we silenced *Nrf2* in HT-22 cells *via* siRNA (Figure 5A). Heme administration inhibited the growth of HT-22 cells, and *Nrf2* silencing further impaired the growth of these cells (Figure 5B). After heme treatment, the 4-HNE and MDA levels were notably increased when *Nrf2* was silenced, but the Fe^{2+} concentration showed no significant difference (Figure 5C-E). The expression of *GPX4*, *SLC7A11*, and *Nrf2* was inhibited by heme, whereas the expression of *HO-1* was increased. Similarly, the expression levels of *GPX4*, *SLC7A11*, *Nrf2* and *HO-1* were significantly decreased when *Nrf2* was silenced *via* heme treatment (Figure 5F). In addition, crystal violet staining analysis revealed that *Nrf2* deficiency diminished the increase in cell growth that was ameliorated by melatonin (Figure 5G). In conclusion, *Nrf2* knockdown aggravated ferroptosis in heme-treated HT-22 cells and subsequently diminished the protective effect of melatonin. We concluded that the *Nrf2*/*HO-1* pathway is crucial for heme-induced neuronal ferroptosis.

Discussion

Ferroptosis is a key pathological feature of ICH injury[25], but the upstream events triggering ferroptosis and neurological damage in ICH are still unclear. Heme, after being released from erythro-

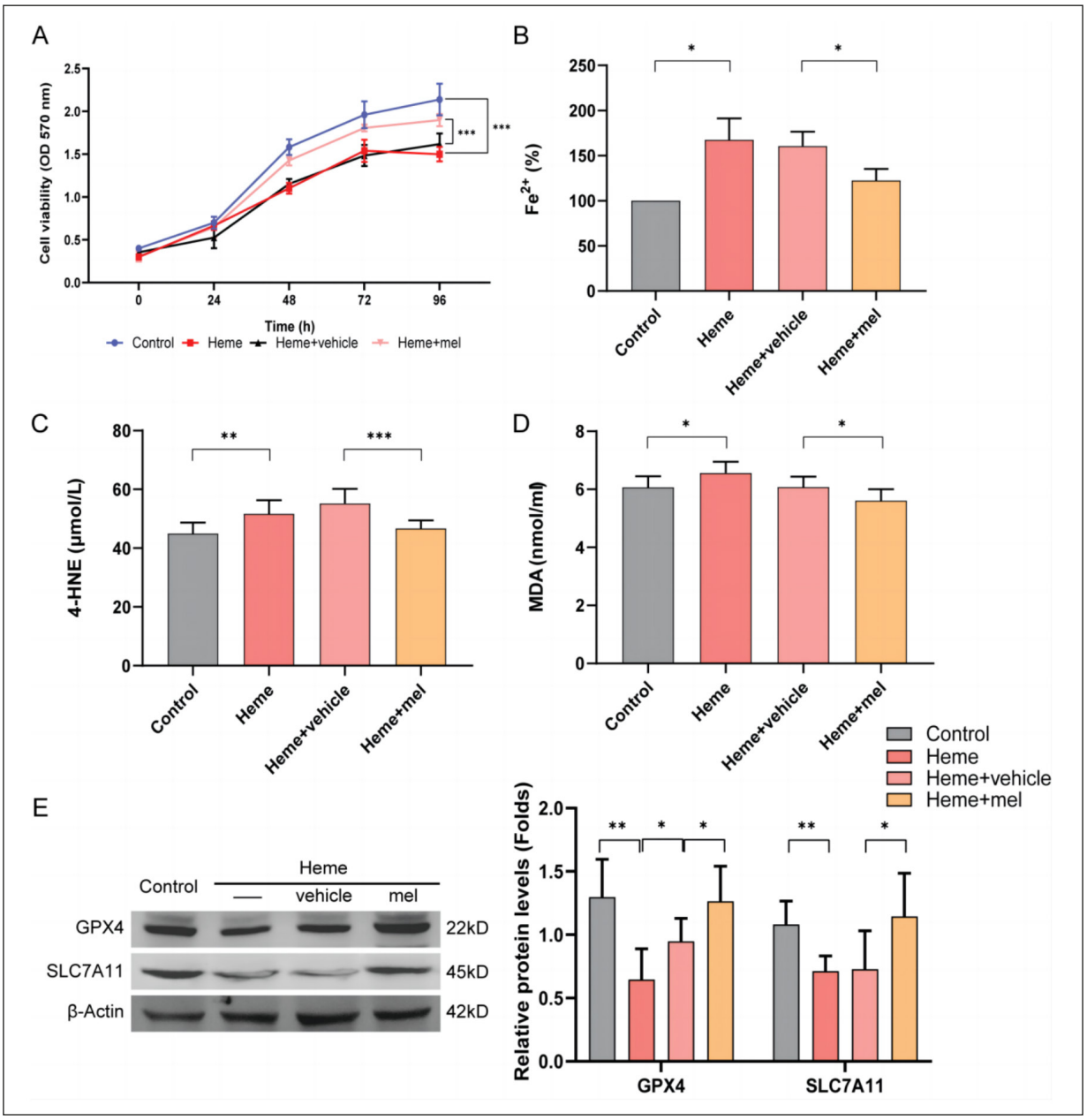

Figure 3. Melatonin alleviates heme-induced ferroptosis in HT-22 cells. HT-22 cells were incubated with heme and then treated with melatonin. **A**, Cell viability was determined by crystal violet staining. **B**, The relative concentration of cellular Fe^{2+} was determined *via* an iron assay kit. **C**, The 4-HNE concentration in cells was determined *via* ELISA. **D**, The level of lipid oxidation-related MDA in the cell supernatants was measured *via* ELISA. **E**, The protein expression of *GPX4* and *SLC7A11* was detected *via* western blotting. The experiments were performed 3 times independently. The data in (**B-E**) were analyzed *via* Student's *t*-test, and the data in (**A**) were analyzed *via* two-way ANOVA. After the analysis, the data were tested *via* Tukey's multiple comparison test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

cytes after ICH, is degraded by heme oxygenase to produce a large amount of free iron ions in the short term[26]. Excessive iron was deposited around the hematoma after 1 day of ICH, which persisted for 2 weeks, and peripheral hematoma edema was also present during this period[27]. These results indicate that iron ions are involved in the formation of cerebral edema around the hematoma. The iron released during erythrocyte lysis can induce lipid peroxidation reactions, produce many free radicals, destroy capillary endothelial cells and the blood-brain barrier (BBB), and cause cerebral

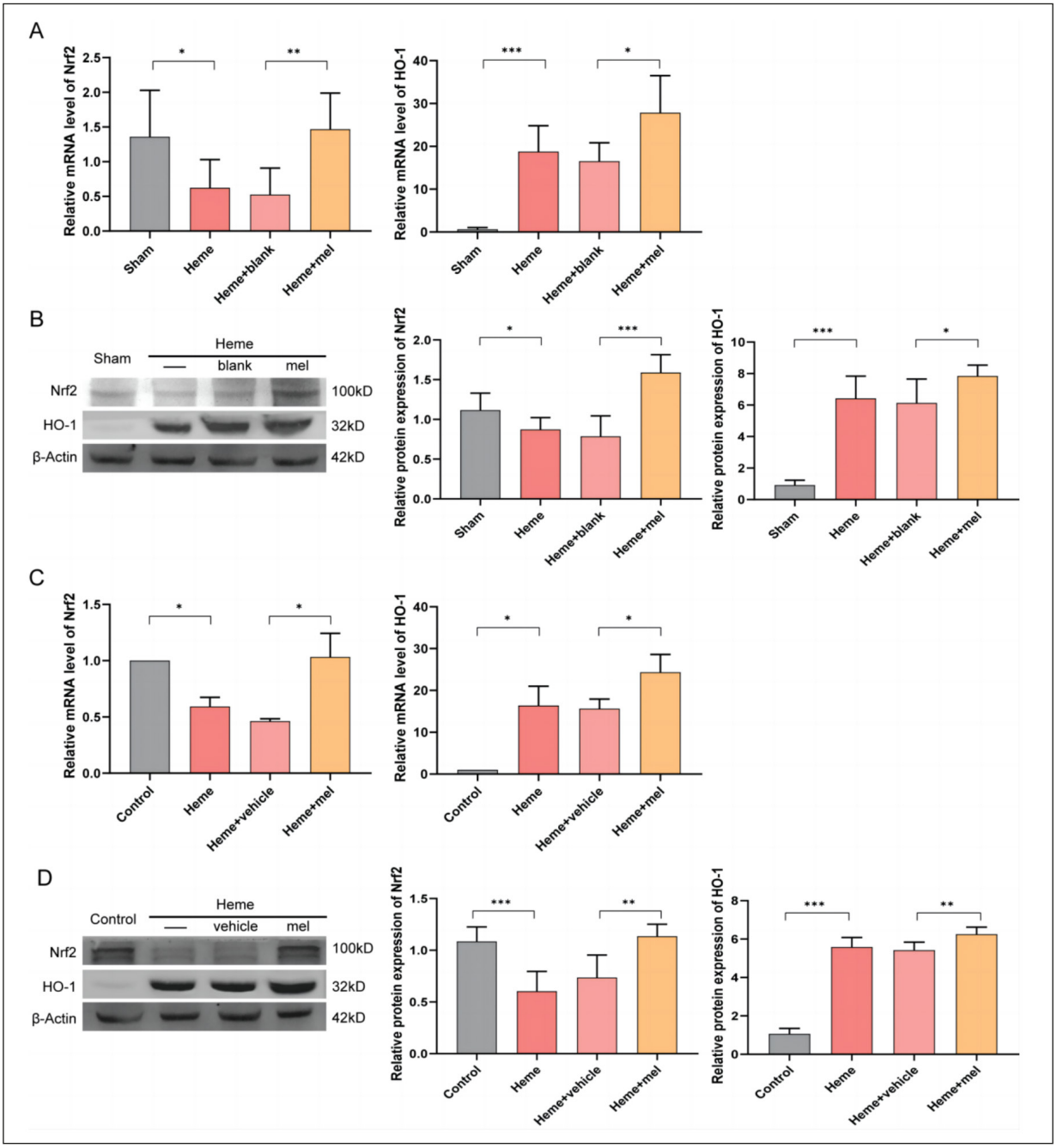

Figure 4. Melatonin promotes the expression of *Nrf2* and *HO-1* in heme-treated neurons. **A**, The mRNA levels of *Nrf2* and *HO-1* in brain tissues were measured *via* qRT-PCR (n = 6). **B**, The expression of *Nrf2* and *HO-1* in heme-injected brain tissues was determined by western blot analysis (n = 6). **C**, The mRNA levels of *Nrf2* and *HO-1* in HT-22 cells were determined by qRT-PCR. **D**, The protein levels of *Nrf2* and *HO-1* in HT-22 cells were determined *via* western blotting. Experiments (**C**) and (**D**) were performed 3 times independently. The data are presented as the means ± SDs and were analyzed *via* Student's *t*-test. After the analysis, the data were tested *via* Tukey's multiple comparison test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

edema[19]. Therefore, we propose that iron ions produced by heme through heme oxygenase trigger iron overload in ICH, resulting in neuronal ferroptosis in the injured area.

To further investigate the role of heme in secondary injury after ICH, heme was intracerebrally injected into the mouse brain. The neurological function deficit score, forelimb placing,

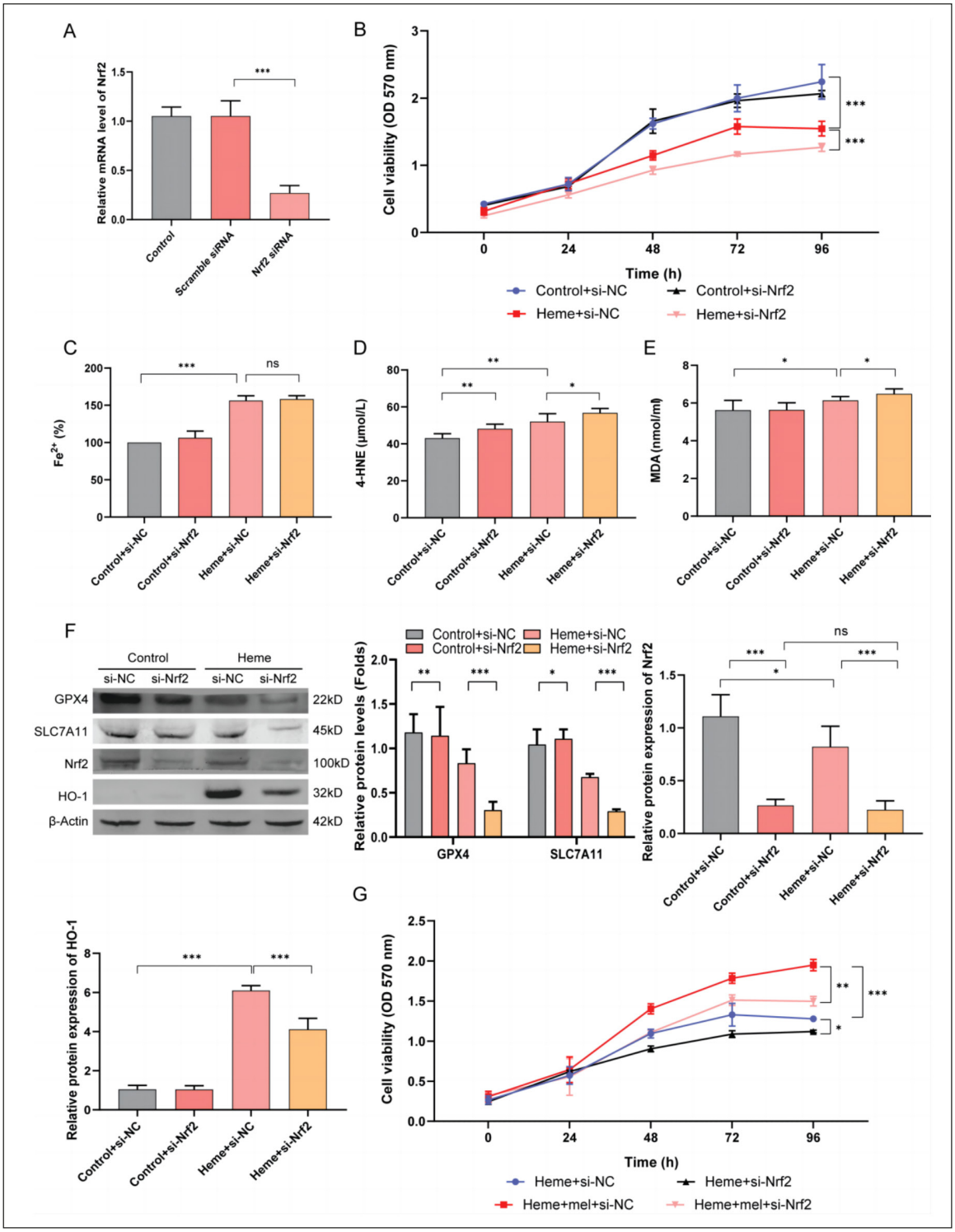

Figure 5. *Nrf2* knockdown exacerbates heme-induced ferroptosis in HT-22 cells. **A**, The silencing efficiency of the siRNA against *Nrf2* was determined *via* qRT-PCR. **B**, The viability of HT-22 cells was determined *via* crystal violet staining. **C**, The relative concentration of Fe^{2+} was determined *via* an iron assay kit. **D**, The 4-HNE concentration in HT-22 cells was determined *via* ELISA. **E**, MDA levels were measured in HT-22 cells. **F**, The protein levels of *GPX4*, *SLC7A11*, *Nrf2,* and *HO-1* were determined *via* western blotting. **G**, The growth of HT-22 cells was determined by crystal violet staining after melatonin treatment. The experiments were performed 3 times independently. The data in (**A**, **C**-**F**) were tested *via* Student's *t*-test, and the data in (**B**, **G**) were analyzed *via* two-way ANOVA. After the analysis, the data were tested *via* Tukey's multiple comparison test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

and corner turn test results all indicated significant neurological deficits. Interestingly, we found that melatonin treatment improved neurological impairment in mice intracerebrally injected with heme. Moreover, evident cerebral edema, neuronal death, intracellular vacuoles, and iron deposition were observed in the brain tissue from the injured site of the intracerebral heme-injected mice and were attenuated by melatonin. Iron overload and lipid oxidation are the key metabolic processes that trigger ferroptosis in ICH. The selenium-containing enzyme *GPX4* is recognized as a central repressor of ferroptosis, and its activity depends on the glutathione produced by the activation of the cystine-glutamate antiporter *SLC7A11*[28]. In the present study, Fe^{2+}, MDA, and 4-HNE levels were consistently increased, whereas the expression of *GPX4* and *SLC7A11* decreased after heme administration. We found that melatonin treatment reversed the process of heme-induced ferroptosis, and similar results were obtained in heme-induced HT-22 cells. These results demonstrated that heme can induce ferroptosis in the mouse brain, which is alleviated by melatonin. Moreover, our *in vivo* and *in vitro* results also revealed that *Nrf2* and *HO-1* were upregulated in neurons after melatonin treatment. *Nrf2* silencing aggravated heme-induced HT-22 cell death and increased 4-HNE and MDA levels, while the expression of *GPX4*, *SLC7A11*, and *HO-1* was inhibited, which inhibited the protective effect of melatonin. Our results initially confirmed that *Nrf2* relieves heme-induced ferroptosis in neuronal cells.

As a neuroendocrine hormone, melatonin has recently been regarded as an effective neuroprotective agent and antioxidant that reaches the central nervous system and multiple peripheral organs to regulate apoptosis, metastasis, angiogenesis, and inflammatory pathways. Melatonin has been regarded as the most promising neuroprotective agent in recent years and can be used in the fields of nutrition, neuroprotection (antioxidant, anti-inflammatory, DNA stabilization, and repair), and neuroplasticity (neurodevelopment, stem cell proliferation, and axonal dendrite formation)[13]. Melatonin alleviates the secondary injury caused by ICH and inhibits neuronal ferroptosis[29]. However, the protective role of melatonin in heme-induced neuronal injury is not understood. In this study, we highlighted that melatonin attenuates heme-induced neuronal ferroptosis by activating the *Nrf2*/*HO-1* pathway.

Melatonin reportedly inhibits ferroptosis by activating the *Nrf2*/*HO-1* pathway[30], and melatonin activates the *Nrf2*/*HO-1* pathway to increase resistance to heme toxicity and oxidative stress in a rat model of ICH[14]. In the present study, *Nrf2* knockdown reduced the therapeutic effect of melatonin, which demonstrated the critical role of *Nrf2* in heme-induced ferroptosis in neuronal cells. *Nrf2* plays an important role in secondary damage caused by ICH by attenuating oxidative stress, inflammation, and neuronal apoptosis, as well as in ferroptosis processes. *HO-1* is the rate-limiting enzyme in heme catabolism[31]. The *Nrf2*/*HO-1* pathway acts as an endogenous antioxidant in multiple organs *via* antagonistic oxidative stress damage[32]. *Nrf2* has been reported to regulate ferroptosis by regulating intracellular iron metabolism through the *Nrf2*/*HO-1* pathway[33]. In line with previous reports[14,30-33], we found that melatonin alleviates neuronal ferroptosis by alleviating heme-induced iron overload and lipid oxide accumulation through the activation of the *Nrf2*/*HO-1* pathway. Notably, in this study, we found that the increase in *HO-1* was influenced by two factors. On the one hand, *HO-1* is directly induced by heme stimulation, and on the other hand, *HO-1* expression is also increased by activated *Nrf2*. Heme-induced *HO-1* expression can attenuate blood-brain barrier disruption after ICH[34]. However, excessive *HO-1* activation can aggravate iron deposition and thus aggravate nerve injury. Therefore, the role of *HO-1* in ICH remains controversial.

In this study, we found that melatonin protected against heme-induced neuronal ferroptosis in ICH by activating the *Nrf2*/*HO-1* pathway. However, the specific metabolic processes of heme and *HO-1* and the detailed protective effects of melatonin in the ICH environment should be further investigated.

Limitations

This study has several limitations that should be acknowledged. First, the pathological conditions of spontaneous ICH in humans may not be fully replicated by using a heme injection model to simulate ICH. While this model is useful for studying certain aspects of ICH, it does not encompass all the complexities of human ICH pathology.

Second, our use of siRNA to inhibit *Nrf2 in vitro* is valuable for understanding the molecular mechanisms involved, but it may not fully reflect

the *in vivo* situation. The knockdown efficiency and potential off-target effects of siRNAs are critical factors that could influence the outcomes of our experiments. We have taken steps to optimize siRNA use and have initially validated its knockdown efficiency in Figure 5A, but these aspects require careful consideration in future studies.

Additionally, our study focused primarily on the role of the *Nrf2/HO-1* pathway in heme-induced ferroptosis and the protective effects of melatonin. However, the specific metabolic processes of heme and *HO-1* and the detailed protective mechanisms of melatonin in the ICH environment need further investigation.

These limitations highlight the need for more comprehensive studies to fully elucidate the mechanisms underlying ICH and the potential therapeutic benefits of melatonin.

Conclusions

In conclusion, our findings suggest that melatonin attenuates heme-induced neuronal ferroptosis by activating the *Nrf2/HO-1* pathway. These findings highlight the potential of melatonin as a therapeutic agent for the treatment of ICH. Despite the limitations related to the experimental models and methods used, this study provides important insights into the molecular mechanisms underlying ICH and the protective role of melatonin. Future studies should aim to address these limitations and further explore the therapeutic potential of melatonin in clinical settings.

Ethics Approval

The experiment was approved by the Biomedical Ethics Committee of the Health Science Center of Xi'an Jiaotong University (No. XJTUAE2023-2166) on October 12, 2023.

Availability of Data and Materials

The data supporting the conclusions of this article are included within the article and its **Supplementary File**, and further inquiries can be sent to the corresponding author.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

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Authors' Contribution

HC: study design, manuscript preparation, data analysis. HC and RH: laboratory work. SG: manuscript revision. LF, YT, JH, BL, and YZ: data research. BY, YH, QZ and SY: literature research.

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AI Disclosure

No artificial intelligence or assisted technologies were used in the production of this study, including the creation of figures.

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