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The neuroprotective effects of carvacrol on ischemia/reperfusion-induced hippocampal neuronal impairment by ferroptosis mitigation

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ABSTRACT

Objective: Cerebral ischemia is the most common type of neuronal injury and is characterized by a reduction in the function and number of hippocampal neurons. Carvacrol has a significant neuroprotective effect in cerebral ischemia. However, the mechanisms by which carvacrol affects cerebral ischemia, especially with respect to the regulation of neuronal damage by iron levels, have never been systematically studied. This study aimed to reveal the mechanisms by which carvacrol protects against hippocampal neuron impairment after ischemic stroke in gerbils.

Materials and methods: The Morris water maze test was performed to evaluate learning and memory impairments. Iron ion content and oxidative stress index were detected by the kit. MTT assay was performed to assess the cell viability. The morphology and molecular characteristics were detected by electron micrographs and western blot.

Results: In the present study, we demonstrated the neuroprotective effects of carvacrol in vivo and in vitro. The Morris water maze test showed that the learning and memory abilities of the gerbils treated with carvacrol were significantly improved. Lipid peroxide injury was evaluated by measuring the levels of lipid peroxide biomarkers; the results indicated that carvacrol decreased the level of lipid peroxide in ischemic gerbil brain tissue. Histopathological examinations and western blotting were performed to evaluate injury in neurons, and carvacrol reduced cell death. Moreover, ferroptosis in the hippocampus was evaluated by measuring the levels of proteins involved in this iron-dependent form of regulated cell death. These results indicated that carvacrol reduced cell death and that carvacrol inhibited ferroptosis by increasing the expression of glutathione peroxidase 4(GPx4). This study showed that carvacrol may be a valuable drug for treating cerebral ischemia.

Conclusion: Carvacrol provides protection for hippocampal neurons against I/R in gerbils by inhibiting ferroptosis through increasing the expression of GPx4.

1. Introduction

Despite decades of intense research, current treatments for neuronal cell damage caused by cerebral ischemia are far from optimal [17]. Early reperfusion is the most effective therapy for acute brain ischemia. However, the reperfusion of tissues may aggravate the injury by causing an increased production of reactive oxygen species (ROS), inflammation, necrosis and apoptosis, which can worsen the prognosis of patients [23]. Moreover, cerebral ischemia/reperfusion (I/R) causes substantial injury to brain tissues during cerebral infarction [41]. Studies have demonstrated that transient ischemic hypoperfusion, especially in the hippocampus; can cause acute brain injury [38,39]. Therefore, reducing cerebral I/R injury is a key challenge in the treatment of cerebral ischemia.

Oxidative stress is characterized by excessive levels of ROS [28], and it has been shown that aberrantly high levels of iron in neurodegenerative diseases can lead to oxidative stress damage [45]. In addition, ROS are produced by high levels of ferrous iron (Fe^{2+}) in cells,

Abbreviations: I/R, ischemia/reperfusion; ROS, reactive oxygen species; GPx4, glutathione peroxidase 4; CAR, Carvacrol

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Table 1
Total carvacrol content in hippocampus.

Group	Carvacrol feeding (mg/kg/day)	Carvacrol in hippocampus (ug/mg)
Sham	0	0
Model	0	0
M + Carvacrol-25	25	0.11 ± 0.02
M + Carvacrol-50	50	0.27 ± 0.02[a]
M + Carvacrol-100	100	0.41 ± 0.03[a]

Data are means of six animals in each group.
[a] $P < 0.01$ compared to M + Carvacrol-25.

which can disturb the homeostasis between ROS scavenging and generation and subsequently excessively augment the generation of hydroxyl radicals [28], thus resulting in cell death.

Cell death, including programmed and non-programmed cell death, is the result of a variety of physical and chemical factors. In the past, studies have found that apoptosis, necrotizing apoptosis, ferroptosis and autophagy are all forms of systemic cell death. In 2002, Cell Magazine reported a new programmed death mechanism called ferroptosis, which is a non-apoptotic programmed death pathway dependent on iron ions and ROS [40]. Ferroptosis can be activated by small molecules or other factors, and its main character is tics are the inhibition of GSH synthesis and the activity of glutathione peroxidase 4 (GPx4) [5]. GPx4 is produced in the cell membrane and can directly reduce peroxidised phospholipid levels [13], and the reduced activity of GPx4 cannot inhibit the production of intracellular ROS, resulting in the accumulation of lipid peroxides and lethal ROS and thus leading to cell death. In addition, Ras selective lethal 3 (RSL3), which is a critical antioxidant enzyme that can reduce lipid hydroperoxide levels within biological membranes, directly binds and inhibits GPx4 [43].Without adequate GPx4 levels, glutathione does not function as a reducing agent in the local peroxidase cycle and thus leads to lipid ROS accumulation and ferroptosis. Both ferroptosis-inducing compounds, eradicator of Ras and ST (erastin) and RSL3, share a common mechanism of cell death that leads to the loss of cellular antioxidant capacity and subsequently to ferroptosis [44]. Recently, researchers have found that many diseases are associated with ferroptosis; for example, ferroptosis was found to be detrimental in melanoma cells with an abnormal expression of ROS and iron ions [2]. Liu and his colleagues showed that puerarin protects against heart failure induced by pressure overload through the mitigation of ferroptosis[22]. In addition, ferroptosis and mitochondrial dysfunction were induced by oxidative stress in PC12 cells [40]. However, the use of iron pools as indicators of iron status and related oxidative stress have not yet been well investigated in ischemia/reperfusion-induced hippocampal neuron impairment, and therapies that specifically target ferroptosis in hippocampal neuron impairment may represent new and useful management strategies.

Carvacrol (CAR), a monoterpenic phenol, is abundant in the essential oil fractions of oregano and thyme. CAR has been widely used as both a food and a food additive in the food industry for a long time [1]. Recently, its multiple functions have been well studied in different fields. It has been demonstrated that the functions of carvacrol include anti-proliferation, anti-apoptosis and neuroprotection [4,9,20,24,38,39]. However, the mechanisms of neuroprotection by carvacrol remain unclear. In this study, we found that carvacrol exerts neuroprotective effects by inhibiting ferroptosis in cerebral ischemia and reperfusion injury in gerbils.

2. Materials and methods

2.1. Animals

A total of 108 gerbils (male; body weight, 70–90 g; age, 12 to 16 weeks) were used in this study. The gerbils were housed in standard environmental conditions at an ambient temperature of 21–24 °C. They were housed 9 per cage with free access to food and water and a natural light cycle. The gerbils were randomly divided into the following five groups: [1] the vehicle-treated group (sham group),which was given an equal volume of physiological saline; [2] the carvacrol (CAR)-treated group (CAR group); [3] the model group, which underwent the ligation of the bilateral carotid artery for 5 min followed by the loosening of the arterial clamp for reperfusion; [4] the model + CAR-treated groups, which included the CAR-treated group and the model + CAR-treated groups that were treated with CAR (25, 50 and 100 mg/kg/day, i.p.) for 2 consecutive weeks [[5,6] and the model + DFO-treated groups that were treated with DFO (150 mg/kg/day, i.p.) for 2 consecutive weeks as the positive drug group [33].

2.2. Morris water maze

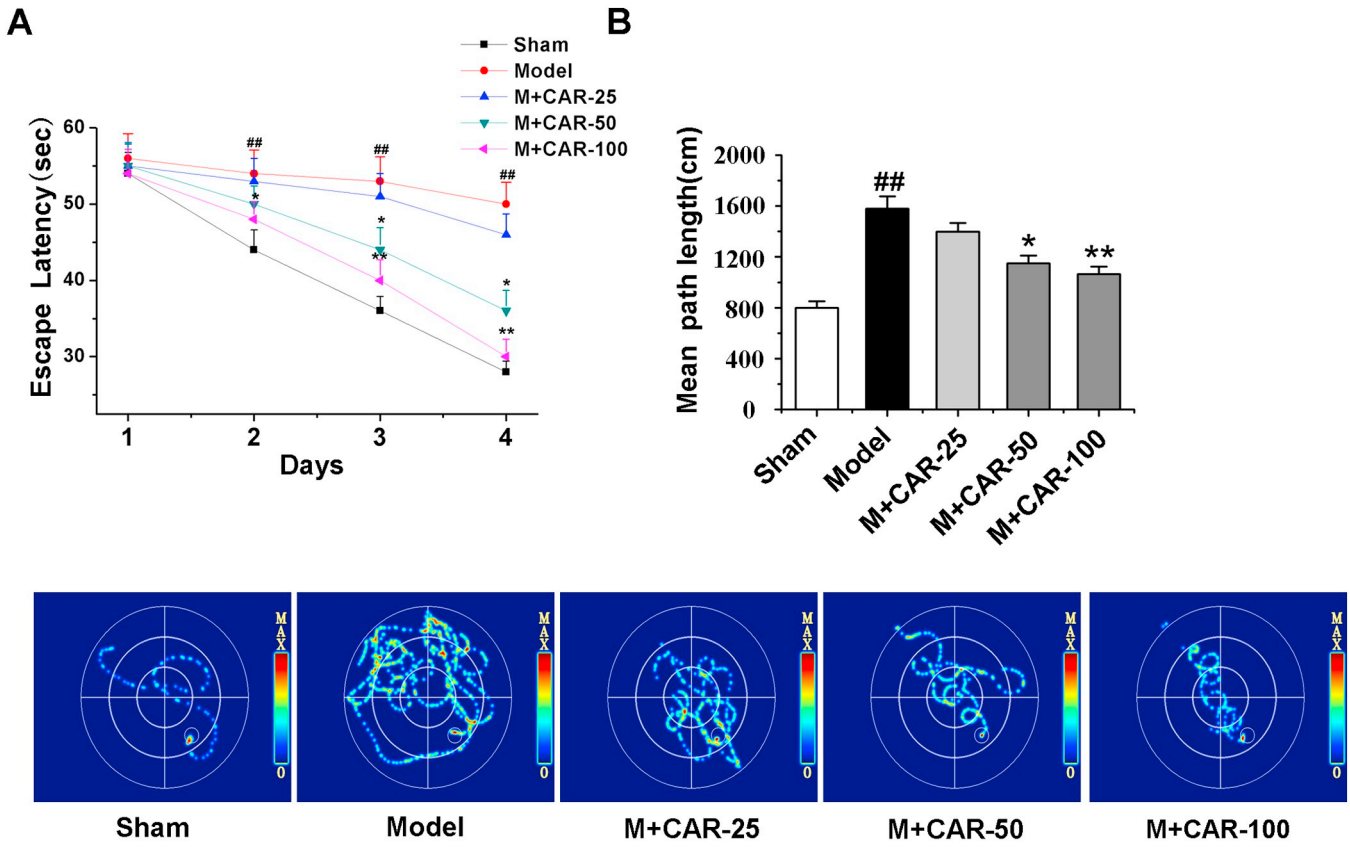
The Morris water maze (MWM) is a convenient and popular test of cognitive function and was used in a previous experiment [35,37]. The MWM apparatus was a 150-cm diameter pool filled to a depth of 35 cm with 23 °C water. It contained a small square 100-cm^2 platform submerged 1.5 cm below the surface of the water. There were large high-contrast visual cues throughout the room and on the walls of the pool. The tracking of the animals and the computation of the dependent measures were performed by an automated water maze system. The pool was divided into four quadrants, and the platform was placed at the midpoint of a quadrant. Two weeks after treatment with carvacrol or saline, spatial learning and memory were assessed using a Morris Water Maze (MWM) task. The MWM test lasted 9 days. On the first day, all gerbils were allowed to swim freely. On the 2nd day through the 4th day, the gerbils were trained to find the platform. If a gerbil located the platform within 60 s, it was allowed to stay on it for 15 s. The gerbils that failed to find the platform within 60 s were manually guided to the platform and allowed to remain on it for 30 s. From the 5th day through the 8th day, the platform was hidden 1.5 cm under the water surface, and each gerbil was subjected to four trials in a maximum of 60 s. Escape latency (the time to find the platform) and path length for the four trials were averaged to obtain each gerbil's visible task score. On the 9th day, the animals underwent a probe trial. The hidden platform was removed; the time spent in the target quadrant and the number of times each gerbil crossed the location on that the hidden platform had previously been located was recorded. The MWM test was conducted by an experimenter blind to the treatment groups.

2.3. Nissl Staining

Sections were immersed in 0.1% cresyl violet at 37 °C for 20 min. After being rinsed with distilled water, the sections were dehydrated, cover slipped and examined with a light microscope. To assess neuronal survival in the CA1 region, neurons with round and palely stained nuclei were considered normal cells. Five brain sections were selected for each animal, and the data are expressed as the number of surviving cells/field [36].

2.4. NeuN immunohistochemistry

An anti-neuronal nuclei marker (NeuN) was used to investigate neuronal damage. The sections were sequentially treated with 0.3%



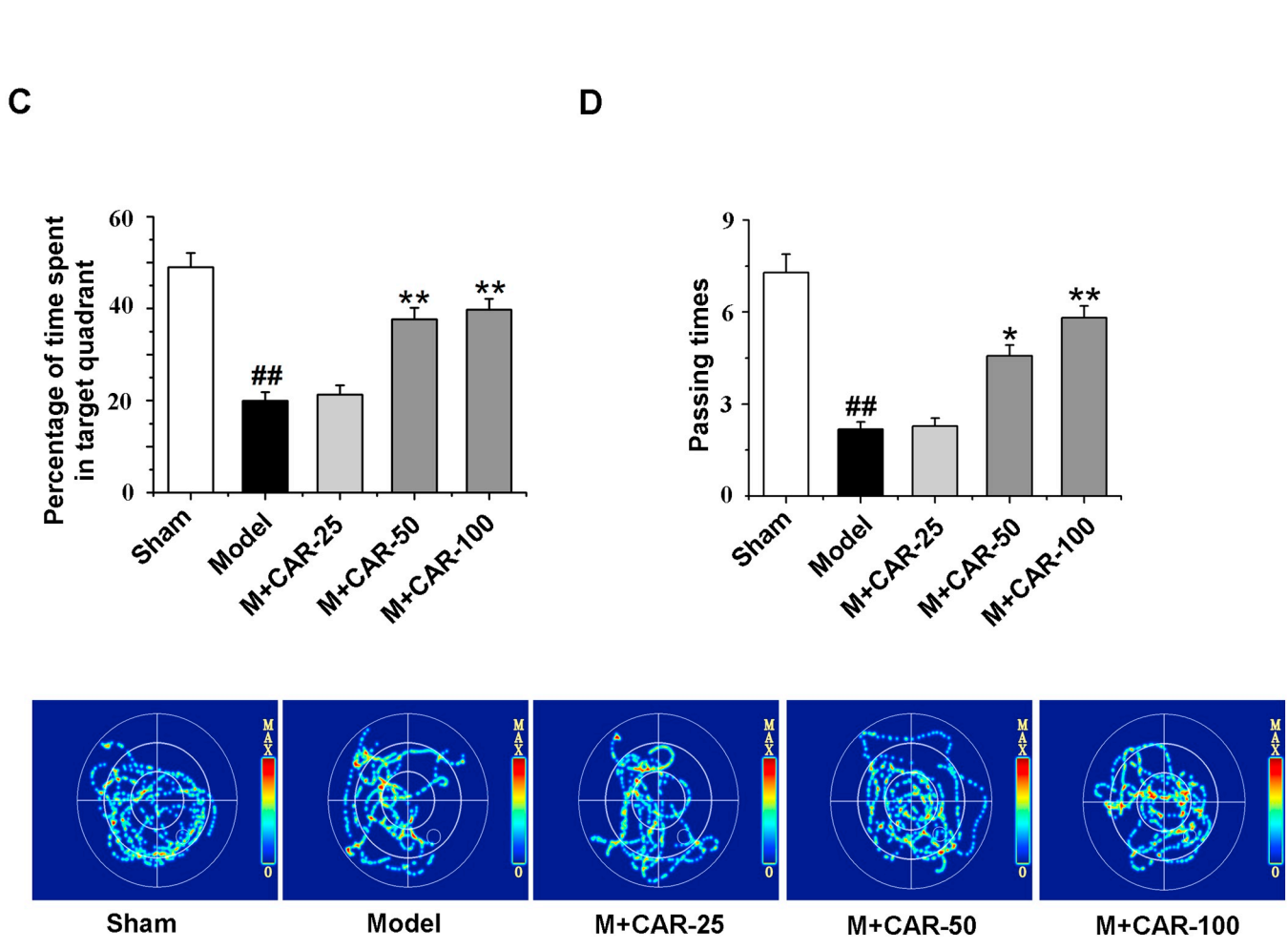


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Fig. 1. Carvacrol ameliorated I/R induced cognitive impairment in gerbils. (a) Escape latency, (b) mean path length, (c) gerbils that stayed in the target quadrant, (d) passing times are shown in the sham, model, and different carvacrol dose groups. M + CAR-25, M + CAR-50, and M + CAR-100 indicate that gerbils underwent the ligation of the bilateral carotid artery for 5 min followed by the loosening of the arterial clamp for reperfusion, and were treated by carvacrol 25, 50, and 100 mg/kg. The data are expressed as the mean ± SEM ($n = 6$ per group). $^{\#\#}P < 0.01$ compared to sham group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared to model group.

H_2O_2 in PBS for 30 min and 10% normal goat serum in 0.05 M PBS for 1 h. The sections were then incubated with diluted mouse anti-NeuN (diluted 1:200, Chemicon International, Temecula, CA) overnight at 4 °C. Thereafter, the tissues were exposed to biotinylated goat anti-mouse IgG (Vector, Burlingame, CA) and a streptavidin peroxidase complex (diluted 1:200, Vector). They were then visualized by staining with 3, 3′-diaminobenzidine (DAB) tetrahydrochloride in 0.1 M Tris-HCl buffer (pH 7.2) and mounted on gelatin-coated slides. The stained sections were observed under a light microscope. The quantification of the number of NeuN-immunopositive cells from 10 CA1 regions per brain sample ($n = 6$) was performed using Image-Pro Plus 6.0 (Media Cybernetics, Bethesda, MA, USA) [18].

2.5. Total carvacrol content analyses in hippocampus of gerbils

Ultraperformance liquid chromatography (UPLC) analysis was performed to detect the carvacrol level of hippocampus in gerbils of different groups using the same method described previously [10].

2.6. Immunofluorescence

TUNEL immunofluorescence staining was performed to explore the colocalization of death cells and neurons. TUNEL staining was conducted with the In Situ Cell Death Detection Kit (Roche, Germany) according to the manufacturer's protocol. The images were captured with a fluorescence microscope (Nikon) [35,37].

2.7. Hippocampal neuron culture

Hippocampal neurons were prepared from 2- to 3-day-old neonatal gerbils. The hippocampuswas dissected and cut into 1-mm^3 pieces under a light microscope. The hippocampal chunks were digested with 0.125% (*w/v*) trypsinase at 37 °C for 10 min and then resuspended in DMEM supplemented with 10% foetal bovine serum (FBS) to stop the trypsin activity. After centrifugation at 14000 × *g* for 5 min, the supernatant was discarded, and the cell pellet was resuspended in DMEM. The cells were cultured in serum-free neurobasal medium with B27 supplement in a 37 °C, 5% CO_2 humidified incubator. Before each experiment, hippocampal neurons were chosen between days 8 and 14 of culture. Then, some cells were treated with carvacrol [31]. The cultured neurons were randomly divided into four groups: [1] the control group; [2] the model group, in which the cells were subjected to OGD for 6 h followed by re-oxygenation for 24 h; [3] the model + carvacrol group, in which the cells underwent re-oxygenation for 24 h and the culture medium was mixed with 1.2 mM carvacrol; and [4] the model + siRNA group, in which the cells were transfected with a GPx4 X-treme GENE small interfering RNA (siRNA) (siGPx4) or a GPx4 scrambled siRNA (scGPx4) [29] and were then exposed to OGD treatment for 6 h followed by re-oxygenation for 24 h, and the culture medium was mixed with 1.2 mM carvacrol.

2.8. MTT assay

Cells were cultured in 96-well plates and incubated for 4 h in medium containing 0.5% MTT. After removing the supernatant, dimethyl sulfoxide (150 μl/well) was added. The plates were then agitated on a plate shaker for 10 min at room temperature. The absorbance was measured at 540 nm using an ELISA microplate reader, and the cell viability was defined as the % of untreated control cells [32].

2.9. Oxidative stress measurements

To determine whether CAR treatment decreases oxidative stress (free radical damage), the enzymatic activities of Cu/Zn-superoxide dismutase (SOD), Mn-SOD, SOD, glutathione peroxidase (GSH-PX), and catalase (CAT), the level of glutathione (GSH) and malondialdehyde (MDA) were evaluated with different detection kits according to the manufacturer's instructions (Nanjing Jian Cheng Bioengineering Institute). One part of the hippocampus was weighed and rinsed with cold saline. Tissue homogenates (10% *w/v*) were prepared by homogenizing the hippocampal tissue in cold saline (pH 7.0). CuZn-SOD and Mn-SOD activities were detected in the hippocampal homogenates. The data were recorded as SOD units/mg of protein. The observation absorbance of CAT was read at 505 nm, and the enzyme activity was calculated as units/mg of protein. The observation absorbance of GSH was read at 420 nm, the content of GSH was expressed as nmol/mg of protein. The level of MDA in the hippocampus was calculated by measuring thiobarbituric acid-reactive substances at 532 nm. The concentration of malondialdehyde is expressed as nmol/mg of protein. The activities of GSH-PX were detected by quantifying the rate of oxidation of reduced glutathione to oxidized glutathione. The levels of enzymatic activity were measured with a spectrophotometer (U-2000, Hitachi) [27].

2.10. Iron measurements

The hippocampus was immediately homogenized in phosphate-buffered saline (PBS). The supernatant was collected after centrifugation. The iron level was determined by the Iron Assay Kit (ab83366, Abcam) according to the manufacturer's instructions.

2.11. Western blotting

The hippocampal tissues were immediately removed and stored at −80 °C until use for western blotting. The samples were treated with a homogenizer, and the homogenate was centrifuged at 13500 rpm for 15 min at 4 °C. An equal amount of protein of different samples were separated by Tris-glycine SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 10% skim milk and then incubated overnight at 4 °C with the following primary antibodies: H2AX (1: 500, Cell Signaling Technology), SOD1 (1: 1000, Cell Signaling Technology), GPx4 (1: 1000, Abcam, Cambridge, MA, USA), ferroportin 1 (Fpn1, 1: 1000, Abcam, Cambridge, MA, USA), transferring receptor (TFR, 1: 1000, Abcam, Cambridge, MA, USA) and amyloid precursor protein (APP, 1:2000, Sigma, St Louis, USA). The NC membranes were washed briefly three times with TBST and then incubated with corresponding HRP-conjugated secondary antibodies for 1 h under normal temperature conditions. After the blots were washed briefly, the protein bands of the immune complexes were scanned by using an Odyssey infrared imaging system (LI-COR, Lincoln, NB). The analysis of the chemiluminescent signals was performed by using Image-Pro Plus version 6.0 (Media Cybernetics, MD, USA) [42].

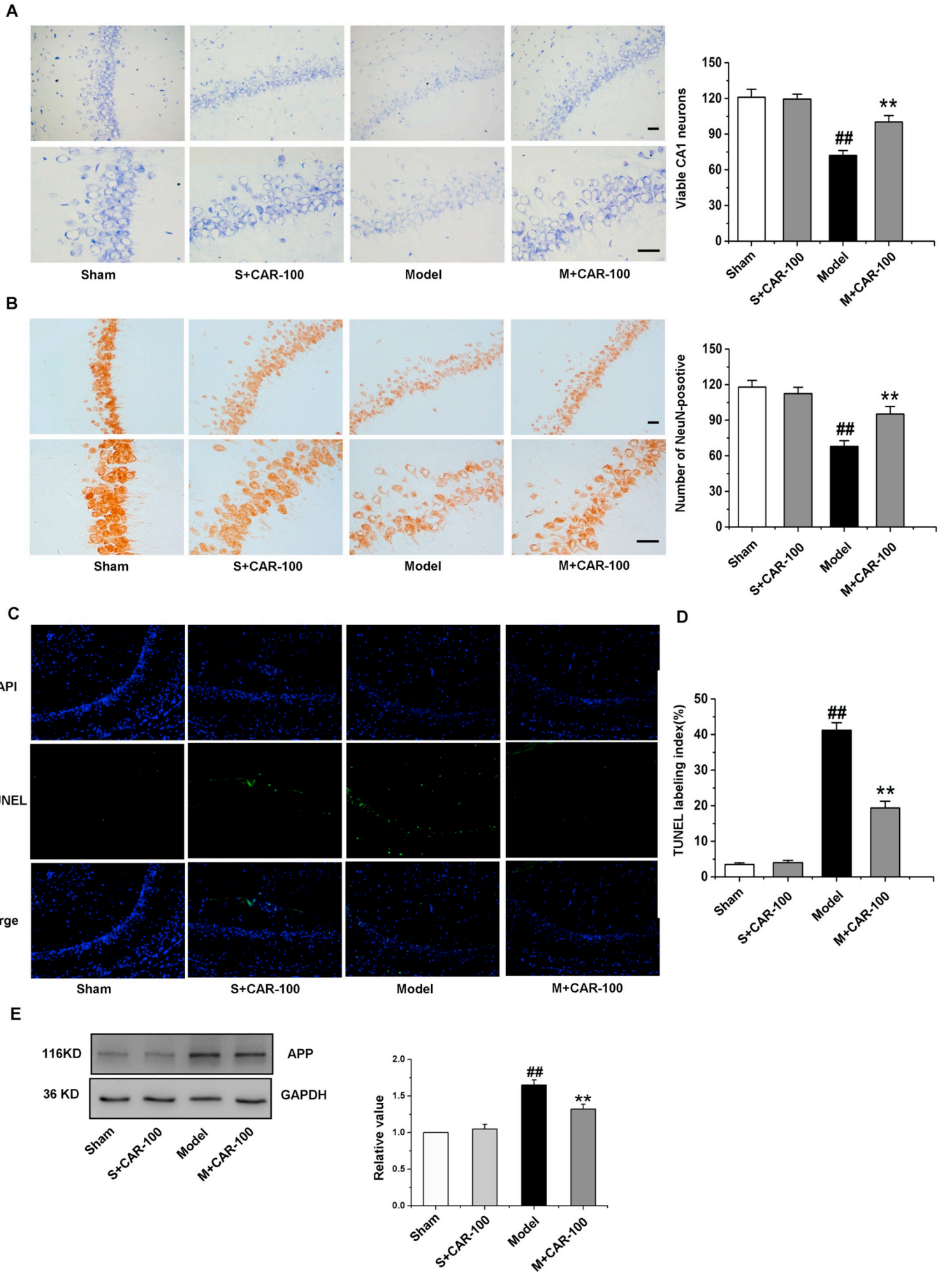

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Fig. 2. The protective effects of carvacrol on I/R induced neurons impairment of the hippocampal CA1 region in gerbils. (a) Representative photomicrographs of Nissl staining for surviving neurons in hippocampal CA1 region, and the statistical analysis of the surviving cells in each group. (b) Representative immunohistochemical photomicrographs of NeuN in gerbils hippocampus, and the statistical analysis of the NeuN-immunopositive cells in each group. There were fewer NeuN and Nissl-positive neurons in the model group than in the sham group. Treatment with different doses of carvacrol, NeuN and Nissl-positive neurons were abundant in the CA1 region compared with the model group. Up panel is lower magnification image (200 x) and down panel is higher magnification image (400 x) of CA1 pyramidal neurons. Scale bar: 50 μm. (c) Representative photographs of TUNEL staining in different groups. TUNEL-positive cells were barely detected in the sham group. In the model group, the TUNEL-positive cells increased markedly compared with the sham group. However, compared with the model group, administration of different doses of carvacrol substantially reduced the number of death cells. (d) Quantification of TUNEL-positive cells. (e) The quantitative analyses of the protein levels of APP in different groups. Scale bar: 100 μm. The data are expressed as the mean ± SEM ($n = 6$ per group). $^{\#\#}P < 0.01$ compared to sham group; $^{**}P < 0.01$ compared to model group.

2.12. Statistical analysis

The data are represented as the mean ± SEM. Statistical analysis was carried out using one-way ANOVA followed by Tukey's test for individual comparisons between the group means. All statistical analyses were performed using SPSS 19.0 software. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Increased carvacrol concentrations in the hippocampus of carvacrol-treated gerbils

To determine whether carvacrol treatment in the I/R-treated gerbils would change the carvacrol content in the hippocampus, we first examined the carvacrol concentrations in the hippocampus in all groups. In the UPLC-MS analysis, we observed that the total carvacrol contents of M + Carvacrol-100 and M + Carvacrol-50 group were increased significantly compared to the M + Carvacrol-25 group ($P < 0.01$). (Table 1).

3.2. Carvacrol rescued the memory and learning abilities of gerbils after I/R

The MWM was used to investigate the effects of carvacrol on learning and memory deficits in gerbils after I/R. Fig. 1A indicates that carvacrol dose-dependently reduced the escape latency ($P < 0.05$, $P < 0.01$). The analysis of the mean path length showed that the memory of the carvacrol-treated gerbils (50 and 100 mg/kg) was significantly rescued after I/R, as described in Fig. 1B. Decreases the time spent in the target quadrant and the number of times the animals crossed the former location of the platform were observed in the model group versus the sham group ($P < 0.05$, $P < 0.01$), and supplementation with carvacrol (50 and 100 mg/kg) dose-dependently reversed the two indices, as shown in Fig. 1C-D ($P < 0.05$, $P < 0.01$). These results suggest that carvacrol can rescue the memory and learning ability of gerbils after I/R, and the 100 mg/kg dose of carvacrol is the most effective.

3.3. Carvacrol protected the Hippocampus of gerbils exposed to I/R

The hippocampus plays an important role in learning and memory [15]; therefore, we detected the effect of carvacrol on hippocampal neurons in gerbils after I/R. The histological examination of Nissl staining revealed a significant loss of neurons in the hippocampal CA1 region, as illustrated in Fig. 2A. The neurons in the sham group did not display any histopathological abnormalities; however, extensively damaged neurons with pyknotic nuclei were observed in the model group. The group treated with carvacrol exhibited significantly increased cell survival and an increased number of cells with palely stained nuclei compared with those in the model group ($P < 0.01$). NeuN immunohistochemistry showed that the number of NeuN-immunopositive cells in the hippocampal CA1 region was markedly decreased in the model group in comparison with the sham group (Fig. 2B). Furthermore, we tested the cells death in different groups by TUNEL staining. The results revealed that 100 mg/kg carvacrol indeed had the ability to mitigate cells death in the hippocampus (Fig. 2C-D). We further detected amyloid precursor protein (APP), an axon-damaged marker protein, and found that carvacrol significantly reduced the expression of APP (Fig. 2E) ($P < 0.01$). These results indicate that carvacrol inhibits the hippocampus impairment of gerbils exposed to I/R.

3.4. Carvacrol decreased ROS and iron ion levels in the hippocampus of gerbils in the model group

To investigate whether carvacrol provides protection against neuronal damage via antioxidant mechanism, biomarkers of oxidative stress were evaluated. As shown in Fig. 3, our results indicated that the model gerbils exhibited reduced activities of antioxidative enzymes, including SOD, Mn-SOD, Cu/Zn-SOD (Fig. 3A-C), GSH-PX (Fig. 3E), CAT (Fig. 3F) and level GSH (Fig. 3D), and increased the level of MDA (Fig. 3G) in comparison with the sham group ($P < 0.01$, $P < 0.05$). Treatment with carvacrol (100 mg/kg) significantly reversed these changes. Similar to the above results, iron, another essential factor for ferroptosis, showed significant accumulation in the model group but not in the sham group or carvacrol treatment groups (Fig. 3H). The concentration of MDA was noticeably increased in the model group compared with the sham group (Fig. 3I) ($P < 0.01$), whereas carvacrol suppressed the level of MDA as same as DFO (Deferoxamine). In addition, the expression of H2AX was decreased by carvacrol and DFO (Fig. 3J) ($P < 0.01$). These findings indicate that I/R induce ferroptosis, suggesting that the protection of carvacrol may involve in hibiting ferroptosis.

3.5. Carvacrol inhibited ferroptosis in the hippocampus of gerbils in the model group

Western blot analysis showed that the expression of the SOD1 protein in the model gerbils was decreased compared with the sham group, and the expression of SOD1 was markedly increased after treatment with carvacrol (Fig. 4A) ($P < 0.01$, $P < 0.05$). Oxidative stress aberrantly elevated the iron ion level; iron overload can generate excessive free radicals and cause neuronal death. To investigate the effects of carvacrol on the iron ion level in the hippocampus, we detected the protein expression levels of GPx4, Fpn1 and TFR1. The results showed that the expression levels of GPx4 and Fpn1 in the model gerbils were decreased but that the TFR1 level was increased compared with the sham group (Fig. 4B-D) ($P < 0.01$, $P < 0.05$); however, carvacrol increased the protein expression levels of GPx4 and Fpn1 but decreased the protein level of TFR1 in the model gerbils. Our results suggest that carvacrol can inhibit ferroptosis in the hippocampus of gerbils in the model group.

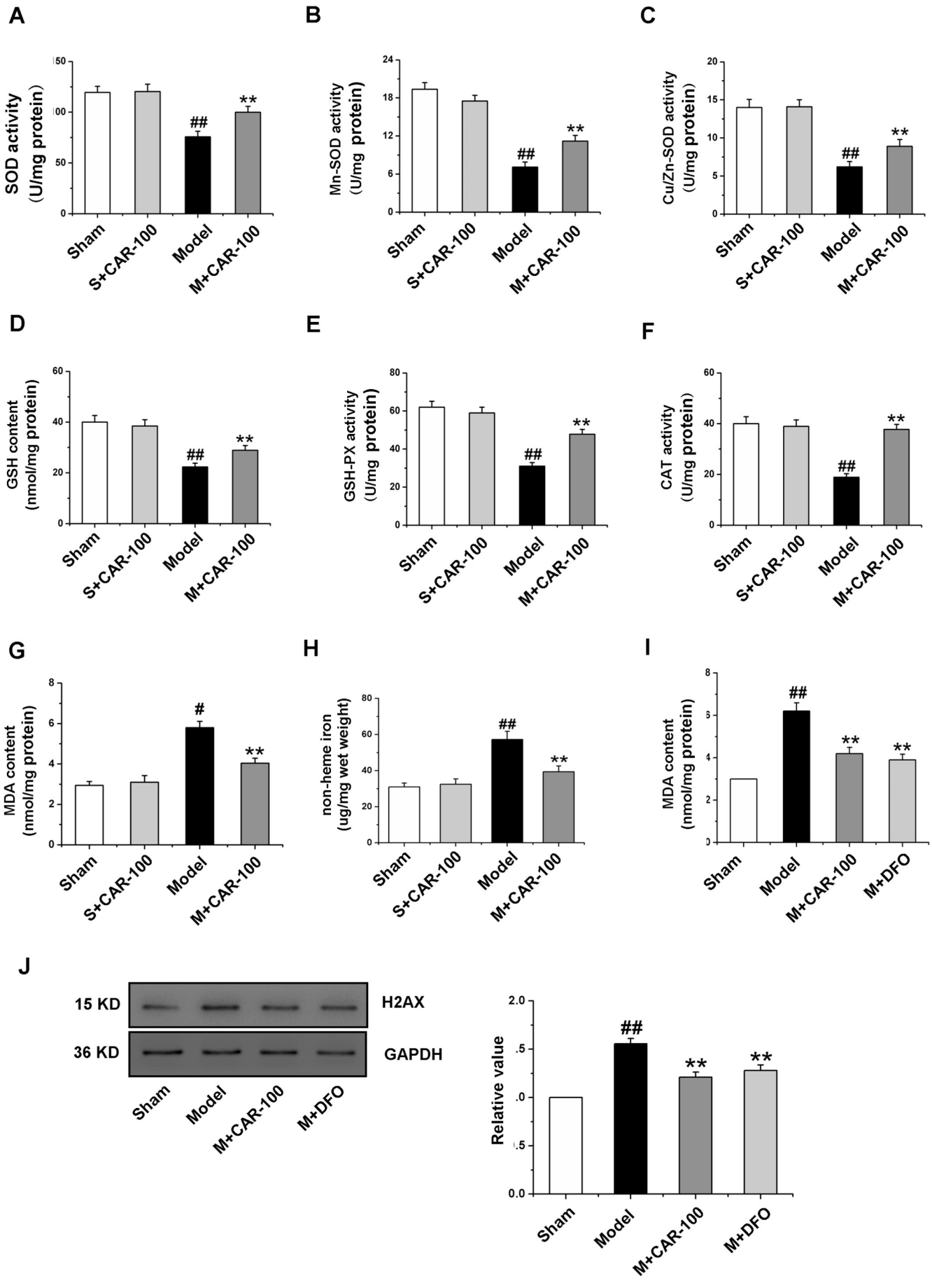

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Fig. 3. Effects of carvacrol on ferroptosis of the hippocampus in I/R gerbils. (a)-(h) The effects of carvacrol on activities of SOD, Mn-SOD, CuZn-SOD, GSH-PX, CAT and levels of GSH, MDA and iron from different group, respectively. (i) Hippocampus were collected to measure the level of MDA. (j) The quantitative analyses of the protein levels of H2AX in different groups. The data are expressed as the mean ± SEM ($n = 6$ per group). $^{\#\#}P < 0.01$ compared to sham group; $^{**}P < 0.01$ compared to model group.

3.6. Carvacrol protected hippocampal neurons by increasing GPx4 expression

To detect the effect of carvacrol against anoxia/reoxygenation in hippocampal neurons, cell viability after different concentrations of carvacrol was explored using the MTT assay. As shown in Fig. 5A, hippocampal neuron viability in the model group was significantly reduced compared with that in the control group ($P < 0.01$). The models treated with 1.2 mM and 2.4 mM carvacrol exhibited significantly increased cell viability compared with the model group ($P < 0.01$); thus, 1.2 mM dose of carvacrol was used in the subsequent experiments. To explore the possible involvement of GPx4 activation in the effects of carvacrol on anoxia/reoxygenation-induced impairment, we first detected GPx4 expression by western blotting analysis. As illustrated in Fig. 5B, GPx4 protein expression was inhibited during anoxia/reoxygenation, while it was remarkably elevated during anoxia/reoxygenation after treatment with 1.2 mM carvacrol ($P < 0.01$). We further investigated the effect of carvacrol on changes in Fe^{2+} levels. The levels of intracellular Fe^{2+} were increased in the model group; interestingly, the carvacrol-treated group exhibited decreased levels of Fe^{2+} (Fig. 5C). We further silenced GPx4 (Fig. 5D) to confirm that the protective effects of carvacrol occur through GPx4. Our results revealed that carvacrol significantly decreased the levels of MDA, H2AX protein expression, and hippocampal neurons impairment compared to those in the anoxia/reoxygenation group, but these effects were reversed by silencing GPx4 (Fig. 5E-H) ($P < 0.01$). Taken together, these findings indicate that carvacrol protects hippocampal neurons by increasing GPx4 expression.

4. Discussion

Accumulating evidence has suggested the potential of carvacrol as a therapeutic agent for cerebral ischemia, and carvacrol has been reported to have neuroprotective functions against neurodegenerative diseases in in vitro and in vivo studies [24,34]. However, whether carvacrol can act on ferroptosis and, if so, the associated mechanism remains unknown. In the present study, we demonstrated that treatment with carvacrol significantly inhibited oxidative stress and alleviated neuronal degeneration and memory deficits in gerbils exposed to ischemia/reperfusion. Moreover, our data also suggested the possible use of carvacrol to inhibit ferroptosis in cerebral ischemia in gerbils.

Determination of behavioural, morphological, chemical, and molecular biological changes have indicated that cerebral ischemia/reperfusion can induce oxidative stress [7] and lead to hippocampal neuronal apoptosis [16], which ultimately results in cognitive dysfunction [25]. Our results showed that carvacrol partially reversed memory and learning deficits in the model group, as indicated by an increase in the number of times the platform location was crossed and a decrease in the escape latency. In addition, Nissl staining, NeuN immunohistochemistry and the expression of APP showed that carvacrol markedly reduced neuronal death in vivo. At the same time, 1.2 mM carvacrol significantly improved the H/R induced reduction in neuronal cell viability in vitro. These results indicate that carvacrol alleviates learning and memory deficits via inhibiting hippocampal neuronal damage.

As an organ that consumes high levels of oxygen, the brain is rich in unsaturated fatty acids, and this provides the pathophysiological basis for reactive oxygen species-mediated hippocampal neuron injury. In addition, abundant non-heme iron in the brain can cause membrane lipid peroxidation through the Fenton reaction under conditions of stress, resulting in encephalo edema, mitochondrial damage, functional impairment, etc. Numerous studies have demonstrated that oxidative damage, an important index for evaluating neuronal injury caused by I/R, plays an important role in neuropathologic lesions [11,30]. In the current study, we investigated the effect of carvacrol on ROS in gerbils exposed to I/R, and the results indicated that carvacrol markedly inhibited oxidative stress-induced damage. Consequently, the reduction of ROS and the enhancement of antioxidative stress may have an important role in the protection against I/R-induced neuronal injury by carvacrol.

The iron ion distribution present in the brains of gerbils exposed to I/R may be involved in the process of inhibiting neuronal ferroptosis, which is an iron-dependent type of cell death [8]. Indeed, to date, the mechanism of ferroptosis is not clear, but iron overload and inflammation are considered hallmarks of ferroptosis [12]. Importantly, researchers have explored ferroptosis as the key mode of cell death in neurodegeneration [14] through the use of iron chelators to prevent hippocampal neuronal death. A number of iron chelators have been reported to have good potency in inhibiting neuronal injury [3,26]. Therefore, it is worth considering whether neuronal populations are important in cognition and may be affected by ferroptosis in the brains of gerbils exposed to I/R and whether carvacrol can inhibit ferroptosis in gerbils under conditions of I/R. As mentioned above, the expression of Fpn1, an iron ion efflux pump was increased, and the expression of TFR, an iron ion importer, was decreased in the brain tissue of carvacrol treated gerbils. Therefore, intracellular iron ion overload in the hippocampus and cortex may be reduced after carvacrol treatment, and iron-dependent cell death may be reduced in the brains of carvacrol-treated gerbils. Carvacrol treatment can also inhibit the generation of ROS and increase the levels of GPx4. In the present study, we found that carvacrol blocked H2AX, and the expression of GPx4 was significantly enhanced after treatment with carvacrol. Taken together, our results indicated that carvacrol treatment enhanced neuronal survival in gerbils exposed to I/R. To investigate the relationship between GPx4 and cell death, we silenced GPx4. From the results, we can conclude that the activation of GPx4 is a possible mechanism of the neuroprotection conferred by carvacrol following I/R exposure.

Ferroptosis, a new form of cell death, was recently discovered, results from iron-dependent lipid peroxide accumulation [8]. Four mechanisms that lead to ferroptosis have been reported: [1] generation of reactive oxygen species [2] depletion of GPX4 [3] accumulation of lipid hydroperoxides [4] transferrin import or iron overload [21]. Surprisingly, when we investigated the protective of carvacrol after I/R-induced neuronal impairment, we found GPX4 is to be a key inhibitor of ferroptosis. Our data analysis was confined to GPx4 effects on neurons; the contribution of other mechanisms cannot be involved. Much more studies on the mechanism of ferroptosis are still required and a deeper understanding of ferroptosis will surely be beneficial to the treatment of relevant diseases.

The results indicated that the protective effects of carvacrol on cognitive dysfunction in gerbils exposed to I/R and hippocampal neuronal impairment may be associated with antioxidative and anti-ferroptosis effects and may be at least in part mediated by GPx4. Because carvacrol is a natural product with a proven record of safe human administration, it is possible that carvacrol may be regarded as a promising drug for clinical therapy.

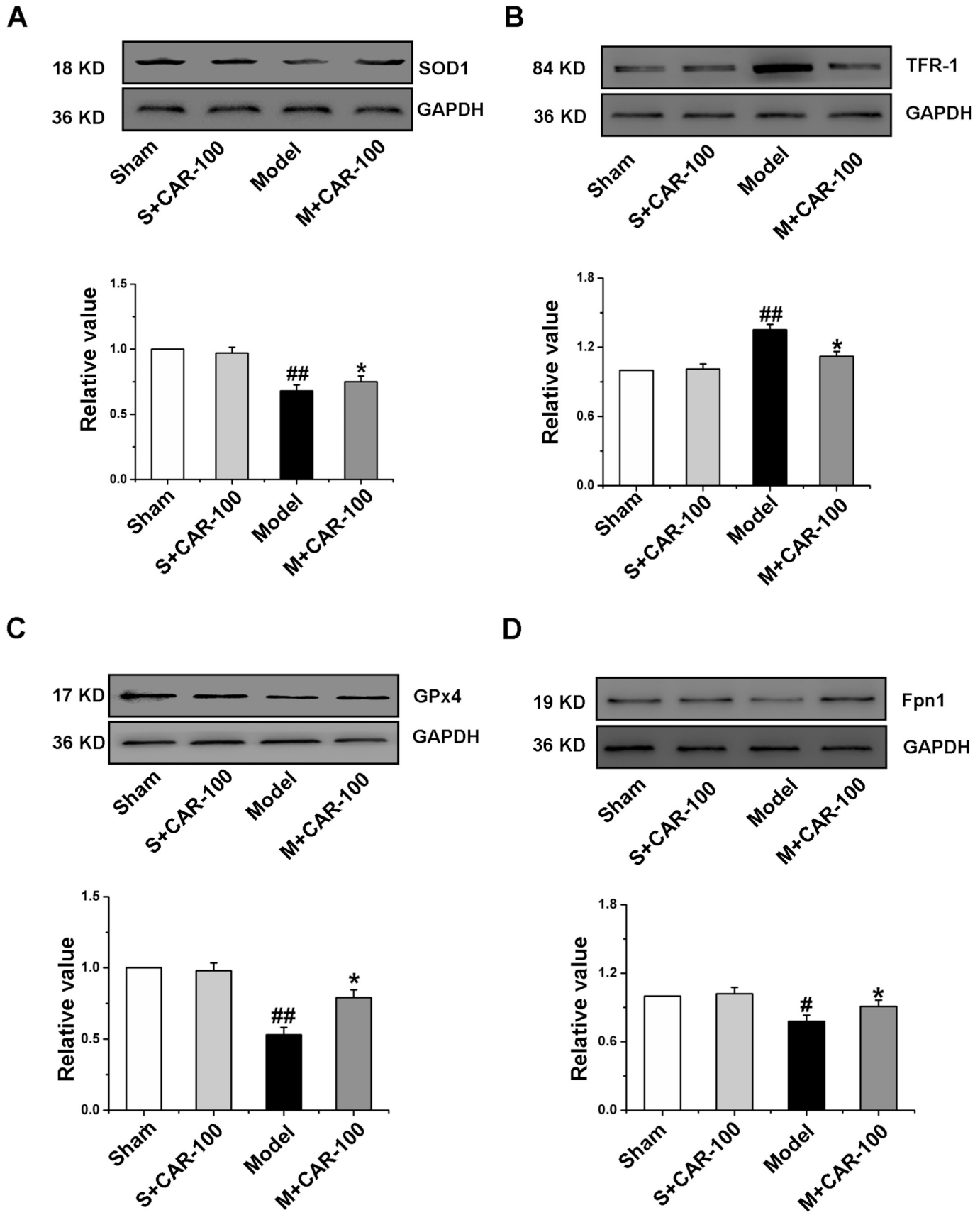

Fig. 4. Carvacrol altered the protein expression of SOD1, TFR-1, GPx4 and Fpn1 of the hippocampus in I/R gerbils. (a)-(g) The quantitative analysis of the protein levels of SOD1, TFR-1, GPx4 and Fpn1 in gerbils hippocampus. The data are expressed as the mean ± SEM ($n = 6$ per group). $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared to sham group; $^{*}P < 0.05$ compared to model group.

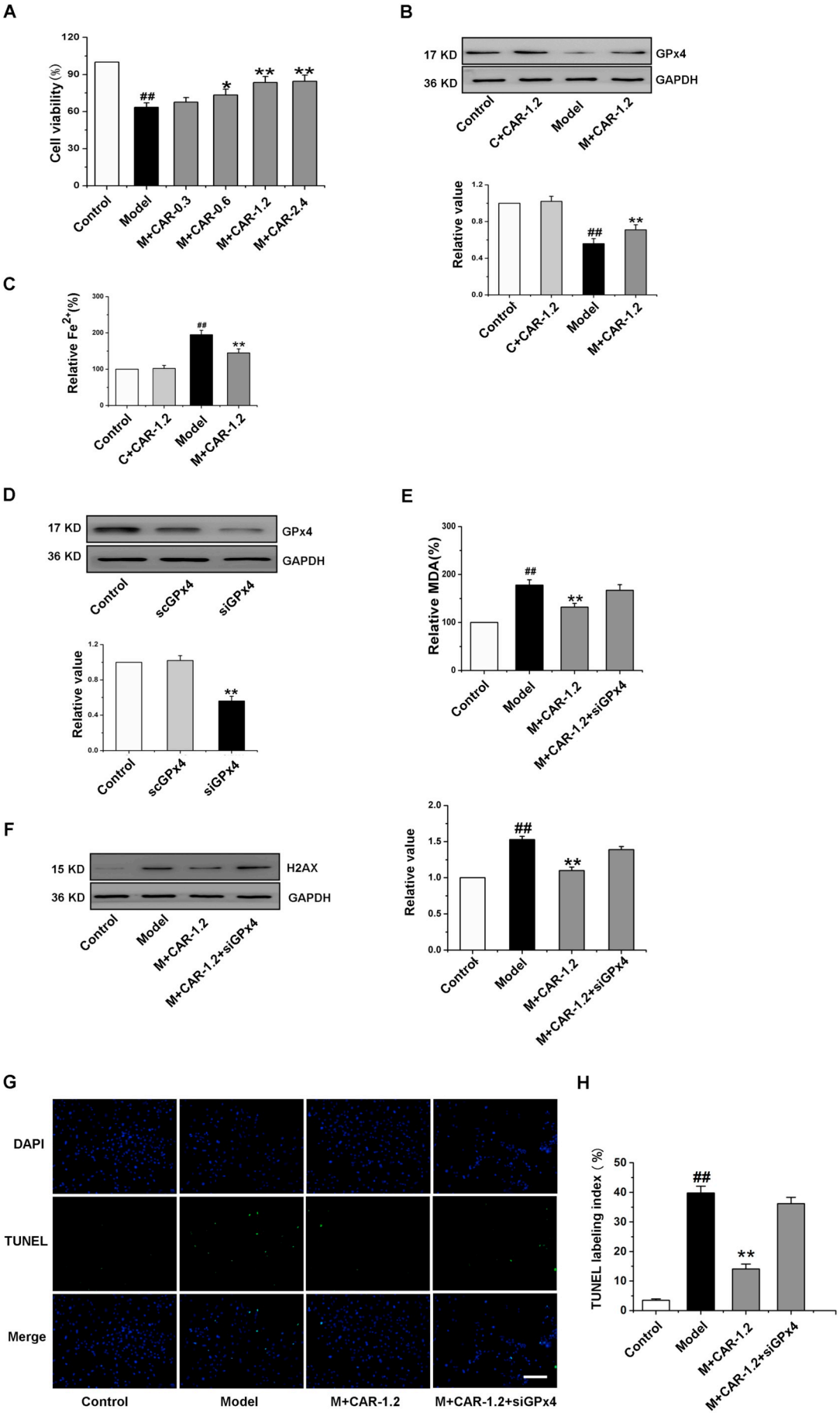

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Fig. 5. Silence GPx4 pathway partially repeals the anti-ferroptosis effects of carvacrol. (a) Effects of carvacrol on cells viability of model group, MTT assay was carried out on the hippocampal neurons. (b) In vitro, the quantitative analyses of the protein levels of GPx4 in different groups. (c) The expression levels of carvacrol influences the Fe^{2+} accumulation in hippocampal neuron cells. (d) The efficiency and specificity of siRNA directed against GPx4. (e) The lipid accumulation was measured by MDA assay. (f) Showed the H2AX protein expression, and (g-h) hippocampal death rate. Carvacrol could inhibit the neurons death induced by oxygen-glucose deprivation. However, when GPx4 was silenced, carvacrol inhibiting cells death effects could be partially repealed. siGPx4: GPx4 was silenced. The data are expressed as the mean ± SEM ($n = 6$ per group). $^{\#\#}P < 0.01$ compared to control group; $^{*}P < 0.05$ and $^{**}P < 0.01$ compared to model group. Scale bar: 100 μm.

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Declaration of competing interest

The authors declare that they have no conflicts of interest to disclose. All authors approved the final article.

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