



Article

A Water Extract of Mixed Mushroom Mycelia Mitigates Cognitive Deficit and Oxidative Stress After Global Cerebral Ischemia–Reperfusion Injury

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Abstract

Background: GMK is a bioactive material newly identified from a water extract of mixed mushroom mycelia (*Phellinus linteus*, *Inonotus obliquus*, and *Ganoderma lucidum*). It has shown protective effects against glutamate-induced excitotoxicity and lipopolysaccharide-triggered neuroinflammation. However, whether GMK can ameliorate global cerebral ischemia–reperfusion injury (GCIRI) and its associated cognitive deficit remains to be elucidated. **Methods:** GCIRI was induced in male Sprague–Dawley rats by bilateral common carotid artery occlusion with hypovolemia (BCCAO/H). GMK (30 or 90 mg/kg, p.o.) was administered once daily for 14 days before surgery. Cognitive functions were evaluated using the Y-maze, Barnes maze, and passive avoidance tests. Hippocampal CA1 neuronal survival and glial activation were analyzed by cresyl violet staining and Iba1/GFAP immunohistochemistry. In parallel, PC12 cells were pretreated with GMK (100 or 200 µg/mL, 24 h) before oxygen–glucose deprivation and reoxygenation (OGD/R), and apoptosis (TUNEL, Bax/Bcl-2), oxidative stress markers (ROS, MDA, and NO), antioxidant enzymes including glutathione peroxidase (GPX) and catalase (CAT), and signaling proteins (p-ERK/ERK, iNOS) were examined. **Results:** GMK significantly ameliorated GCIRI-induced learning and memory impairments, protected CA1 pyramidal neurons, and reduced microglial and astrocytic activation. In OGD/R-challenged PC12 cells, GMK attenuated apoptosis, suppressed ROS, MDA, and NO production, normalized GPX and CAT activities, and favorably regulated p-ERK and iNOS pathways. **Conclusions:** These findings suggest that GMK confers dose-dependent behavioral and histopathological protection against GCIRI, potentially by modulating redox- and apoptosis-related signaling (Bax/Bcl-2, GPX/CAT, and ERK/iNOS pathways), with more consistent effects at a higher dose.



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Keywords: GMK; global cerebral ischemia–reperfusion injury; cognitive impairment; hippocampal CA1; oxidative stress; anti-apoptotic; PC12 cells; BCCAO/H model

1. Introduction

Global cerebral ischemia–reperfusion injury (GCIRI) can follow transient whole-brain hypoperfusion caused by cardiac arrest, profound hypotension, or systemic hypoxia [1,2]. Although reperfusion is required for survival, it may exacerbate secondary damage, and therapies that consistently preserve cognition after global ischemia are still unavailable [3,4]. Selective vulnerability of hippocampal circuitry—especially CA1 pyramidal neurons—is a hallmark of GCIRI; delayed neuronal loss in this region often manifests as enduring learning and memory deficits [5–8]. Such vulnerability likely arises from the combined impact of energy failure, excitotoxicity, oxidative injury, and inflammatory amplification during the post-ischemic phase [9–12].

Oxidative stress plays a central role in this process: reactive oxygen species (ROS) generated during reperfusion impair mitochondrial function, drive lipid peroxidation, and engage pro-apoptotic signaling [10,13]. Concurrently, activated microglia and reactive astrocytes may prolong injury through cytokine release and reactive nitrogen/oxygen species, further perturbing neurovascular and synaptic homeostasis [10–12]. Because GCIRI is multifactorial, agents with broad antioxidant and anti-inflammatory actions remain of substantial interest.

Natural products—including medicinal mushrooms—are rich in bioactive constituents (e.g., polysaccharides, polyphenols, terpenoids, and sterols) reported to influence redox balance, immune pathways, and neuronal survival in models of neurological injury [14,15]. The mushroom species *Phellinus linteus*, *Inonotus obliquus*, and *Ganoderma lucidum* have each shown neuroprotective potential in preclinical studies, such as reducing oxidative stress-related neuronal damage and improving cognitive outcomes in neurodegenerative or ischemic paradigms [16–18]. However, variability in extract composition and batch-to-batch reproducibility highlights the need for standardized preparations in mechanistic and translational work.

In earlier work, aqueous fractions from mixed mushroom mycelia grown on a solid barley substrate protected PC12 cells from glutamate-induced excitotoxicity and reduced infarct burden while improving function in focal cerebral ischemia–reperfusion injury models [19]. We also observed benefits in Alzheimer’s disease (AD)-related settings, including attenuation of amyloid- β toxicity and improved cognition in transgenic models [20]. Those findings were encouraging but did not determine whether protection extends to global ischemia.

To enhance consistency and biosafety, we subsequently optimized and standardized a refined water extract from the same strains, termed GMK. In cellular models of excitotoxicity and lipopolysaccharide-driven neuroinflammation, GMK improved cell survival and dampened apoptotic and inflammatory signaling; metabolomic analyses suggested candidate neuroactive metabolites associated with antioxidant and anti-apoptotic pathways [21]. Nonetheless, whether GMK can counteract GCIRI-associated diffuse neuronal injury and severe cognitive decline remains unknown. Compared with focal ischemia, global ischemia imposes broader metabolic and inflammatory stressors, providing a stringent testbed for neuroprotective candidates [22].

Here, we examined the hypothesis that GMK pretreatment preserves hippocampal integrity and cognitive function following GCIRI. GMK was evaluated in a rat bilateral common carotid artery occlusion with hypovolemia (BCCAO/H) model using complementary behavioral tests (Y-maze, Barnes maze, and passive avoidance) together with

histopathological assessment of CA1 neuronal survival and glial reactivity [23–25]. In parallel, to interrogate redox- and apoptosis-related mechanisms under controlled conditions, we used an oxygen–glucose deprivation/reoxygenation (OGD/R) paradigm in PC12 cells and quantified oxidative stress indices, antioxidant enzyme activities, and apoptosis-related endpoints [26].

2. Materials and Methods

2.1. Preparation of the Aqueous Extract of GMK

The GMK was obtained by Giunchan Co., Ltd. (Cheonan, Republic of Korea) using a previously reported method, with the following steps [21]. A blend of *Phellinus linteus*, *Inonotus obliquus*, and *Ganoderma lucidum* mycelia, first cultivated in liquid medium, was transferred to sterilized barley solid substrate at 3% (*w/w*). This composite mycelium was incubated for 50–55 days at 25–30 °C and 40–60% humidity, then dried for 24 h at 57–60 °C. The dried material was mixed with purified water at a 1:10 ratio and extracted at 75–80 °C. After filtration to remove solids, the solution was concentrated at 55–60 °C and subsequently freeze-dried. The resulting GMK powder was stored at –20 °C until use. The tentative chemical composition of GMK was previously characterized by UHPLC–QTOF–MS/MS in both positive and negative electrospray ionization modes, and a summary of the chromatographic profiles and putative compound annotations from that analysis is provided in the Figure S1 [21].

2.2. Animals

Male Sprague-Dawley rats (220–250 g, 8 weeks old) were purchased from Samtako (Osan, Republic of Korea). The animals were housed under controlled conditions (22–23 °C, 40–60% humidity, 12:12 h light/dark cycle) with free access to food and water [27]. All experimental procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, 8th edition, 2011) and approved by the Institutional Animal Care and Use Committee (IACUC) of Konyang University (Daejeon, Republic of Korea; approval code: P-24-30-A-01; approval date: 8 November 2024) [22,28].

2.3. In Vivo Experimental Plan

After a 7-day acclimatization period, rats were randomly divided into four groups (*n* = 5 per group): the Control group (sham-operated, vehicle only), the OP group (BCCAO/H with vehicle), the OP + GMK(L) group (BCCAO/H with low-dose GMK, 30 mg/kg), and the OP + GMK(H) group (BCCAO/H with high-dose GMK, 90 mg/kg). GMK was dissolved in normal saline (used as the vehicle) and administered by intragastric gavage at doses of 30 or 90 mg/kg in a final volume of 1 mL once daily for 14 consecutive days prior to BCCAO/H surgery, while the Control and OP groups received the same volume of vehicle. After surgery, all animals underwent behavioral tests (Y-maze, Barnes maze, and passive avoidance), followed by sacrifice for histological and biochemical analyses. The timeline of the in vivo experimental schedule is shown in Figure 1A. Group allocation was carried out using a computer-generated random sequence. Behavioral testing and histological quantification were carried out by investigators blinded to group assignment, and group codes were revealed only after data analysis. The same animals were used across all behavioral paradigms in the fixed order shown in Figure 1A, with at least 24 h between tasks.

2.4. BCCAO/H

GCIRI was induced using the BCCAO/H procedure as previously described, with minor modifications [29]. Briefly, rats were anesthetized with 1.5% isoflurane in a

30:70 mixture of oxygen and nitrous oxide, and their body temperature was maintained at 37 ± 0.5 °C using a heating pad connected to a rectal probe (JD-DT-08-06, Jeong-do B&P, Seoul, Republic of Korea). After a midline cervical incision, (1) the right common carotid artery (CCA) was transiently occluded with an aneurismal clip. (2) The left CCA was permanently ligated with 4-0 black silk, and a vascular catheter was inserted rostral to the ligation site to allow controlled withdrawal of arterial blood while preventing bleeding. (3) Arterial blood was withdrawn through the catheter to induce systemic hypotension and reinfused after approximately 8 min to restore blood pressure. Afterward, the aneurismal clip on the right CCA was removed to allow reperfusion, the surgical wounds were closed, and the rats were returned to their home cages.

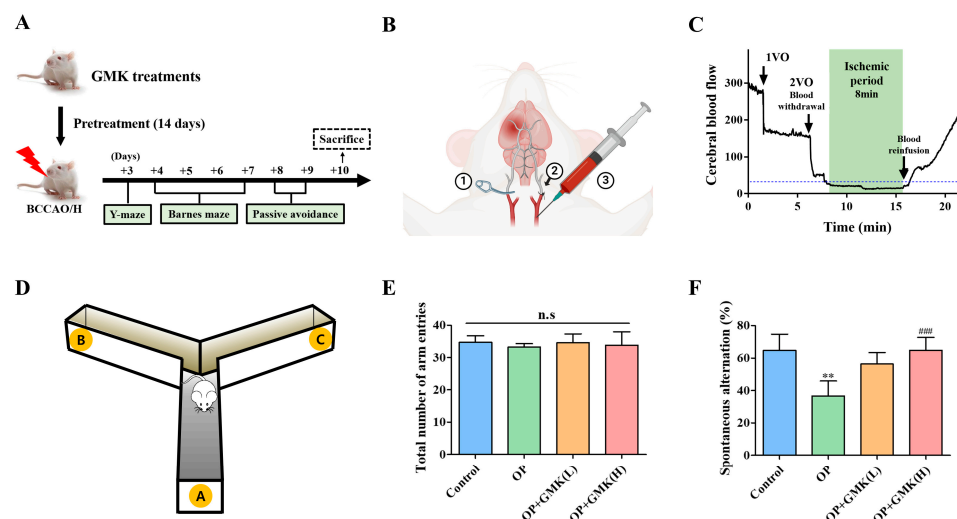


Figure 1. Effects of GMK on GCIRI-induced cognitive deficits. (A) Timeline illustrating the in vivo experimental design. (B) Schematic overview of the surgical procedure for GCIRI induction, comprising the sequential steps of (1) transient occlusion of the right common carotid artery, (2) permanent ligation of the left common carotid artery, (3) controlled arterial blood withdrawal to induce systemic hypotension, followed by reinfusion of the withdrawn blood to allow reperfusion. (C) Representative Doppler flowmetry traces of cerebral blood flow recorded throughout the surgical period, demonstrating a pronounced decrease during the ischemic period (approximately 8 min) and restoration upon reperfusion. (D) Schematic diagram of the Y-maze apparatus consisting of three distinct arms (A, B, and C). (E) Total number of arm entries and (F) spontaneous alternation behavior assessed in the Y-maze test. Data in panels (E,F) are expressed as the mean $\pm$ SEM (** $p < 0.01$ vs. Control; ### $p < 0.001$ vs. OP; n.s., not significant). BCCAO/H, bilateral common carotid artery occlusion with hypovolemia.

2.5. Y-Maze Test (Y-MT)

The Y-MT was carried out to assess spatial learning, memory, and locomotor activity [30]. At postoperative day (POD) 3, each rat was placed in the center of a black plastic maze with three arms (30 cm long, 8 cm wide, 15 cm high) arranged at 120° angles. The sequence and number of arm entries were recorded for 5 min. Two parameters were analyzed: (1) the total number of arm entries (indicator of locomotor activity); (2) the percentage of spontaneous alternation behavior, calculated as the ratio of actual alternations to possible alternations (total arm entries $- 2$) $\times 100$. All tests were carried out under dim lighting, and the maze was cleaned with 70% ethanol after each trial.

2.6. The Barnes Maze Test

The Barnes maze test, used to evaluate learning and spatial memory, was carried out on POD 4–7 [31]. The apparatus consisted of a circular platform, 100 cm in height and 122 cm in diameter, with 20 evenly spaced holes around its perimeter; one hole contained a black

refuge box (20 × 15 × 12 cm). During trial sessions (POD 4–6), each rat was placed on the platform under bright light and allowed 120 s to locate and enter the refuge box (one trial per day). On the probe session (POD 7), the refuge box was removed, and the time spent in the target quadrant (where the box had been located) was recorded for 120 s. Exploratory behaviors—including distance traveled and latency to locate the refuge box during trial sessions, and time spent in the target quadrant during the probe session—were recorded using a video camera connected to an EthoVision XT9 system (Noldus, Wageningen, The Netherlands). The maze was cleaned with 70% ethanol after each trial.

2.7. Passive Avoidance Test

At POD 8 and 9, the passive avoidance test was carried out to assess memory function using a two-chamber apparatus, each chamber measuring approximately 20 × 40 × 20 cm, one chamber illuminated (50 W lamp), the other dark [32]. In the training session, each rat was placed in the illuminated chamber, and the step-through latency (time to enter the dark chamber) was recorded. Upon entering the dark chamber, the rat received a mild foot shock (0.5 mA, 3 s) via stainless steel rods. The test session was carried out 24 h later: each rat was again placed in the illuminated chamber, and the step-through latency to re-enter the dark chamber was quantified for up to 60 s. The apparatus was cleaned with 70% ethanol after each trial.

2.8. Tissue Processing and Cresyl Violet (C-V) Staining

After the completion of behavioral tests, rats were anesthetized and transcardially perfused with 4% paraformaldehyde (PFA). Brains were removed, post-fixed in 4% PFA for 48 h, dehydrated in graded ethanol, embedded in paraffin, and sectioned at 5 µm using a microtome (RM2255; Leica Biosystems, Wetzlar, Germany). Sections containing the hippocampal CA1 region were selected, deparaffinized in xylene, and stained with 0.1% cresyl violet solution (Sigma-Aldrich, St. Louis, MO, USA) [33]. Stained sections were examined under a light microscope (DM4; Leica Biosystems). Images were captured at 50× magnification for overview, 200× for regional analysis, and 1000× for insets. Within the CA1 region, the sublayers *stratum oriens* (S.O), *stratum pyramidale* (S.P), and *stratum radiatum* (S.R) were identified. For quantification, three randomly selected fields from each section were analyzed, and neurons with clearly visible nucleoli were counted as viable. The average number of viable neurons per field was then calculated for each group.

2.9. Immunohistochemistry

Paraffin-embedded brain sections containing the hippocampal CA1 region were deparaffinized in xylene, rehydrated through a graded ethanol series, and rinsed twice with distilled water. Endogenous peroxidase activity was quenched with 1% H₂O₂ for 1 h at ambient temperature. The sections were then incubated with primary antibodies—rabbit anti-Iba1 (1:200; Wako, Richmond, VA, USA) and mouse anti-GFAP (1:200; Invitrogen, Rockford, IL, USA)—in a humidified chamber for 24 h at 4 °C. After washing, the sections were incubated with biotinylated secondary antibodies (anti-rabbit or anti-mouse IgG, 1:250; Vector Laboratories, Burlingame, CA, USA) for 2 h at ambient temperature, followed by treatment with an avidin-biotin complex (VECTASTAIN ABC kit; Vector Laboratories) for 1 h. Positive signals were visualized using 3,3'-diaminobenzidine tetrahydrochloride (DAB; Vector Laboratories) [34]. Three randomly selected sections per group were examined under a light microscope (DM4; Leica) at 200× magnification, with selected insets captured at 1000×. For microglia, the number of Iba1-positive cells were counted in each high-power field (HPF) within the CA1 region and averaged per group. For astrocytes, the GFAP-positive area was quantified as a percentage of the total HPF area and averaged per group using ImageJ software (v1.54f; NIH, Bethesda, MD, USA).

2.10. Cell Culture

The PC12 cells, derived from rat pheochromocytoma, were obtained from the Korean Cell Line Bank (Seoul, Republic of Korea) [35]. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (*v/v*) fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO₂. All cell culture reagents were obtained from HyClone (Logan, UT, USA).

2.11. Cell Viability Assay

PC12 cells were seeded in 96-well plates at a density of 1×10^4 cells/well and cultured overnight to allow attachment. For baseline cytotoxicity assessment, cells were treated with increasing concentrations of GMK (0–1600 µg/mL) for 24 h, and cell viability was assessed using the Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) following the manufacturer's instructions. Cell viability was expressed as a percentage relative to the control.

2.12. OGD/R

PC12 cells were seeded into 96-well plates at a density of 1×10^4 cells per well and cultured overnight to allow attachment. To determine the optimal duration of OGD [27], the cells were rinsed twice with phosphate-buffered saline (PBS), and the culture medium was replaced with glucose-free DMEM (Welgene, Gyeongsan, Republic of Korea). The plates were then immediately transferred to a hypoxia incubator chamber (STEM-CELL Technologies, Vancouver, BC, Canada) and exposed to a gas mixture of 95% N₂ and 5% CO₂ at 37 °C for various durations (0–3 h). After hypoxic exposure, the cells were returned to normoxic conditions, and the medium was replaced with glucose-containing DMEM supplemented with 10% FBS and 1% P/S. The cultures were then incubated for 24 h to allow reoxygenation, completing the OGD/R cycle. Cell viability was subsequently evaluated using the Cell Counting Kit-8 (Dojindo). Based on these preliminary experiments, an OGD duration of 2 h was selected as optimal. In the subsequent experiments designed to assess the neuroprotective effects of GMK, cells were pretreated with GMK (100 µg/mL or 200 µg/mL) for 24 h prior to 2 h of OGD induction, followed by 24 h of reoxygenation. Throughout all experiments, control cells were maintained in glucose-containing DMEM under normoxic conditions.

2.13. TUNEL Assay

Apoptotic cell death was assessed using a TUNEL assay kit (Promega, Madison, WI, USA). PC12 cells were seeded at a density of 1×10^5 cells per well in 12-well plates, each containing a sterilized 15 mm coverslip, and cultured overnight to allow attachment. The cells were then subjected to OGD/R with or without pretreatment with GMK (100 or 200 µg/mL), as described above. Following OGD/R induction, cells were incubated with the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) reaction mixture for 60 min at 37 °C in a humidified chamber, briefly rinsed with PBS, and counter-stained with DAPI (Sigma-Aldrich) for 15 min at ambient temperature to visualize nuclei. Coverslips were mounted using antifade mounting medium (Fluoroshield, Sigma-Aldrich) and observed under a confocal laser scanning microscope (LSM900; Carl Zeiss, Oberkochen, Germany). TUNEL-positive apoptotic cells were quantified by analyzing at least three randomly selected microscopic fields (approximately 40–50 cells per condition, 200×) for each experiment. All experiments and image analyses were carried out in triplicate.

2.14. Enzyme-Linked Immunosorbent Assay (ELISA)

Oxidative stress-related parameters were quantified using enzyme-linked immunosorbent assay (ELISA)-based methods. Malondialdehyde (MDA) levels were quantified using the thiobarbituric acid-reactive substances (TBARS) assay kit (Cayman Chemical, Ann Arbor, MI, USA), while intracellular nitric oxide (NO) production, glutathione peroxidase (GPX) activity, and catalase (CAT) activity were assessed using specific assay kits (Abcam, Cambridge, UK), according to the manufacturers' protocols. All assays were carried out in 96-well ELISA plates, and absorbance was quantified at the respective wavelengths using a microplate spectrophotometer (BioTek Instruments, Winooski, VT, USA). The obtained values were normalized to total protein content and expressed as percentages relative to the control group.

2.15. DCF-DA Assay

ROS generation was quantified using 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) [36]. PC12 cells were seeded in 12-well plates at a density of 1×10^5 cells per well and cultured overnight to allow attachment. The cells were pretreated with GMK (100 or 200 $\mu\text{g/mL}$) for 24 h, followed by OGD for 2 h. Afterward, the cells were reoxygenated for 30 min under normoxic conditions (95% air/5% CO_2 , 37 °C). Following the treatment, cells were incubated with 100 μM DCF-DA in glucose-containing DMEM for 30 min at 37 °C, then rinsed with PBS to remove excess probe. Fluorescence images were captured using a confocal laser scanning microscope (LSM900). At least 20 randomly selected microscopic fields (approximately 10–30 cells per field at 400 $\times$ magnification) were analyzed, and DCF-DA-positive cells were counted among approximately 300 DAPI-stained nuclei per condition. All experiments and image analyses were carried out in triplicate.

2.16. Western Blot Analysis

PC12 cells (1×10^6 cells per well) were pretreated with GMK (100 or 200 $\mu\text{g/mL}$) for 24 h and then subjected to OGD/R as described above. After treatment, cells were rinsed three times with cold PBS and lysed in RIPA buffer supplemented with protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA). The lysates were centrifuged at 14,000 rpm for 10 min at 4 °C, and protein concentrations were assessed using a bicinchoninic acid (BCA) protein assay kit (Sigma-Aldrich). Equal amounts of protein (20 μg) were resolved on 10% SDS–polyacrylamide gels and transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA) for 2 h. The membranes were blocked with 5% skim milk (BD Biosciences, San Jose, CA, USA) for 1 h at ambient temperature, followed by overnight incubation at 4 °C with primary antibodies against Bax, Bcl-2, phosphorylated ERK (p-ERK), total ERK, inducible NO synthase (iNOS), and β -actin (loading control). After washing with TBST buffer (Tris-buffered saline containing 0.05% Tween-20; Sigma-Aldrich), membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at ambient temperature. Protein bands were visualized using an enhanced chemiluminescence (ECL) detection system (Bio-Rad), and images were captured with a chemiluminescence imaging system (ChemiDoc MP, Bio-Rad).

2.17. Statistical Analysis

All data are presented as the mean $\pm$ standard error of the mean (SEM), as specified in the figure legends. Statistical analyses were carried out using GraphPad Prism (v6; GraphPad Software, San Diego, CA, USA). One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was carried out for comparisons among multiple groups. For behavioral trial session data, two-way ANOVA with Bonferroni correction was applied,

whereas probe test data were analyzed using one-way ANOVA followed by Tukey's post hoc test. Statistical significance was defined as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. Non-significant differences were denoted as ns. Statistical significance versus the control group is indicated above the error bars, and significance between groups is shown between the error bars. Normality was assessed using the Shapiro–Wilk test and homogeneity of variance was evaluated using the Brown–Forsythe test; all datasets met the assumptions for the applied parametric tests.

3. Results

3.1. GMK Improves Cognitive Performance After GCIRI

To investigate the effects of GMK on GCIRI-induced cognitive dysfunction, rats were assigned to the Control, OP, OP + GMK(L), and OP + GMK(H) groups following a 14-day pretreatment period with GMK or vehicle and were subjected to BCCAO/H procedure. Behavioral assessments were subsequently performed according to the experimental timeline (Figure 1A). The BCCAO/H procedure was conducted as illustrated schematically (Figure 1B), and cerebral blood flow changes during the entire surgical process were monitored by Doppler flowmetry, demonstrating a marked reduction during occlusion and restoration upon reperfusion (Figure 1C).

Spatial working memory and locomotor activity were first evaluated using the Y-MT (Figure 1D). The total number of arm entries did not differ significantly among the Control, OP, OP + GMK(L), and OP + GMK(H) groups, indicating comparable locomotor activity (Figure 1E). Spontaneous alternation performance was significantly reduced in the OP group compared with the Control group. Although the OP + GMK(L) group did not exhibit a statistically significant increase in alternation behavior relative to the OP group, a significant improvement was observed in the OP + GMK(H) group (Figure 1F).

Spatial learning and reference memory were further assessed using the Barnes maze test (Figure 2A). Representative movement trajectories recorded during the final trial session revealed disorganized and diffuse search patterns in the OP group, whereas the OP + GMK(H) group displayed more directed movement paths comparable to those of the Control group; the OP + GMK(L) group exhibited a pattern intermediate between the OP and OP + GMK(H) groups (Figure 2B). During the final trial session, the OP group exhibited prolonged escape latency and increased total distance traveled compared with the Control group. Although the OP + GMK(L) group did not show statistically significant reductions in escape latency or travel distance, the OP + GMK(H) group demonstrated significant decreases in both parameters (Figure 2C,D). In the probe test, representative trajectories indicated reduced exploration of the target quadrant in the OP group, whereas increased target quadrant occupancy was observed in the OP + GMK(H) group (Figure 2E). Quantitative analysis confirmed that the time spent in the target quadrant was significantly decreased in the OP group and significantly increased only in the OP + GMK(H) group, while the OP + GMK(L) group showed a modest, nonsignificant increase (Figure 2F).

Long-term memory retention was assessed using the passive avoidance test (Figure 3A). No significant differences were observed among the Control, OP, OP + GMK(L), and OP + GMK(H) groups during the training session (Figure 3B). However, during the test session conducted 24 h later, step-through latency was significantly reduced in the OP group. Both the OP + GMK(L) and OP + GMK(H) groups exhibited prolonged step-through latencies compared with the OP group; notably, a statistically significant increase was observed in the OP + GMK(H) group compared with the OP + GMK(L) group, indicating a dose-dependent improvement in memory retention (Figure 3C).

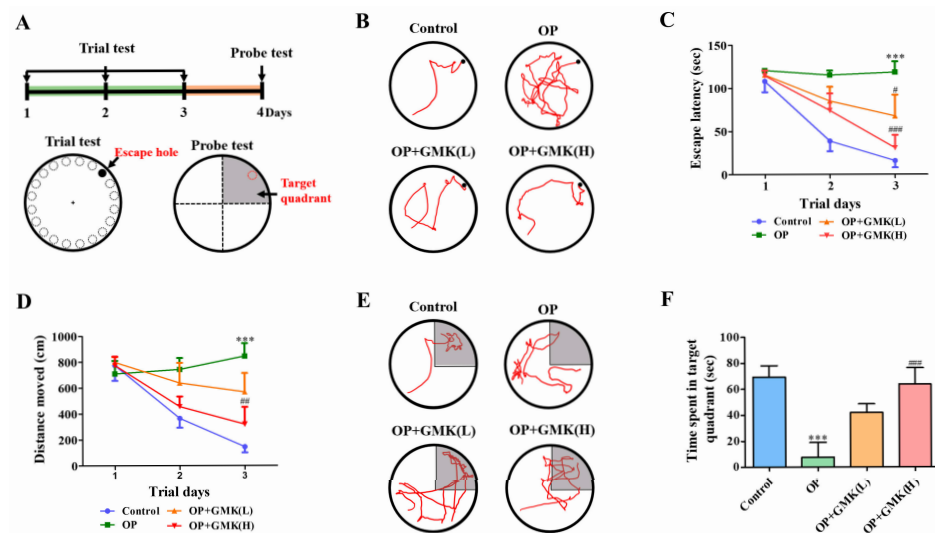


Figure 2. Effects of GMK on GCIRI-induced impairments in spatial learning and memory as evaluated by the Barnes maze test. (A) Timeline and schematic representation of the Barnes maze paradigm, consisting of three consecutive days of trial sessions followed by a probe test. (B) Representative movement paths obtained during the final trial session for each experimental group. (C) Escape latency across the trial sessions. (D) Total distance traveled during the trial sessions. (E) Representative movement trajectories recorded during the probe test, with the target quadrant highlighted. (F) Time spent in the target quadrant during the probe test. Data in panels (C,D,F) are presented as the mean $\pm$ SEM (** $p < 0.001$ vs. Control; # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.001$ vs. OP).

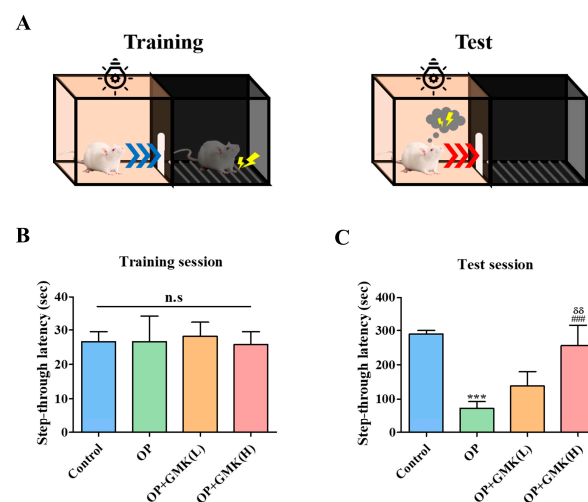


Figure 3. Effects of GMK on GCIRI-induced memory impairment assessed by the passive avoidance test. (A) Schematic illustration of the passive avoidance test paradigm. (B) Step-through latency measured during the training session. (C) Step-through latency measured during the test session performed 24 h after training. Data in panels (B,C) are presented as the mean $\pm$ SEM (** $p < 0.001$ vs. Control; ### $p < 0.001$ vs. OP; $\delta\delta$ $p < 0.01$ vs. OP + GMK(L); n.s., not significant).

3.2. GMK Preserves Hippocampal CA1 Neurons and Attenuates Glial Activation After GCIRI

To determine whether GMK mitigates histopathological alterations induced by GCIRI, neuronal survival and glial responses in the hippocampal CA1 region were examined using C-V staining and immunohistochemistry. Representative C-V-stained sections revealed intact and densely packed pyramidal neurons of the Control group, whereas the OP group exhibited marked neuronal loss and morphological disruption in the CA1 region (Figure 4A). In contrast, GMK-treated groups showed preservation of pyramidal neurons, with a more pronounced effect observed in the OP + GMK(H) group. Quantitative analysis

confirmed a significant reduction in the number of surviving CA1 pyramidal neurons in the OP group compared with the Control group (Figure 4B). However, GMK administration significantly increased neuronal survival in both treatment groups.

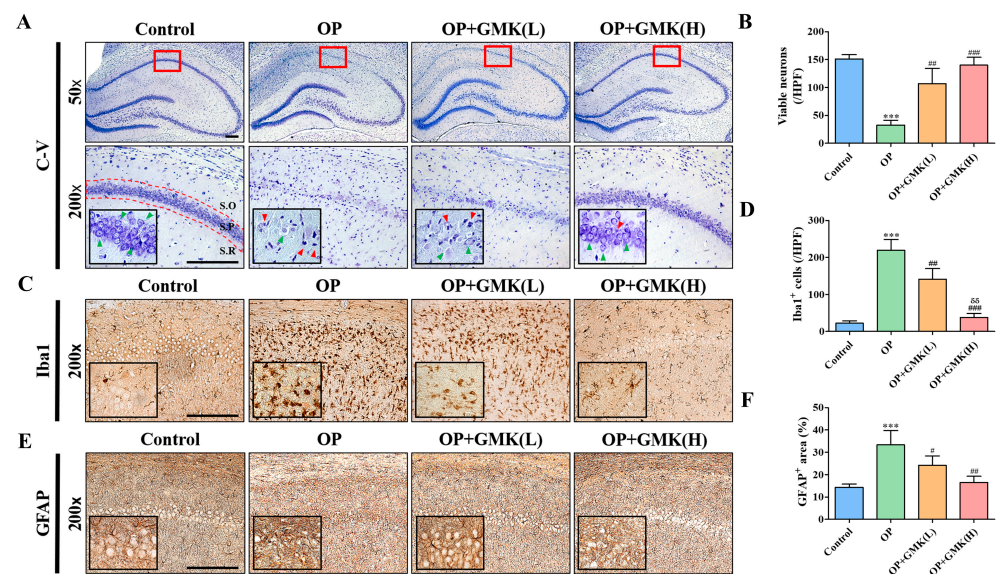


Figure 4. GMK alleviates neuronal loss and glial activation in the hippocampal CA1 region following GCIRI. Representative images of (A) cresyl violet-stained pyramidal neurons, (C) Iba1-immunoreactive microglia, and (E) GFAP-immunoreactive astrocytes in the hippocampal CA1 region from each experimental group. Quantitative analyses of (B) surviving CA1 pyramidal neurons, (D) Iba1-positive microglial cells, and (F) GFAP-positive area. In panel (A), regions of interest are indicated by red rectangular boxes; Green arrowheads indicate viable neurons with intact nucleoli, whereas red arrowheads indicate degenerating neurons with pyknotic nuclei. Panels (A,C,E) include higher-magnification images shown in the corresponding insets. Scale bar = 200 μ m. Data in panels (B,D,F) are expressed as the mean $\pm$ SEM (** p < 0.001 vs. Control; # p < 0.05, ## p < 0.01, and ### p < 0.001 vs. OP; $\delta\delta$ p < 0.01 vs. OP + GMK(L)). C-V, cresyl violet; Iba1, ionized calcium-binding adaptor molecule 1; GFAP, glial fibrillary acidic protein; S.O, stratum oriens; S.P, stratum pyramidale; S.R, stratum radiatum.

Microglial activation in the CA1 region was evaluated by Iba1 immunostaining. Representative images demonstrated sparse, ramified Iba1-positive microglia in the Control group, whereas the OP group displayed an increased number of Iba1-immunoreactive cells with hypertrophic morphology (Figure 4C). GMK treatment reduced microglial density and activation, with a more evident attenuation observed in the OP + GMK(H) group. Quantitative assessment showed a significant increase in Iba1-positive cells in the OP group relative to the Control group, which was significantly reduced by GMK treatment; the reduction was significantly greater in the OP + GMK(H) group compared with the OP + GMK(L) group (Figure 4D).

Astrocytic activation in the CA1 region was evaluated by GFAP immunoreactivity. The Control group exhibited minimal GFAP staining, whereas the OP group showed pronounced astrocytic hypertrophy and an expanded GFAP-positive area (Figure 4E). In contrast, both GMK-treated groups displayed markedly reduced astrocytic activation. Quantitative analysis confirmed a significant increase in GFAP-positive area in the OP group compared with the Control group, which was significantly reduced by GMK treatment in a dose-dependent manner (Figure 4F).

3.3. GMK Protects PC12 Cells Against OGD/R-Induced Cytotoxicity

To define appropriate *in vitro* experimental conditions, the baseline cytotoxicity of GMK and the optimal duration of OGD/R were first examined in PC12 cells (Figure 5). GMK treatment for 24 h did not induce cytotoxicity at concentrations up to 200 $\mu\text{g/mL}$ (Figure 5A). Accordingly, 100 and 200 $\mu\text{g/mL}$ were selected as the low and high concentrations of GMK, respectively, for subsequent experiments. PC12 cells exposed to increasing durations of OGD exhibited a time-dependent reduction in cell viability (Figure 5B), and the LT50 (time to 50% loss of viability), indicated by the red dotted line, was reached at approximately 2 h of OGD exposure. Based on these findings, an OGD duration of 2 h followed by reoxygenation, together with GMK concentrations of 100 and 200 $\mu\text{g/mL}$, was applied in all subsequent *in vitro* experiments. Under OGD/R conditions, PC12 cells displayed pronounced morphological alterations and reduced cell density compared with control cells (Figure 5C). In contrast, GMK pretreatment preserved cellular morphology in a concentration-dependent manner. Quantitative analysis further confirmed that the significant reduction in cell viability induced by OGD/R was markedly attenuated by GMK pretreatment, with greater preservation observed at the higher concentration (Figure 5D).

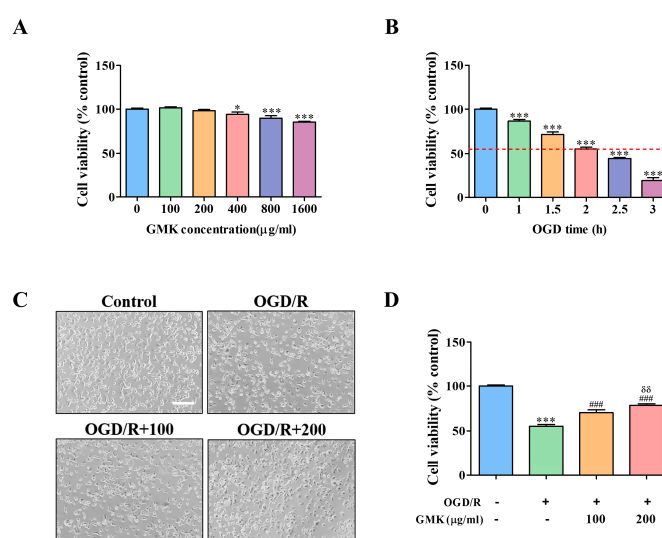


Figure 5. Evaluation of the protective effects of GMK against OGD/R-induced cytotoxicity in PC12 cells. (A) Effects of various concentrations of GMK on PC12 cell viability under basal conditions. Because GMK did not exert cytotoxic effects at concentrations up to 200 $\mu\text{g/mL}$, 100 and 200 $\mu\text{g/mL}$ were chosen for subsequent *in vitro* experiments. (B) Time-dependent reduction in PC12 cell viability following oxygen–glucose deprivation (OGD). The red dotted line denotes the LT50 (time to 50% loss of viability) during OGD exposure. On the basis of this analysis, an OGD duration of 2 h was selected for subsequent oxygen–glucose deprivation/reoxygenation (OGD/R) experiments. (C) Representative phase-contrast images of PC12 cells under control conditions, OGD/R alone, and OGD/R in the presence of GMK pretreatment (100 or 200 $\mu\text{g/mL}$). Scale bar = 200 μm . (D) Quantitative assessment of cell viability following OGD/R with or without GMK pretreatment. Data are presented as the mean $\pm$ SEM (* $p < 0.05$ and *** $p < 0.001$ vs. control; ### $p < 0.001$ vs. OGD/R; $\delta\delta$ $p < 0.01$ vs. OGD/R with 100 $\mu\text{g/mL}$ GMK).

3.4. GMK Attenuates OGD/R-Induced Apoptosis and Oxidative Stress in PC12 Cells

To evaluate the effects of GMK on OGD/R-induced apoptotic cell death, TUNEL staining and immunoblotting analyses were performed in PC12 cells. Representative fluorescence images demonstrated a marked increase in TUNEL-positive cells following OGD/R exposure compared with control cells, whereas GMK pretreatment reduced the number of apoptotic cells (Figure 6A). Quantitative analysis confirmed a significant increase in TUNEL-positive cells in the OGD/R group, which was significantly decreased

by GMK pretreatment in a concentration-dependent manner (Figure 6B). The expression of apoptosis-related proteins was further examined by Western blot analysis. OGD/R exposure resulted in increased Bax expression and decreased Bcl-2 expression compared with control cells (Figure 6C). GMK pretreatment altered the expression levels of these proteins under OGD/R conditions. In parallel, lipid peroxidation was assessed by measuring MDA levels. MDA levels were significantly elevated following OGD/R exposure and were significantly reduced by GMK pretreatment, with a greater reduction observed at the higher concentration (Figure 6D).

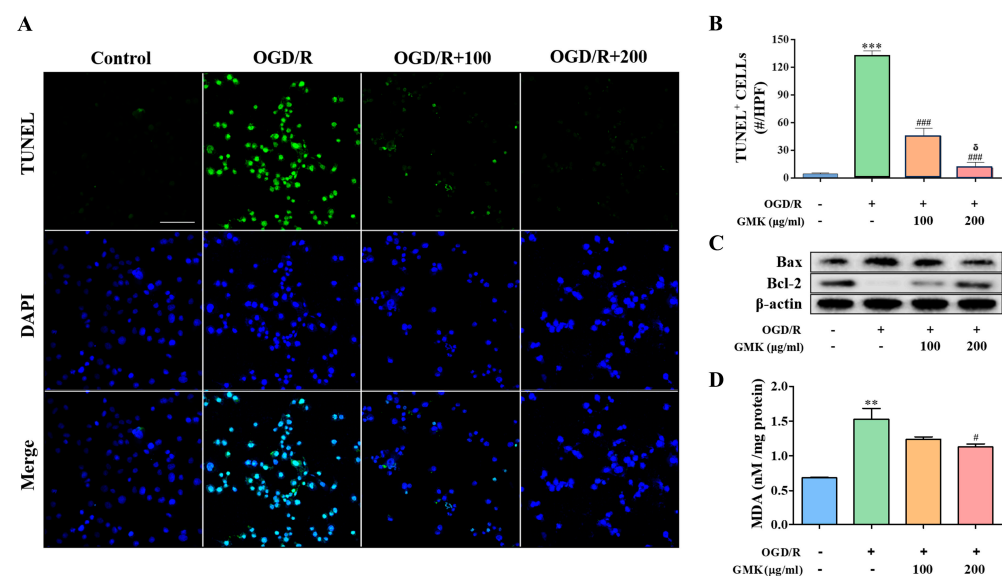


Figure 6. GMK attenuates OGD/R-induced apoptosis and oxidative damage in PC12 cells. (A) Representative fluorescence images showing TUNEL-positive apoptotic cells (green) and DAPI-stained nuclei (blue) under control conditions, OGD/R alone, and OGD/R with GMK pretreatment (100 or 200 µg/mL). Scale bar = 200 µm. (B) Quantitative analysis of TUNEL-positive cells expressed as the number of apoptotic cells per high-power field (HPF). (C) Representative immunoblots of Bax and Bcl-2, with β-actin used as a loading control. (D) Quantification of malondialdehyde (MDA) levels following OGD/R with or without GMK pretreatment. Data are presented as the mean ± SEM (** $p < 0.01$ and *** $p < 0.001$ vs. control; # $p < 0.05$ and ### $p < 0.001$ vs. OGD/R; δ $p < 0.05$ vs. OGD/R with 100 µg/mL GMK).

Intracellular oxidative stress was further assessed using DCF-DA staining. Representative fluorescence images showed an increased number of ROS-positive cells following OGD/R exposure, which was reduced by GMK pretreatment (Figure 7A). Quantitative analysis demonstrated a significant increase in DCF-DA-positive cells in OGD/R-exposed cells, which was decreased in GMK-treated cells in a dose-dependent manner (Figure 7B). Consistent with increased oxidative stress, NO levels were significantly elevated following OGD/R exposure and were reduced by GMK pretreatment (Figure 7C). In addition, OGD/R exposure significantly decreased the amounts of major intracellular antioxidant enzymes, i.e., GPX and CAT, compared with control cells, whereas GMK pretreatment significantly restored the levels of both enzymes (Figure 7D,E). As key upstream regulators of NO production, the expression of p-ERK and iNOS was further examined by Western blot analysis. OGD/R exposure downregulated the expression of p-ERK and iNOS, with corresponding reversals observed in GMK-pretreated cells (Figure 7F).

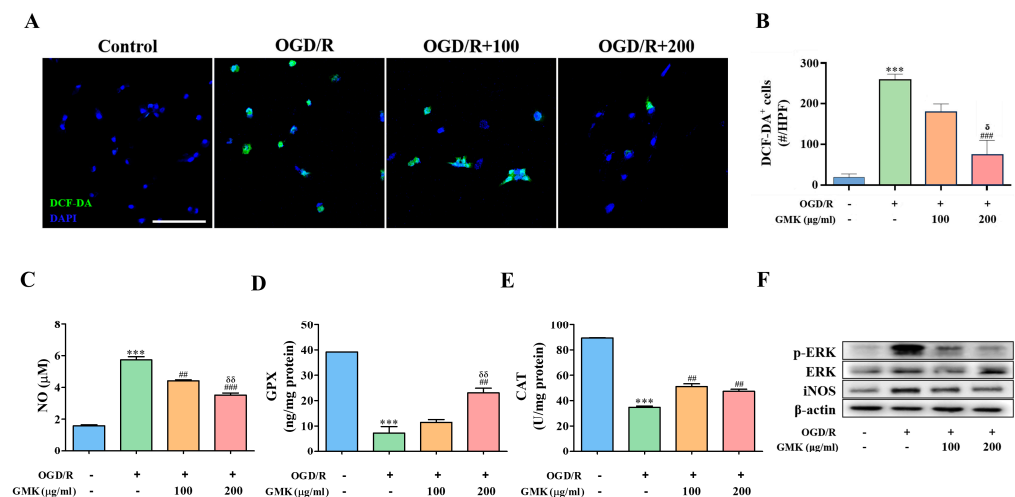


Figure 7. Effects of GMK on OGD/R-induced oxidative stress-related parameters in PC12 cells. (A) Representative fluorescence images showing intracellular ROS detected by DCF-DA staining (green) with DAPI counterstaining (blue) under control conditions, OGD/R alone, and OGD/R with GMK pretreatment (100 or 200 μg/mL). Scale bar = 200 μm. (B) Quantitative analysis of DCF-DA-positive cells expressed as the number of ROS-positive cells per high-power field (HPF). (C) Nitric oxide (NO) levels measured by ELISA under the indicated experimental conditions. (D) Glutathione peroxidase (GPX) activity and (E) Catalase (CAT) activity determined under the indicated experimental conditions. (F) Representative immunoblot images showing the expression of phosphorylated ERK (p-ERK), total ERK, and inducible nitric oxide synthase (iNOS), with β-actin used as a loading control. Data are presented as the mean ± SEM (***) $p < 0.001$ vs. control; ### $p < 0.01$, and δδδ $p < 0.001$ vs. OGD/R; δ $p < 0.05$ and δδ $p < 0.01$ vs. OGD/R with 100 μg/mL GMK).

4. Discussion

In this study, GMK pretreatment was linked to better performance in selected cognitive tasks after GCIRI, preservation of hippocampal CA1 pyramidal neurons, and attenuation of microglial and astrocytic activation. Behavioral improvements were more consistently observed at the higher dose, whereas the lower dose showed nonsignificant trends for certain outcomes. In the complementary PC12 OGD/R model, GMK reduced oxidative and apoptotic injury markers and restored antioxidant defenses, supporting a redox- and apoptosis-associated component of its protective profile.

The BCCAO/H paradigm is commonly used to model global forebrain ischemia with delayed, selective CA1 degeneration and cognitive impairment, and it recapitulates key features of post-resuscitation brain injury [37]. In our experiments, BCCAO/H produced marked CA1 neuronal loss and impaired task performance, and these outcomes were mitigated by GMK administration. Locomotor activity in the Y-maze was comparable across groups, making it less likely that gross motor differences account for the cognitive results; however, because dedicated anxiety/motivation assays were not included, subtle behavioral confounds cannot be fully excluded.

From a mechanistic perspective, ischemia–reperfusion injury is strongly influenced by ROS overproduction, followed by lipid peroxidation and engagement of apoptotic signaling [38,39]. In line with this framework, OGD/R increased ROS, NO, and MDA levels and shifted the Bax/Bcl-2 balance toward apoptosis in PC12 cells. GMK blunted these changes and partially restored GPX and CAT activities, consistent with reports that mushroom-derived bioactives can support redox homeostasis and cell survival under stress [19–22,40,41]. These in vitro findings provide plausible mechanistic support but should be viewed as complementary, not definitive, evidence for in vivo mechanisms.

We also observed that GMK pretreatment modulated p-ERK and iNOS under OGD/R conditions. ERK signaling can have context-dependent roles during ischemic stress, and changes

in ERK activity may shape downstream inflammatory and nitrosative pathways [42–44]. The associated alterations in NO production and iNOS expression suggest that GMK may influence upstream regulators of nitrosative stress, although additional pathway-level analyses will be required to establish directionality and causality.

Several limitations should be acknowledged. (i) In vivo mechanistic validation was largely restricted to histological endpoints; we did not quantify cytokines/glial phenotypes or directly assess hippocampal antioxidant enzymes, and therefore mechanistic interpretation relies mainly on the in vitro analyses. (ii) The in vitro experiments used a single neuronal-like cell line (PC12) and thus do not capture neuron–glia interactions. (iii) The in vivo sample size ($n = 5$ per group) and the lack of an a priori power calculation reduce sensitivity to detect small effects, particularly at the lower dose. (iv) Dedicated behavioral control assays (e.g., open-field) were not included, and we did not evaluate pharmacokinetics/brain penetration, toxicity, or long-term outcomes. (v) Although GMK is a standardized extract and tentative UHPLC–QTOF–MS/MS-based annotations are provided in the Supplementary Materials, the specific bioactive constituent(s) driving the observed neuroprotection have not been definitively identified or quantified; isolation, targeted quantification, and activity-guided fractionation will be required. (vi) Behavioral and histological outcomes were assessed only within the acute/subacute period after GCIRI, so sustained neuroprotection and long-term recovery (e.g., 28 days and beyond) were not evaluated. Future studies with larger cohorts (guided by power calculations), broader cellular models, and in vivo molecular validation will be important for defining translational relevance.

5. Conclusions

The present study shows that GMK pretreatment is associated with improved cognitive performance and preservation of hippocampal CA1 neurons after GCIRI, and that GMK reduces oxidative stress- and apoptosis-related injury markers in a complementary PC12 OGD/R model. These preclinical findings support further studies to validate mechanisms in vivo and to evaluate exposure, safety, and long-term efficacy.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/cimb48020151/s1>. Figure S1. Tentative identification of compounds in GMK extracts by UHPLC–QTOF–MS/MS analysis.

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Institutional Review Board Statement: The animal study protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Konyang University (Daejeon, Republic of Korea; approval code: P-24-30-A-01; approval date: 8 November 2024) and was conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (8th edition, 2011).

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in this study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

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Conflicts of Interest: Jong Yea Park and Hyun Min Kim are employed by the company Giunchan Co., Ltd. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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