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*Original Article*

# Protective roles of compound porcine cerebroside and ganglioside injections against damage induced by intracerebral hemorrhaging

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**Abstract:** The present study aimed to investigate the effects of compound porcine cerebroside and ganglioside injections (CPCGI) on injuries induced by intracerebral hemorrhaging (ICH). To perform this study, an experimental ICH mouse model was created by autologous arterial blood injections in C57BL/6 mice. Cultured primary mouse cortical neurons/microglial cells were exposed to oxyhemoglobin/erythrocyte lysis to mimic ICH *in vitro*. Next, the mice and neurons were treated with CPCGI. After specifying treatment with CPCGI, neurological scores and brain water content levels of the mice were measured. Levels of inflammation cytokines (TNF-α, IL-6, and IL-1β) were measured by ELISA. Additionally, viability and apoptosis levels of the neurons were determined after treatment with CPCGI. Furthermore, apoptosis-related genes (GADD45α, Bcl-2, and Bax) were determined using Western blot assay and qRT-PCR, respectively. *In vivo* experiments showed that increased neurological scores and brain water content levels induced by ICH were reduced by CPCGI administration. CPCGI treatment significantly decreased levels of TNF-α, IL-6, and IL-1β induced by ICH. Moreover, apoptosis levels of the neurons significantly increased after ICH. This increase was eliminated by CPCGI treatment. In addition, CPCGI treatment decreased levels of GADD45α and Bax, while increasing Bcl-2 levels in neurons. In conclusion, current data demonstrates the protective effects of CPCGI on brain injuries induced by ICH.

**Keywords:** Compound porcine cerebroside and ganglioside injections, intracerebral hemorrhage, brain injury, apoptosis

## Introduction

Intracerebral hemorrhaging (ICH) is a common clinical nervous system disease, accounting for about 30% of acute cerebrovascular disease in China. It is more common in the elderly. One of the major causes of death and disability in China due to high morbidity, disability, and mortality rates, ICH seriously endangers the lives of people [1, 2]. The main risk factors for ICH include cerebral atherosclerosis, cerebrovascular malformations and hypertension [3, 4]. Basic pathological changes of ICH include primary and secondary injuries [5, 6]. Primary injuries mainly include mechanical damage caused by tissue destruction, hematoma enlargement, and increased intracranial pressure after hemorrhaging [7]. Mechanisms of secondary injuries include apoptosis, inflammatory reactions, necrosis, erythrocyte lysis, and brain edema formation [8]. Of these, apoptosis is an important factor in secondary brain injuries. Although significant progress has been achieved in clinical treatment of ICH, outcomes of current treatment strategies for ICH remain unsatisfactory [9, 10].

Compound porcine cerebroside and ganglioside injections (CPCGI, drug approval H220-26472; Buchang Pharmaceutical Group Ltd., Jilin, China) are compounds consisting of polypeptides, gangliosides, and hypoxanthine. One milliliter of CPCGI contains 3.2 mg of polypeptides, 0.24 mg of monosialotetrahexosyl ganglioside (GM-1), and 0.125 mg of hypoxanthine. GM-1 is a neurotrophic factor found in the central nervous system that promotes differentiation, survival, axonal stability, neurodegeneration, and brain regeneration [11-15]. Hypoxanthine, identified as a bio-marker for cardiac ischemia [16], can improve the body's material and energy metabolism, accelerate repair of

damaged tissues, and promote the recovery of normal physiological function of pathological cells and hypoxic tissues [17, 18]. In addition, carnosine, a dipeptide consisting of histidine and β-alanine, protects nerve damage by regulating oxidative stress, apoptosis, and autophagy, as well as other cellular processes [19-23]. At present, in China, CPCGI is widely used for treatment of strokes, nerve injuries, Alzheimer's disease, and functional disorders induced by brain-related diseases [24-26]. However, the precise roles of CPCGI in ICH remain unclear.

Therefore, the present study aimed to investigate whether CPCGI provides protective effects on brain injuries caused by ICH, exploring the underlying mechanisms.

## Materials and methods

### *Animals*

Male C57BL/6 mice (20-24 g, 8-10 weeks old) were housed in individual cages. They ate and drank freely. The present study was approved by the Animal Ethics Committee of the Second Hospital of Hebei Medical University. All experimental procedures were performed according to Recommended Guidelines for the Care and Use of Laboratory Animals issued by the Chinese Council on Animal Research.

### *ICH model establishment*

An ICH mouse model was established, in accord with a previous study [27]. Briefly, the mice were anesthetized by intraperitoneal injections with 10% chloral hydrate (150 mg/kg), then fixed on a mouse stereotaxic frame (Stoelting). Autologous non anticoagulated blood (20 μl) of the mice was harvested from the tail veins. The blood was then injected into the caudate nucleus at a speed of 2 μl/minute, according to the following stereotactic guidance with respect to the coordinates of the bregma in the 10-minute period: Front 0.8 mm, left 2 mm, and depth 3.5 mm. After injections, the needle was fixed for 10 minutes. When the blood coagulated, the micro-syringe was pulled out. Bone wax was then used to seal the craniotomy and sutures were used to close the scalp. Throughout the experiment, the body temperature of the mice was maintained at 37°C. They were free to obtain water and food after waking. A total of 18 mice participated in this investigation. Mice that died (two) due to anesthesia were excluded. Finally, a total of 15 mice were randomly divided into three groups (n=5), including sham, ICH, ICH + vehicle (ICH-V), and ICH + CPCGI (ICH-C) (1 mL/kg/d) groups. The mice received CPCGI treatment (1 mL/kg/d; intraperitoneal injections) for 3 consecutive days, beginning one hour after ICH induction. The ICH + vehicle group received an equal amount of saline. CPCGI was provided by Jilin Buchang Pharmaceutical Group Co., Ltd (Jilin, China).

### *Neurological score evaluations*

Neurological scores were evaluated, as previously described [27]. Sensory, reflex, exercise, and balance tests were performed to determine neurological scores. Neurological scores ranged from 1 to 18. A score of 1 was obtained due to the inability to test or the lack of a test reflex. Higher scores indicate more severe injuries (2-3 is normal and 18 is the highest).

### *Brain water content detection*

After ICH, brain water content levels were measured in the brain tissues. The mice were randomly selected from each group and anesthetized with chloral hydrate intraperitoneally. Cerebral tissues were then removed. Filter paper was used to absorb the water on the surface of the brain tissues. Subsequently, the samples were immediately weighed using an electronic analytical balance, obtaining wet weights. To obtain dry weights, the samples were dried for 24 hours at 100°C. The following formula was used to calculate brain water content levels: Brain water content (%) = (wet weight - dry weight)/wet weight × 100% [28].

### *Primary cell culturing and treatment*

Primary mouse cortical neurons were obtained and cultured, as previously described [29]. Cortical neurons were extracted from C57BL/6 mouse embryos (E16), then treated with Trypsin-EDTA solution for 5 minutes at 37°C. Extracted neurons were seeded into plates (Corning, USA) coated with 0.1 mg/ml poly-D-lysine (Sigma, USA) and grown in Neurobasal-A medium containing 0.5 mM GlutaMAX™-I (Gibco) and 2% B-27. Cells were incubated at 37°C with 5% $CO_2$ for about 14 days. Half of the culture media was exchanged every 2 days. To conduct an *in vitro* ICH model, enriched neurons were exposed to OxyHb for 48 hours [20]. The neurons were divided into following groups:

Control group (control), OxyHb exposure group (OxyHb), OxyHb exposure + vehicle group (OxyHb-V), and the OxyHb exposure + CPCGI group (OxyHb-C).

Primary microglial cells were obtained and cultured, as previously described. An *in vitro* ICH model of microglial cells was established by exposure to erythrocyte lysis for 48 hours, in accord with a previous study [27]. Microglial cells were divided into four groups, including the control group (control), erythrocyte lysis exposure group (EL), erythrocyte lysis exposure + PBS (EL-P), and erythrocyte lysis exposure + CPCGI group (EL-C).

*Western blot analysis*

Forty-eight hours after treatment, protein levels of Bcl-2, Bax, and GADD45α were detected by Western blotting. Total cell proteins were extracted from neurons using lysis buffer (Cell Signaling Technology). BCA assays (Thermo Fisher Scientific, Inc.) were carried out to measure protein concentrations. Equal amounts of protein samples (30 μg per lane) were separated by 10% SDS-PAGE, then transferred onto polyvinylidene difluoride membranes. After blocking with 5% skim milk at room temperature for 2 hours, the membranes were incubated with the primary antibody against Bcl-2, Bax, or GADD45α (all 1:1,000; all Cell Signaling Technology) overnight at 4°C. Next, the membranes were incubated with a secondary antibody at room temperature for 2 hours. β-actin (1:5,000; Cell Signaling Technology) served as the internal control. Finally, to visualize protein bands, an enhanced chemiluminescence detection system (Super Signal West Dura Extended Duration Substrate; Pierce Chemical) was utilized.

*Quantitative reverse transcription polymerase chain reaction (qRT-PCR)*

Total RNA from the cells was extracted by using TRIzol Reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to manufacturer protocol. RNA concentrations and quantification were measured using a nanodrop spectrophotometer (Thermo Fisher Scientific, Inc.) via calculating 260 nm/280 nm. A PrimeScript reverse transcription reagent kit (Takara Biotechnology Co., Ltd., Dalian, China) was used to generate cDNAs, according to manufacturer instructions. Subsequently, these cDNAs were analyzed using a TaqMan Universal PCR Master Mix kit (Thermo Fisher Scientific, Inc.). Amplification conditions were as follows: 95°C for 10 minutes, followed by 40 cycles of 95°C for 10 seconds and 60°C for 60 seconds. All primer sequences used for qPCR were obtained from GeneScript (Nanjing, USA), as required. GAPDH was used as an internal control. The $2^{-\Delta\Delta Cq}$ method was used for relative gene expression quantification [30].

*ELISA assay*

Detecting levels of TNF-α, IL-1β, and IL-6 in cell culture supernatants, ELISA assays were used, according to manufacturer instructions.

*MTT assay*

An MTT assay was performed to determine cell viability. After the indicated treatments, $1 \times 10^5$ neurons were seeded in a 96-well plate and incubated with MTT (20 μl; 5 mg/mL; Sigma-Aldrich) for 4 hours. The culture medium was then removed and 150 μl dimethyl sulfoxide was added. Optical density (OD) levels of each sample at 490 nm were measured using a micro-plate reader (Bio-Rad).

*Cell apoptosis analysis*

To analyze cell apoptosis, an annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Oncogene Research Products, Boston, MA) was used, according to manufacturer instructions. Briefly, the neurons were stained with Annexin V-fluorescein isothiocyanate (0.5 mg/ml) and propidium iodide (0.6 mg/ml) after being washed twice with ice-cold PBS, according to manufacturer instructions. After incubation for 15 minutes at room temperature without light, a flow cytometer (Beckman Coulter, USA) was used to analyze cell apoptosis.

*Statistical analysis*

Data are expressed as mean ± SD. Variations between groups were statistically examined using Student's t-test or ANOVA, followed by Student-Neuman-Keuls (SNK) testing. *P*-values < 0.05 indicate statistical significance.

## Results

*CPCGI treatment relieved brain damage in ICH*

As shown in **Table 1**, previous studies have indicated that CPCGI and its main components pro-

**Table 1.** Main components of CPCGI and corresponding roles

| | | Roles | References |
|---|---|---|---|
| CPCGI | | Protective effect in stroke, nerve injuries, Alzheimer's disease, and functional disorders induced by brain related diseases | [24-26] |
| Main components of CPCGI (1 ml) | Polypeptides (3.2 mg) | Neuroprotective effect; Regulation of oxidative stress, apoptosis, and autophagy | [19-23] |
| | GM-1 (0.24 mg) | Regulation of differentiation, survival, axonal stability, neurodegeneration and brain regeneration, etc | [11-15] |
| | Hypoxanthine (0.125 mg) | Biomarker for cardiac ischemia, involved in body's material and energy metabolism, etc | [16-18] |

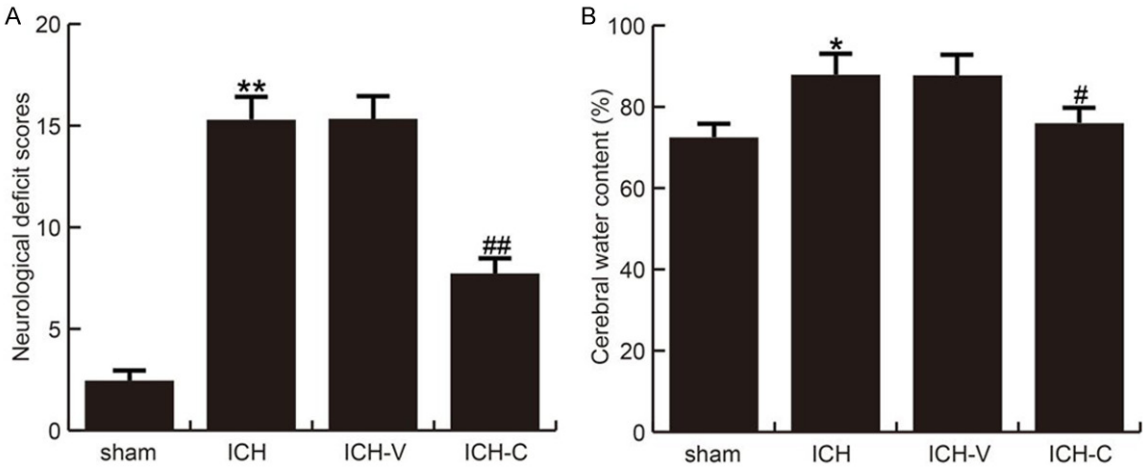


**Figure 1.** CPCGI treatment reduces brain damage in ICH. A: Neurological deficit tests were determined by neurological severity scores; B: Cerebral water content of the mice was analyzed. These experiments were performed three times. Data are displayed as the mean ± SD. *$p < 0.05$.

vide substantial effects on brain-related diseases [11-26]. The current study investigated the effects of CPCGI on injuries induced by ICH. After subjection to ICH for 48 hours, determining brain damage levels in the mice, water content levels in the brains were measured. Additionally, neurological deficit tests were performed. As shown in **Figure 1**, compared with the control group, ICH significantly increased neurological injuries and water content levels in mice brains. These increases were inhibited by CPCGI treatment.

*CPCGI treatment reduced ICH induced microglia inflammation*

Previous studies have reported that ICH can cause neuronal damage by promoting the activation of microglia and release of pro-inflammatory mediators [31]. Present results showed that levels of TNF-α, IL-6, and IL-1β were significantly enhanced after ICH. These enhancements were suppressed by CPCGI administration (**Figure 2**).

*CPCGI treatment reduced ICH induced neuronal injury*

To further explore the effects of CPCGI on ICH induced neuronal injuries, cell viability and apoptosis levels of the neurons were determined. Results showed that ICH significantly repressed cell viability and induced apoptosis levels of the neurons. However, CPCGI treatment notably inhibited ICH-induced cell viability repression (**Figure 3A**) and cell apoptosis (**Figure 3B** and **3C**).

*CPCGI treatment affected levels of GADD45c-taBax and Bcl-2*

To confirm the roles of CPCGI-mediated apoptosis in neuroprotection, expression levels of apoptosis-related genes (GADD45α, Bcl-2, and Bax) were determined, using qRT-PCR and Western blotting, respectively. As shown in **Figure 4**, after exposure to OxyHb for 48 hours, mRNA and protein levels of Bcl-2 significantly decreased. However, levels of GADD45α and

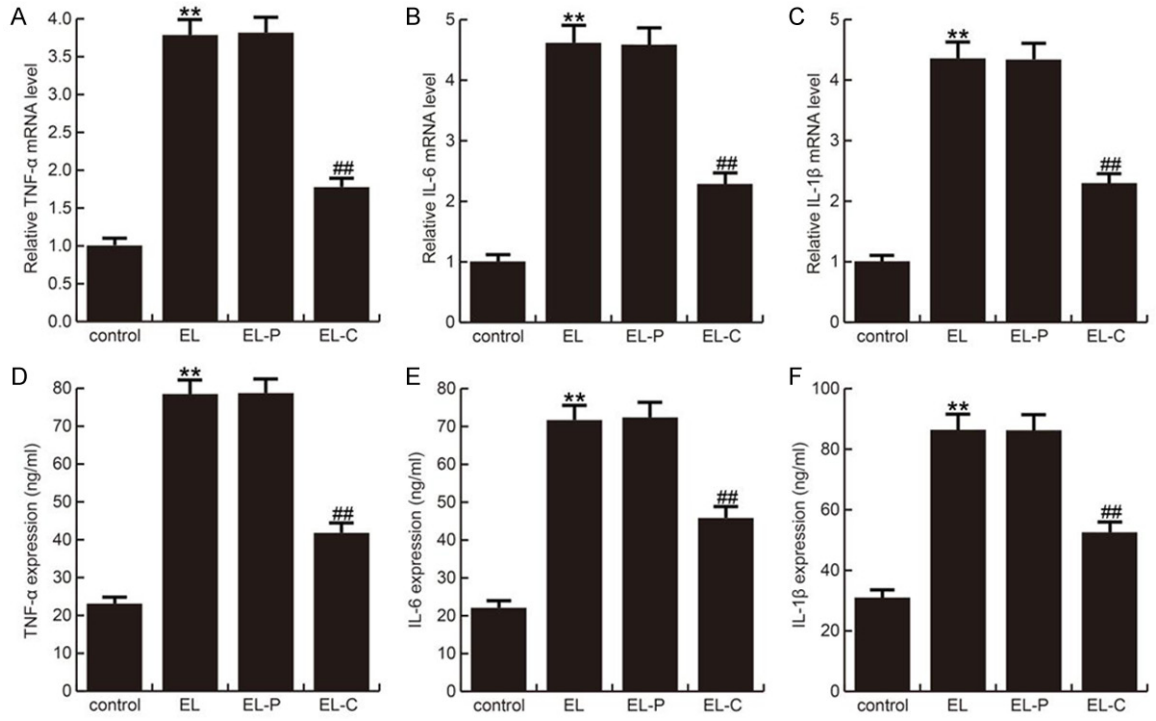


**Figure 2.** CPCGI treatment reduces erythrocyte lysis-induced microglia inflammation. (A-F) After treatment for 48 hours, mRNA (A-C) and protein (D-F) levels of TNF-α, IL-6, and IL-1β were determined. The experiments were performed three times. Data are displayed as the mean ± SD. *p < 0.05.

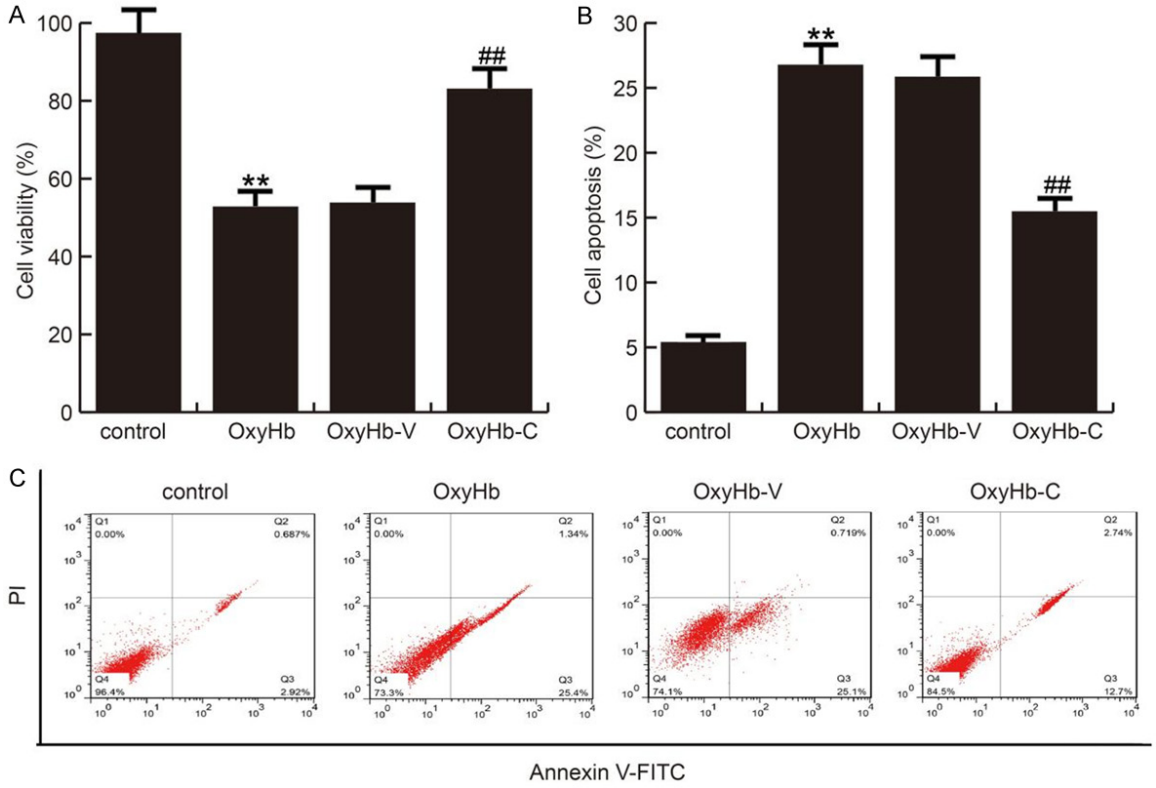


**Figure 3.** CPCGI treatment reduces OxyHb induced neuronal injuries. After treatment for 48 hours, cell viability (A) and apoptosis (B, C) levels of the neurons were determined. The experiments were performed three times. Data are displayed as the mean ± SD. *p < 0.05.

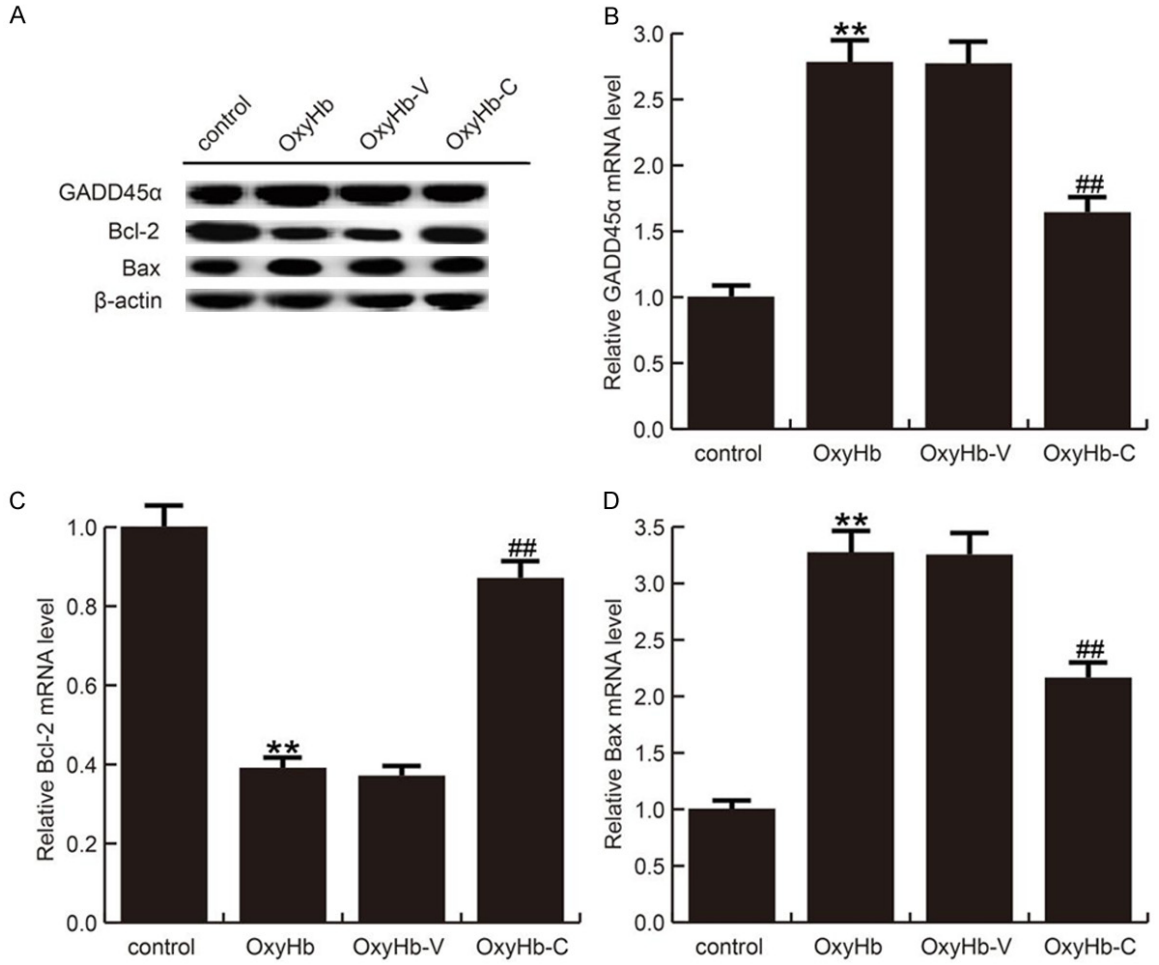


**Figure 4.** CPCGI treatment affected expression of GADD45α, Bcl-2, and Bax in neurons. After treatment for 48 hours, protein (A) and mRNA (B-D) expression levels of GADD45α, Bcl-2, and Bax in the neurons were determined. The experiments were performed three times. Data are displayed as the mean ± SD. *$p < 0.05$.

Bax increased. These changes were eliminated by administration of CPCGI.

## Discussion

A common disease with high mortality and morbidity rates, ICH affects more than 2 million people worldwide each year. It accounts for almost 20% of all stoke patients. In recent years, ICH research has received increased attention [32, 33]. Due to the unclear mechanisms of brain damage after ICH, there are still no effective clinical treatment methods for ICH. CPCGI is a neurotrophic drug used to treat certain functional disorders of the brain. Despite extensive usage throughout China, the exact effects of CPCGI on ICH remain unknown. The current study is the first to test the therapeutic potential of CPCGI against ICH induced brain injuries. Results indicated that CPCGI treatment significantly decreased neurological injuries and brain water content levels caused by ICH. However, this study did not present morphological data of the mice brains before and after CPCGI treatment. This may be a limitation of the current study. Increased levels of TNF-α, IL-6, and IL-1 in microglial cells caused by ICH were significantly inhibited by CPCGI treatment. Moreover, it was found that CPCGI markedly inhibited ICH induced cell viability repression and cell apoptosis. Present results suggest that CPCGI could reduce brain damage caused by ICH.

CPCGI is a compound composed of polypeptides, gangliosides, and hypoxanthine. CPCGI is neuroprotective and can restore damaged nerve tissues. CPCGI reduces cerebral isch-

emia-reperfusion injuries in rats by activating mitochondrial autophagy [25]. One of the main components of CPCGI, carnosine plays critical roles in preventing neuronal apoptosis following acute-cerebral ischemia [34, 35]. A recent study suggested that CPCGI exhibits neuroprotective properties in rats with middle cerebral artery occlusion injuries by inhibiting apoptosis and improving synaptic and mitochondrial function [24]. However, to the best of our knowledge, the roles of CPCGI in ICH remain unclear. Therefore, the present study was conducted.

To explore the impact of CPCGI on ICH, an experimental ICH model was conducted by autologous arterial blood injections in C57BL/6 mice. Cultured primary mouse cortical neurons/microglial cells were exposed to oxyhemoglobin/erythrocyte lysis to mimic ICH *in vitro*. Next, the mice and cells were treated with CPCGI. The current study measured water content levels of mice brain tissues and assessed neurological injuries by detecting neurological severity scores. Data indicated that CPCGI treatment significantly inhibited ICH induced neurological injuries and increased water content levels in mice brains. Increasing evidence has indicated that inflammatory response plays a key role in secondary brain injuries induced by ICH [32, 36-38]. Moreover, ICH promotes the activation of microglia and releases pro-inflammatory mediators that lead to neuronal damage [31]. The current study then investigated the effects of CPCGI on ICH-induced microglia inflammation. Results indicated that CPCGI significantly reduced levels of TNF-α, IL-6, and IL-1β, which were enhanced by ICH. Moreover, the effects of CPCGI on ICH-induced neuronal injuries were explored. Cell viability and apoptosis levels of the neurons were investigated. Findings suggest that ICH significantly represses cell viability and induces apoptosis of neurons. These changes were inhibited by CPCGI treatment. In addition, expression levels of apoptotic genes (Bcl-2, Bax, and GADD45α) were determined. Levels of Bcl-2 significantly decreased, while levels of Bax increased after ICH. These changes were eliminated by CPCGI. In addition, GADD45α, a protein shown to activate apoptosis [39, 40], was also inhibited by CPCGI.

Taken together, the present study suggests that CPCGI treatment reduces ICH induced brain damage, microglia inflammation, and neuronal injuries. Therefore, CPCGI plays a protective role in ICH-induced damage. However, this was a preliminary study concerning the effects of CPCGI on ICH. The effects of CPCGI on ICH mice was not completely clarified, requiring further study.

## Disclosure of conflict of interest

None.

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