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Investigation of the protective effect of heparin pre-treatment on cerebral ischaemia in gerbils

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ABSTRACT

Context: The interruption of cerebral blood circulation may cause stroke characterized by high neurological deficits (NDs) as a result of neuronal dysfunction or destruction. Heparin may exert a neuroprotective effect against cerebral ischaemia/reperfusion injury.

Objective: The objective of this study was to investigate the mechanism underlying the effects of heparin pre-treatment on cerebral injury in the gerbil.

Materials and methods: A total of 80 healthy Mongolian gerbils were randomly divided into four groups to establish cerebral ischaemia model by bilateral carotid artery occlusion: control (no anaesthesia and surgery), sham (no occlusion), non-anticoagulation (occlusion), and anti-coagulation treatment groups (50 IU/100 g heparin pre-treated, occlusion). Gerbils were anesthetized with 40 mg/kg pentobarbital sodium through intraperitoneal injection before operation except for the control group. Then, the ND and histopathological damage (HD) scores were determined. The percentage of tumour necrosis factor (TNF)-α- and interleukin (IL)-1β-positive cells were calculated based on immunohistochemical results. The mRNA and protein levels of caspase-9, caspase-8, FasL, and calpain were evaluated with real-time polymerase chain reaction (RT-PCR) and western blotting, respectively.

Results: Compared with non-anticoagulation group, heparin pre-treatment (50 IU/100 g) delayed the onset of dyspnoea ($p < 0.05$), and showed a significant decrease in ND ($p < 0.01$), mortality rate ($p < 0.05$), HD ($p < 0.01$) and percentage of positive cells for TNF-α, IL-1β ($p < 0.01$) in cerebral ischaemia gerbils. Besides, the expression levels of caspase-9, caspase-8, FasL, and calpain were reduced after pre-treatment with 50 IU/100 g heparin.

Discussion and conclusions: The damage caused to gerbil brain was reduced upon pre-treatment with heparin, possibly through the amelioration of neuronal cell apoptosis and expression of TNF-α and IL-1β. These findings are expected to provide a new breakthrough in the study and treatment of cerebral ischaemia.

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Anticoagulation; apoptosis; histopathological damage

Introduction

The brain is almost entirely dependent on the continuous and stable flow of glucose and oxygen for oxidative phosphorylation, which serves as the energy operation mechanism (Zhang et al. 2013). Any interruption in the cerebral blood circulation may cause stroke, characterized by high neurological deficits (NDs) owing to neuronal dysfunction and/or destruction. The disruption in blood flow leads to the accumulation of lactic acid through anaerobic glycolysis and reduces the production of cellular adenosine triphosphate (ATP). The decrease in cellular energy may result in impairment in important cellular functions, including the activity of ATPase, thereby promoting Ca^{2+} overload (Kao et al. 2006). Increased intracellular Ca^{2+} concentration causes cellular damage and eventually death (Kao et al. 2006). The rehabilitation ability of an organism after ischaemic stroke is weak and demands improvement, thereby putting a huge medical burden in developing countries (Feng et al. 2012).

An increasing number of natural products from animals, plants, and marine organism have been developed for brain disease and tumours (Pejin et al. 2013, 2015; Pejin and Glumac 2018). Low molecular weight heparin (LMWH), a natural anticoagulant with neuroprotective effects, may serve as a potential therapeutic agent for Alzheimer's disease based on its ability to reduce A β25-35-induced neurotoxicity (Hao et al. 2011). In addition, LMWH is considered as a preferred agent of anticoagulation for the patients with primary brain tumours or secondary brain metastasis for its known safety and effectiveness (Lin et al. 2018). A previous study has indicated that LMWH attenuates ischaemia–reperfusion injury in rats through the reduction in cerebral infarction, ND, malondialdehyde content, resulting in the inhibition of Ca^{2+} accumulation and an increase in superoxide dismutase (SOD) activity in the ischemic brain (Zhang et al. 2007).

Furthermore, LMWH has the ability to regulate cellular apoptosis (Yu et al. 2008). LMWH reduces leucocyte recruitment at

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the site of injury or inflammatory stimuli through the downregulation of the expression of cytokines, tumour necrosis factor (TNF)-α, endotoxins, human leukocyte elastase (HLE), and heparinase (Lever and Page 2012). The administration of LMWH was shown to significantly decrease ND scores and mediate a neuroprotective effect against cerebral ischaemia–reperfusion injury through improvement in energy metabolism, inhibition of apoptosis, and attenuation of inflammatory responses (Zhang et al. 2013). However, the protective effect of LMWH pre-treatment on ischaemia–reperfusion injury and the underlying mechanisms remain unclear.

In the present study, a transient ischaemia model was generated to evaluate the ND score, histopathological damage (HD) score, and recovery time of movement after the opening of the artery. In addition, the percentage of cells expressing TNF-α and interleukin (IL)-1β was calculated based on immunohistochemistry results. The mRNA and proteins levels of caspase-9, caspase-8, FasL, and calpain were evaluated with real-time polymerase chain reaction (RT-PCR) and western blot analysis, respectively.

Materials and methods

Subjects

This study was approved by the medical ethics committee of the People's Hospital of Ningxia Hui Autonomous Region. A total of 80 healthy Mongolian gerbils (SPF grade, weighing 60–80 g) were obtained from the Animal Experiments laboratory of Beijing Capital Medical University (Beijing, China). All gerbils were housed at a constant temperature (15–25 °C) and humidity (45–55%) and had free access to purified water.

Grouping

A total of 80 gerbils were randomly assigned into four groups, namely, a control group that was neither anesthetized nor operated (Group I, $n = 10$); sham group in which the bilateral carotid artery was not closed after anaesthesia and operation (Group II, $n = 10$); non-anticoagulation group, wherein the bilateral carotid artery was closed for 10 min without anticoagulation treatment after anaesthesia and operation (Group III, $n = 30$); anti-coagulation group pre-treated with heparin, followed by bilateral carotid artery closure for 10 min after anaesthesia and operation (Group IV, $n = 30$).

Establishment of a cerebral ischaemia model

All gerbils were randomly divided into four groups. The gerbils from group I were fed with free water and euthanized to obtain histological specimens for the determination of ND score. Gerbils from groups II to IV were anaesthetized with 40 mg/kg pentobarbital sodium through an intraperitoneal injection. Body temperature was maintained at 37 °C using a heating pad. A midline neck incision was made to expose both the sides of the common carotid arteries (CCAs). A transient focal cerebral ischaemia was produced by clamping two CCAs for 10 min. Gerbils from group II were similarly treated except that the arterial occlusion was not performed. For the gerbils from group III, two CCAs were initially exposed for 10 min, and the gerbils were subsequently injected with 0.01 mg/kg adrenaline. Gerbils from group IV were pre-treated with 50 IU/100 g heparin before ligation, and the subsequent treatment was consistent with that for gerbils from group III.

Post-cerebral ischaemia management

The vein was ligated after drug administration, and the incision was stitched after arterial exposure for 10 min. The gerbils were placed in a 50% oxygen box to intermittently observe changes in various indicators. Gerbils were excluded based on the following criteria: (1) bleeding of ≥0.8–1 mL during operation; (2) death during anaesthesia or operation. In particular, to assess ND and HD scores, the gerbils with survival time ≤72 h after cerebral blood flow (CBF) test were excluded. The carotid arteries were exposed for 72 h, and ND score was evaluated for each group according to the rat asphyxia model ND scoring criteria (Katz et al. 1995). The gerbils were anesthetized with 40 mg/kg pentobarbital sodium by an intraperitoneal injection and subjected to intubation and mechanical ventilation (1 mL/g, 80 times/min). A total of 20 mL of a fixative solution (10% paraformaldehyde, 5 mL; 25% glutaraldehyde, 1 mL; 0.2 M phosphate buffer, 25 mL; double-distilled water, 19 mL) was infused in the aorta. The right atrium was cut into a continuous infusion until the right heart drainage fluid became clear. The brain tissues were harvested and fixed for 24 h in 4% paraformaldehyde (pH = 7.4 and temperature of 4 °C), and then embedded in paraffin. According to the anatomical atlas of the rat brain provided by (Paxinos and Watson 1986), 6 μm thick paraffin sections were prepared from the coronal region across the 19, 29, 36, 42, and 63 regions. Nissl staining was performed to observe neuronal loss and damage to the CA1 and CA3 regions of the hippocampus in samples from groups III and IV. Histological damage to the hippocampus, cortex, thalamus, shell caudate nucleus, and cerebellum was assessed and scored using the Laurence Katz HD scoring system and standards (Katz et al. 1995). After cerebral perfusion, two gerbils were selected from each group and the hippocampus (CA1-3) was retrieved from the same area, followed by preparation of 1 mm^3 thick samples for electron microscopy.

Sample preparation for light microscopy

The brain tissues were fixed with 10% neutral formalin for 24 h and passed through a graded series of ethanol for dehydration. The tissues were soaked in xylene, embedded in paraffin, and sliced. The samples were dewaxed in xylene and dehydrated in decreasing concentrations of ethanol. Nissl staining was performed to evaluate any neuronal loss in these samples. After rehydration with ethanol, the samples were sectioned and subjected to immunohistochemical staining. The cells expressing TNF-α and IL-1β in the neurons of the hippocampus (CA1-3) were evaluated to assess the extent of damage to the brain tissue.

Sample preparation for electron microscopy

A 1-mm^3 thick hippocampus sample was retrieved and fixed in 2.5% glutaraldehyde, followed by treatment with 1% osmium tetroxide. The sample was dehydrated in a graded series of ethanol and soaked in epoxy resin. The sample was embedded, sliced, and stained with plumbum. H-600-4 transmission electron microscope was used to observe the changes in the ultrastructure of hippocampus cells.

Observation of indicators

The time of coma and dyspnoea after carotid artery occlusion was observed for animals from groups III and IV. The time from the opening of the artery to the stability of consciousness, reflex,

foraging behaviour, and movement was also reported. The ND scores in these four groups were evaluated before treatment and at 20 min, 1, 24, 48, and 72 h after the opening of the artery, as per the standards provided by Katz et al. (1995).

HD assessment

Optical and electron microscopies were used to assess HD scores according to the standards provided by Katz et al. (1995). All the neurons (normal and ischaemic) in the respective brain region were counted (in a range of 0.25×0.05 mm at 200× magnification) using the three-grid method. The proportion of ischaemic neurons in total neuron count of the region was expressed as a percentage. The average HD score of the ischaemic neurons was obtained from five different cerebral regions in each brain tissue section. The cells expressing TNF-α and IL-1β in the neurons of the hippocampus (CA1-3) were analyzed with immunohistochemistry. All positively and negatively stained neurons (normal and ischaemia) from each side of the corresponding brain regions were counted using the three-grid counting method (0.25×0.05 mm range at 200× magnification). The number of gerbils surviving for 72 h from the cardio-pulmonary resuscitation (CPR) test in groups III and IV was recorded.

Primary culture of neuronal cells

Neuronal cells (2×10^5/mL) were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, UT) supplemented with 10% foetal bovine serum (FBS; HyClone, Logan, UT) in a 6-cm dish pre-treated with D-poly-lysine. The cells were maintained in an incubator with 5% CO_2 and 95% air at 37 °C. After cultivation for 6 d *in vitro*, the cells were divided into two groups as follows: hypoxia and heparin treatment groups. The neurons from the hypoxia group were placed in an anoxic jar (5% CO_2, 95% N_2) at 37 °C for 4 h and then immediately transferred into an incubator for cultivation. The neurons from the heparin group were treated with 0.2 mg/L heparin added to the medium, while the rest of the treatment was similar to that for the hypoxia group. The neuronal cells were recovered after 3 and 6 d, and the RNA and proteins were extracted.

RT-PCR

Total RNA was isolated from neuronal cells and its concentration determined. cDNA was generated from RNA with RevertAid First strand PrimeScript RT Reagent Kit (Takara, Tokyo, Japan). The PCR reaction conditions were as follows: denaturation at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 5 s and annealing at 60 °C for 34 s, and a final extension at 72 °C for 5 min. The PCR products were verified with 2% agarose gel electrophoresis.

Western blot analysis

Proteins were extracted and lysed with radioimmunoprecipitation assay (RIPA) buffer supplemented with phenylmethylsulfonyl fluoride (PMSF; Beyotime Biotechnology, Beijing, China). The protein concentration was assessed with the Bradford method. The samples were separated with sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and the separated bands were transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were incubated with primary antibodies at 4 °C overnight, followed by treatment with secondary antibodies for 2 h at room temperature. The protein expression was detected with Las-4000 developer (Fujifilm, Tokyo, Japan).

Statistical analysis

All statistical analysis was performed using SPSS11.5 (SPSS Inc., Chicago, IL) statistical software. The difference in the time of coma and dyspnoea after carotid artery occlusion between groups III and IV was estimated with one-way analysis of variance (ANOVA). The differences in the time from the opening of the artery to stability in consciousness, reflex, foraging behaviour, and movement between the groups III and IV were also evaluated with ANOVA. The Kruskal–Wallis test was applied to analyze the variance in ND and HD scores among the four groups. The differences in ND and HD scores at different time points between groups III and IV were evaluated with the Kruskal–Wallis test. All data were presented as mean ± standard deviation (SD). The chi-square test was used to evaluate the difference in mortality rate between groups III and IV. The correlation between ND and HD scores in groups III and IV was assessed using Spearman's analysis. A value of $p < 0.05$ was considered statistically significant.

Results

Time for recovery of major neurological functions and ND scores

We failed to observe any significant differences in coma time between groups III and IV ($p > 0.05$). The time of dyspnoea was significantly earlier in group III than in group IV ($p < 0.05$). Other indicators, including conscious recovery, righting reflex, movement, foraging behaviour, and smooth movement, were significantly shorter in group III than in group IV (Figure 1(A)). Before the experiment, the ND score of these four groups was 0; however, the ND score obviously reduced in group IV as compared to that in group III after the exposure of the carotid arteries for 20 min, 60 min, 24 h, 48 h, and 72 h ($p < 0.01$, Figure 1(B)).

Percentage of positive cells and HD scores

A total of 13 and 5 gerbils died in groups III and IV, respectively, during the experiment. A difference in mortality rate was observed between gerbils from groups III (43%) and IV (16.7%) ($p < 0.05$, Figure 1(C)). The results of the immunohistochemical analysis revealed a significant reduction in the percentage of IL-1β- and TNF-α-positive cells in group IV as compared to that in group III ($p < 0.01$, Figure 1(C)). Moreover, the HD scores of hippocampus, cortex, thalamus, cerebellum, and shell–caudal nucleus were significantly lower in gerbils from group IV than in those from group III ($p < 0.01$, Figure 1(D)), however, we failed to note any significant difference in HD score between gerbils from groups I and II.

Growth of hippocampus and neuronal cells

After the experiment, the structure of the nerve cells of the hippocampus (CA1-3) retrieved from the animals from groups I and II was normal, as evaluated with light and electron microscopies (Figure 2(A,B)). The hippocampus neurons (CA1-3) from

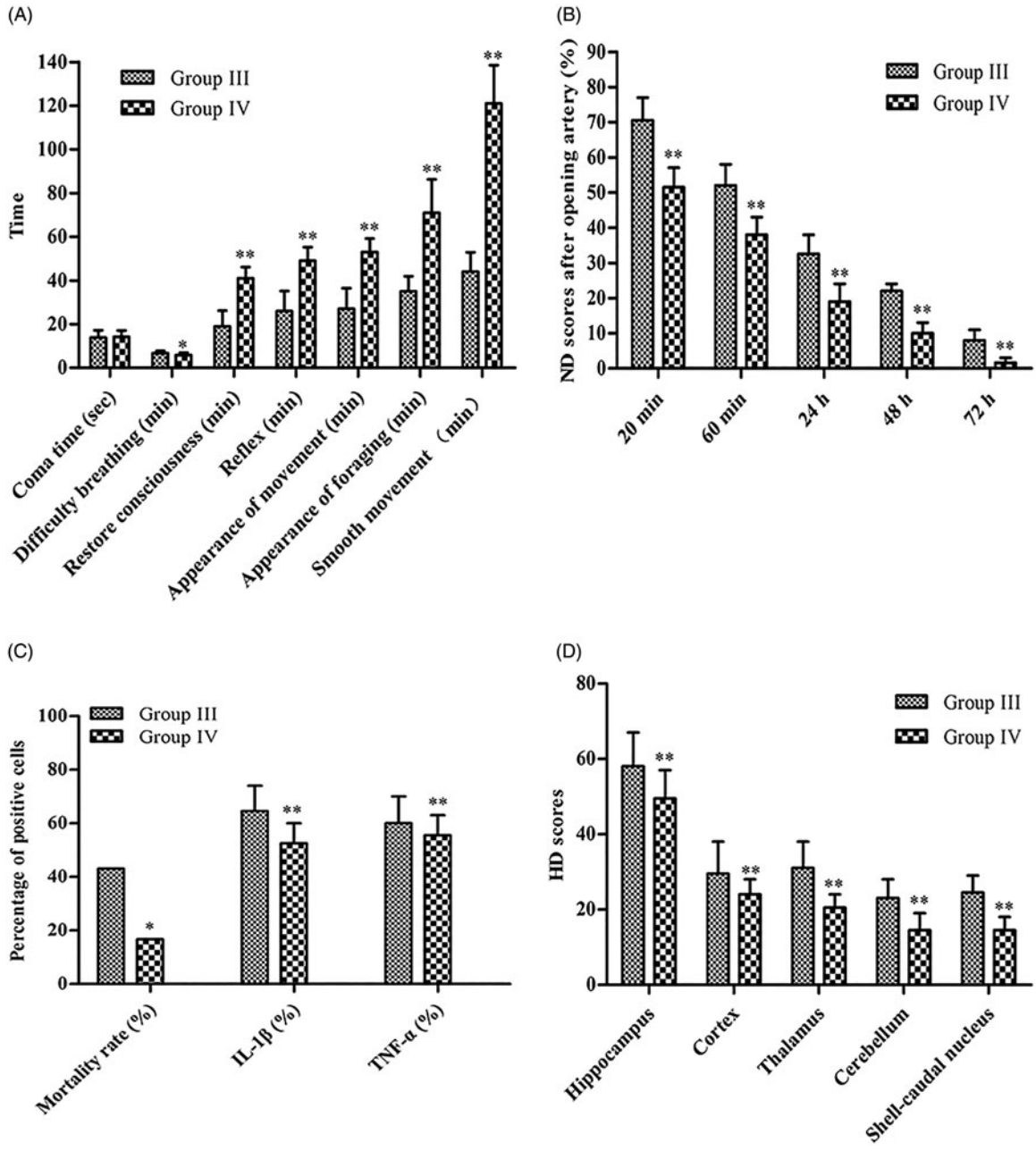

Figure 1. Effect of heparin pre-anticoagulation treatment on injury and recovery after ischaemia. (A) The onset time of behavioural difficulties after carotid artery occlusion. (B) ND score after the opening of artery. (C) Percentage of positive cells. (D) HD scores of different regions in brain tissue slices. ND: neurological deficit; HD: histopathological damage; $^{*}p < 0.05$; $^{**}p < 0.01$.

groups III and IV presented different degrees of damage after treatment, and the extent of damage was more serious in the animals from group III (Figure 2(C,D)).

The primary neurons in the hippocampus showed a good growth trend before hypoxia. The neurons of the hippocampus of gerbils from groups III and IV presented different degrees of damage. Furthermore, heparin pre-treatment (50 IU/100 g) significantly improved the growth of neuronal cells after resuscitation for 3 and 6 d. Thus, heparin pre-treatment (50 IU/100 g) exerts a protective effect on primary neurons of the hippocampus to allow adaptation to the hypoxic environment (Figure 3).

Expression of TNF-α and IL-1β

Immunohistochemical staining results revealed the absence of any expression of TNF-α (Figure 4(A,B)) and IL-1β (Figure 5(A,B)) in the hippocampus (CA1-3) of the gerbils from groups I and II. However, the positive expression of TNF-α (Figure 4(C,D)) and IL-1β (Figure 5(C,D)) was detected in the hippocampus (CA1-3) of gerbils from groups III and IV. The rate of positive expression of TNF-α and IL-1β significantly increased in gerbils from group III as compared with those from group IV ($p < 0.01$).

RT-PCR

The expression of the genes encoding FasL, caspase-9, caspase-8, and calpain after 50 IU/100 g heparin pre-treatment for 3 and 6 d under hypoxic environment was significantly different from that observed in non-heparin pre-treatment group. Heparin pre-treatment (50 IU/100 g) obviously reduced the mRNA levels of

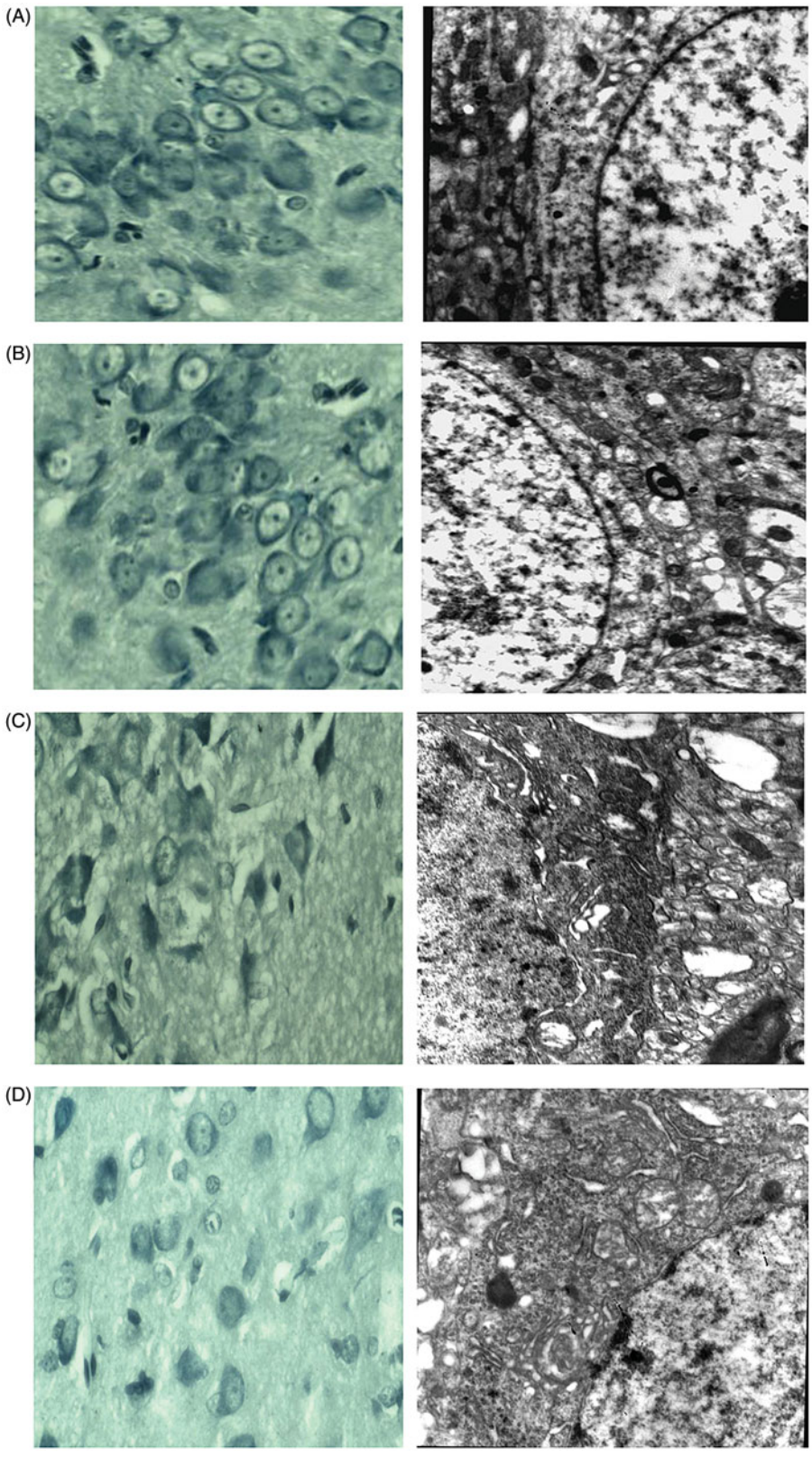

Figure 2. Results of cell structure in the hippocampus (CA1-3) under light and electron microscope. A: Group I; B: Group II; C: Group III; D: Group IV. Magnification under light microscope, 400×; magnification under electron microscope, 15,000×.

caspase-9, caspase-8, and calpain after 3 d (Figure 6(B,C,D), $p < 0.05$), and the expression of caspase-9 and caspase-8 was inhibited by heparin pre-treatment at day 6 (Figure 6(B,C), $p < 0.05$). Furthermore, heparin pre-treatment (50 IU/100 g) promoted the mRNA expression of FasL at day 3 as well as that of calpain at day 6 (Figure 6(A,D), $p < 0.05$).

Western blot analysis

Western blot analysis results revealed the reduction in the levels of calpain and caspase-9 following heparin pre-treatment (50 IU/100 g) after resuscitation for 3 d (Figure 7(A,C,E)). The levels of caspase-8, calpain, and caspase-9 were inhibited by

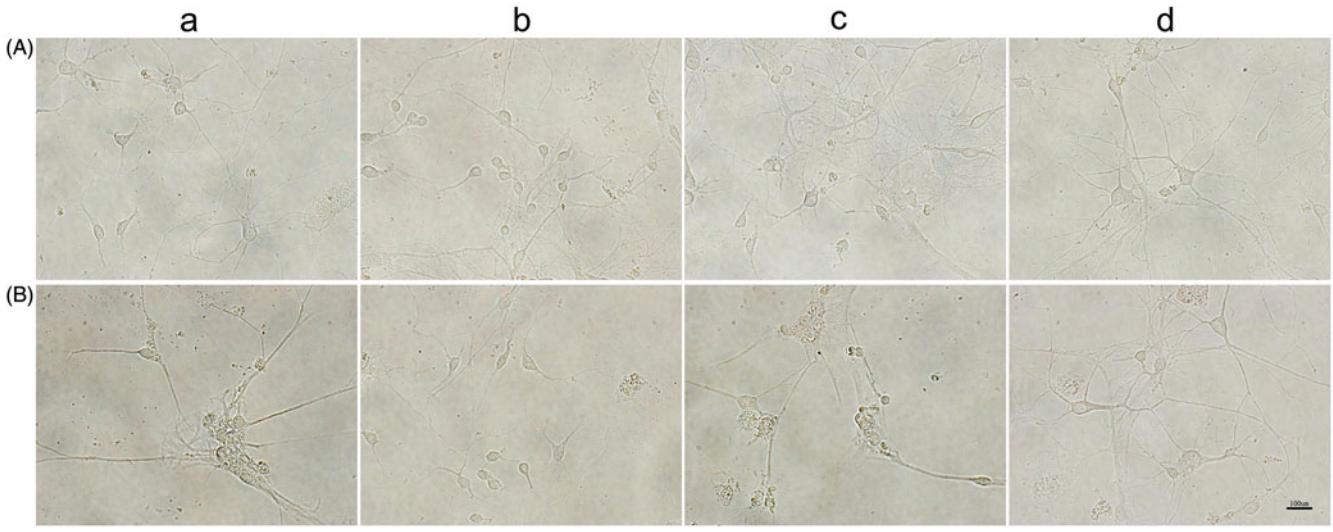

Figure 3. Growth of neuronal cells under hypoxia. (A) Before low oxygen treatment. (B) After resuscitation; a, non-heparin treatment group with resuscitation for 3 d; b, heparin treatment group with resuscitation for 3 d; c, non-heparin treatment group with resuscitation for 6 d; d, heparin treatment group with resuscitation for 6 d. Scale bar: 100 μm.

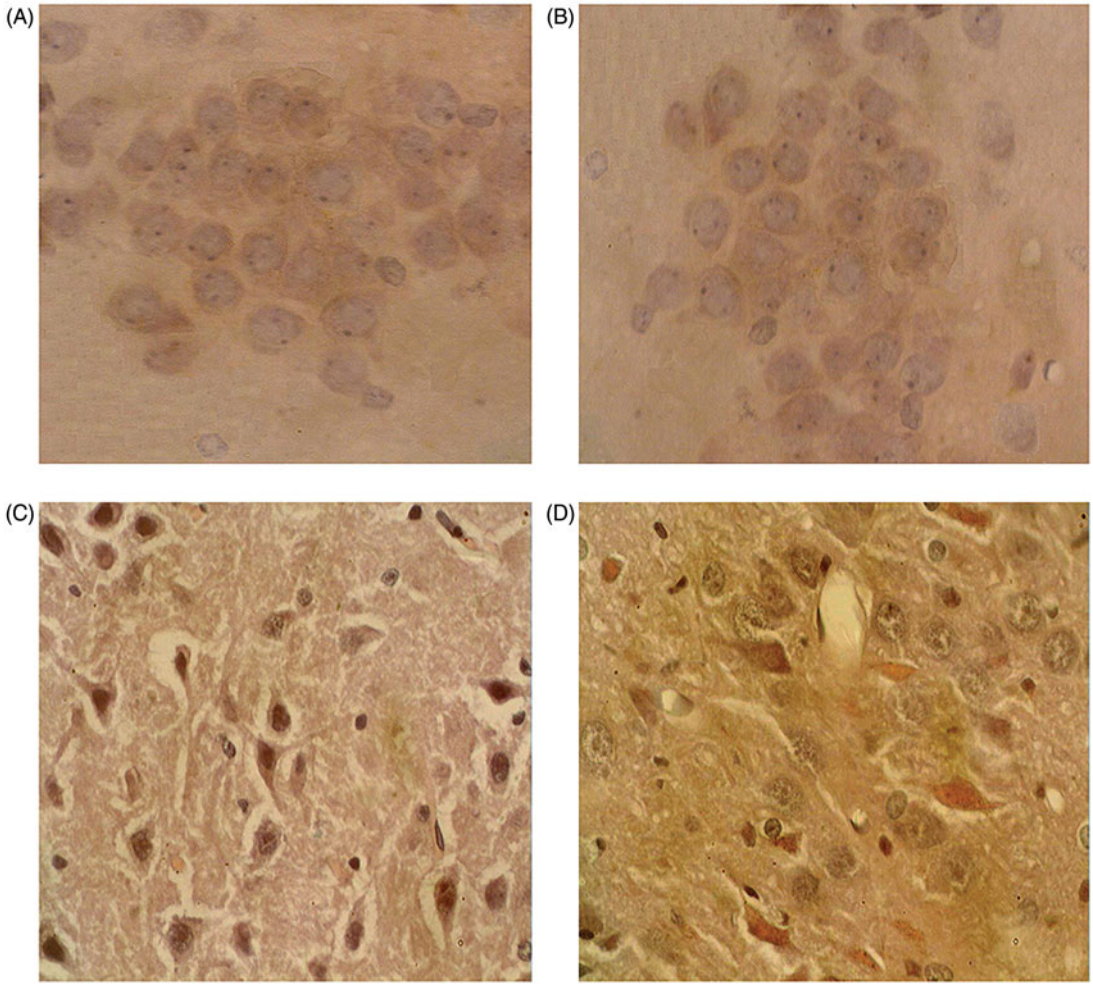

Figure 4. Results of immunohistochemistry for TNF-α expression detection in the hippocampus (CA1-3). A: Group I; B: Group II; C: Group III; D: Group IV. Magnification, 400×.

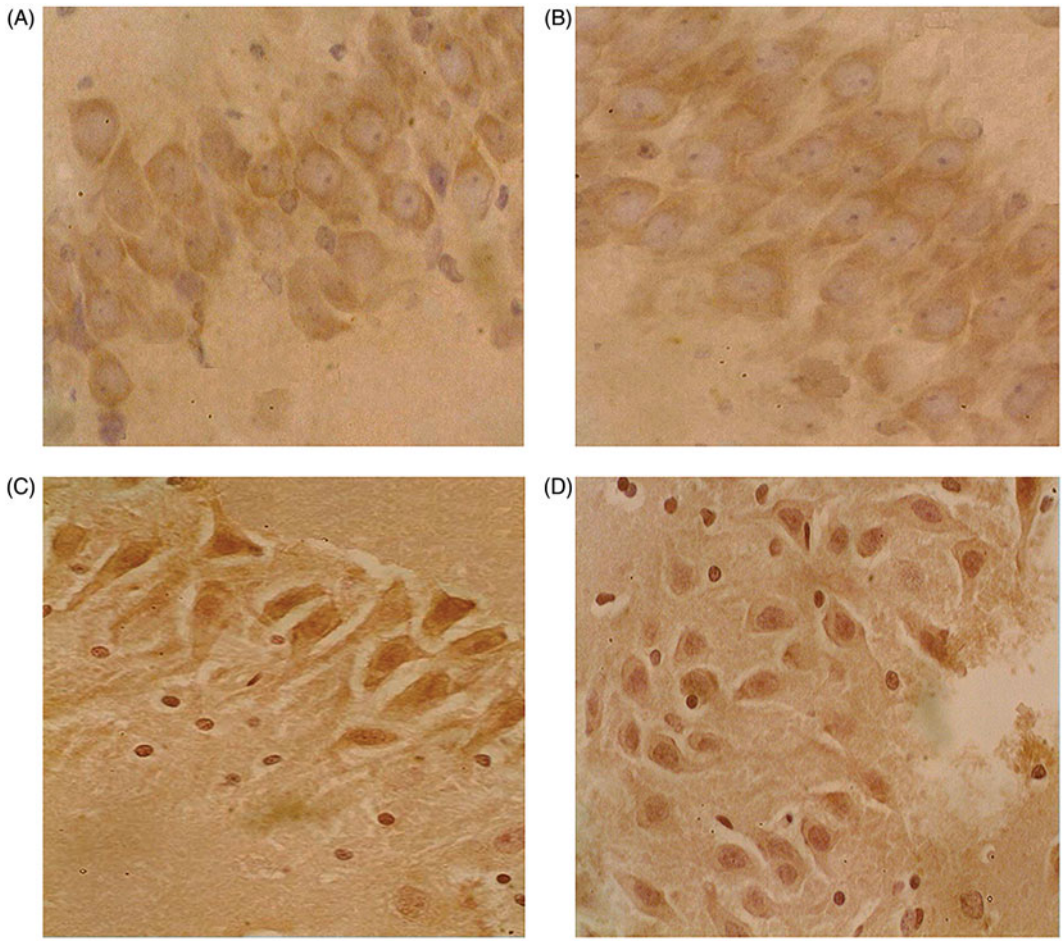

Figure 5. Results of immunohistochemistry for IL-1β expression detection in the hippocampus (CA1-3). A: Group I; B: Group II; C: Group III; D: Group IV. Magnification, 400×.

heparin pre-treatment (50 IU/100 g) after resuscitation for 6 d (Figure 7(A,B,C,E)).

Discussion

The present study suggests that the clamping of the bilateral carotid artery may cause cerebral ischaemic damage to gerbils and that heparin pre-anticoagulation may delay the onset of dyspnoea. The recovery time of neurological function was significantly shortened in gerbils subjected to heparin anticoagulation treatment after the opening of arteries, indicating that heparin pre-anticoagulation improves the recovery of the nervous system function. The mortality rate was obviously lower in the gerbils subjected to heparin pre-anticoagulation treatment than in those without heparin pre-treatment, indicating that the prognosis of brain injury may be changed with heparin pre-anticoagulation. Furthermore, heparin pre-anticoagulation reduced neurological impairment after cerebral ischaemia and promoted the recovery of neurological functions. The HD scores correlated with ND scores and showed improvement following heparin pre-anticoagulation treatment. Anticoagulation alleviated the damage to neuronal cells and reduced the percentage of cells expressing TNF-α and IL-1β after cerebral ischaemia. Thus, heparin pre-anticoagulation mediated a protective effect on the central nervous system. The expression levels of caspase-9, caspase-8, FasL, and calpain reduced upon heparin pre-treatment under hypoxia.

Inflammation is considered as a crucial factor in the pathogenesis of ischaemic stroke. Although reperfusion is effective for thrombolysis, spontaneous and therapeutic reperfusion is associated with inflammatory responses and may lead to brain damage (Jean et al. 1998). A previous study has shown that the expression of TNF-α and signal transducer and activator of transcription 3 (STAT3)/suppressor of cytokine signalling 3 (SOCS3) pathway was upregulated in a transient middle cerebral artery occlusion (MCAO) model (Wang et al. 2014). TNF-α is implicated in the pathogenesis of acutely ischaemic brain injury (Galasso et al. 2000), and promotes neuronal apoptosis, activates glial cells, stimulates the proliferation of astrocytes, and induces neurotrophic factors in IL-6-expressing cells and astrocytes (Ye et al. 2013). TNF-α is known to be an apoptosis activator that potentiates Fas expression and induces neuronal apoptosis (Northington et al. 2005). TNF-α is produced upon injury to the spinal cord. The upregulation in the expression of TNF-α in glial cells is related to the expression of the p55 receptor on adjacent neurons (Ohtori et al. 2004). This association may induce the expression of several cytokines and immediate early genes in the dorsal root ganglion and spinal cord neurons via the TNF signalling pathway (Ohtori et al. 2004). Injection of IL-1β plays an important role in nerve tissue injury by reducing the aggregation of peripheral macrophages and activating microglia (Bethea 2000). The expression levels of IL-1β and TNF-α increased in gerbils with cerebral ischaemia reperfusion, suggestive of the involvement of IL-1β and TNF-α in the mechanism underlying

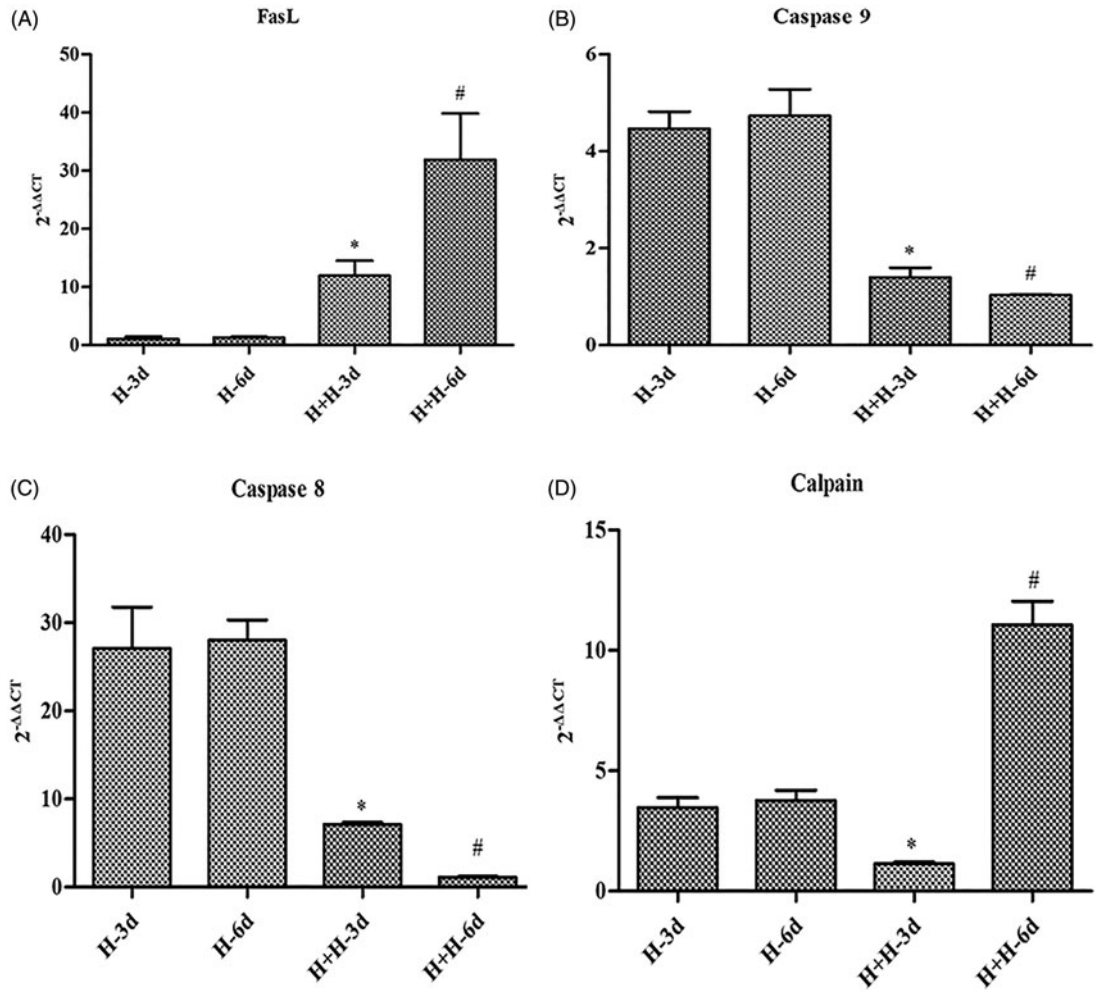

Figure 6. Results of RT-PCR. H-3d: non-heparin treatment group with resuscitation for 3 d; H-6d: non-heparin treatment group with resuscitation for 3 d; H + H-3d: heparin treatment group with resuscitation for 3 d; H + H-6d: heparin treatment group with resuscitation for 6 d. $^{*}p < 0.05$ as compared with H-3d; $\#p < 0.05$ as compared with H-6d.

cerebral ischaemia reperfusion injury (Li et al. 2016). In the present study, the expression levels of TNF-α and IL-1β as well as the damage to neuronal cells in gerbils with the ischaemic brain injury obviously increased, but these effects were ameliorated after heparin pre-treatment. We speculate that heparin pre-anticoagulation alleviated the damage to the nervous system caused by cerebral ischaemia through the reduction in the expression levels of TNF-α and IL-1β.

Caspase-8, a member of the caspase family, coordinates cell apoptosis, necrosis, and inflammation. Caspase-8 is activated by dimerization and causes a conformational change in zymogen. Caspase-8 is thought to prevent neuronal survival and regeneration in the central nervous system of an adult mammal (Monnier et al. 2011). The mRNA level of caspase-8 was shown to be increased in the ischaemic cortex of MCAO model (Harrison et al. 2001). Caspase-8 plays a pivotal role in the neuropathology of ischaemia and neurons after cerebral infarction (Morita-Fujimura et al. 2001). Activated caspase-8 may cause the cleavage and activation of Bid, which targets the mitochondrial membrane to change the conformation of Bax and, finally, activate caspase-3. Inhibition of caspase-8 expression protects neurons through the selective killing of activated microglia or regulation of the TNF death-receptor signalling (Inoue et al. 2006; Fricker et al. 2013).

The current study indicates that heparin pre-treatment reduced the expression of caspase-8 under hypoxia. Caspase-9 is a member of the caspase family and is required for the changes in the morphology of mitochondrion as well as for the production of reactive oxygen species (ROS). Caspase-9 is also required for normal hematopoietic development and is activated by the cytochrome released from mitochondria. Caspase-9 is associated with the mitochondrion-dependent apoptotic pathway (Kawamoto et al. 2016). Intriguingly, caspase-3 can be activated by caspase-9 (Ferrer and Planas 2003). The expression of FasL was increased during early reperfusion after MCAO, and the activated Fas receptor initiated the cell-death receptor pathway by binding to FasL (Ferrer and Planas 2003). FasL may serve as a death receptor for neuronal cells and participate in the death-receptor pathway following ischaemia (Felderhoff-Mueser et al. 2000). Calpain/caspase-12 activation induced by endoplasmic reticulum (ER) stress may activate caspase-3.

We found that the expression levels of caspase-8, caspase-9, and calpain were downregulated after heparin pre-treatment; however, the expression of FasL was upregulated in the injured brain under hypoxia. The corresponding four genes were associated with cellular apoptosis. The injury to neurons was reduced after heparin pre-treatment. Therefore, we speculate that heparin pre-treatment may inhibit neuronal apoptosis through the regulation of the expression of caspase-8, caspase-9, calpain, and FasL under hypoxia, thereby playing a protective role in the nervous system.

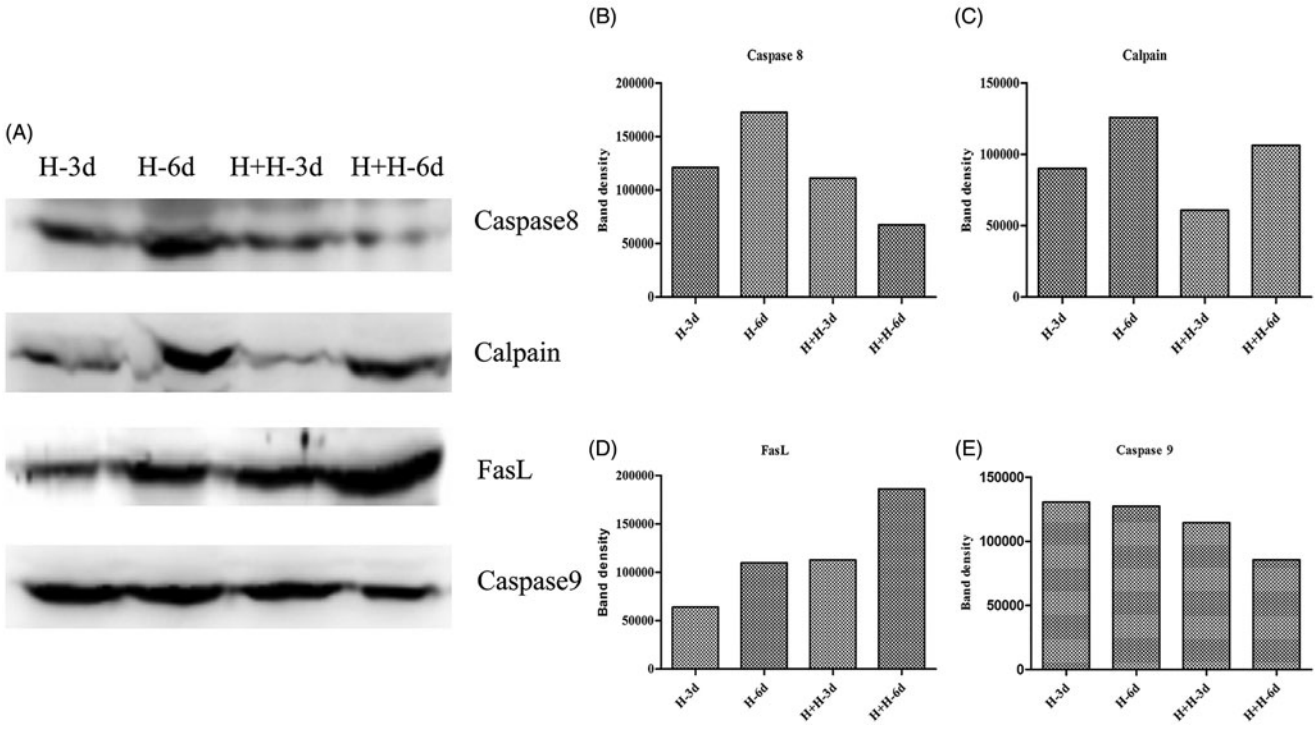

Figure 7. Expression level of proteins. (A) Western blot analysis for four proteins. (B) Expression of caspase-8 before and after resuscitation. (C) Expression of calpain before and after resuscitation. (D) Expression of FasL before and after resuscitation. (E) Expression of caspase-9 before and after resuscitation. H-3d: non-heparin treatment group with resuscitation for 3 d; H-6d: non-heparin treatment group with resuscitation for 3 d; H + H-3d: heparin treatment group with resuscitation for 3 d; H + H-6d: heparin treatment group with resuscitation for 6 d.

The present study has a few limitations. For instance, the neuronal cells used *in vitro* were injured by hypoxia; however, we used a cerebral ischaemia gerbil model for the *in vivo* study. We would verify the conclusion established herein using neuronal cells isolated from the hippocampus injured with cerebral ischaemia in the future. In addition, we did not monitor apoptosis in the nerve and neuronal cells.

In summary, the present study demonstrates that heparin pre-treatment exerted a protective effect on cerebral ischaemia in gerbils through the downregulation of the expression of TNF-α and IL-1β and reduction in the apoptosis of neuronal cells. This finding provides a theoretical basis for the study of the mechanism underlying the effects of heparin on the injured brain.

Disclosure statement

No potential conflict of interest was reported by the authors.

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