学位論文

Intravenous edaravone plus therapeutic hypothermia offers limited neuroprotection in the hypoxic-ischaemic newborn piglet

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Intravenous edaravone plus therapeutic hypothermia offers limited neuroprotection in the hypoxic-ischaemic newborn piglet

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Abstract

Background: Therapeutic hypothermia is a standard therapy for neonatal hypoxic-ischaemic encephalopathy. One potential additional therapy is the free radical scavenger edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one).

Objectives & Methods: This study aimed to compare the neuroprotective effects of edaravone plus therapeutic hypothermia with those of therapeutic hypothermia alone after a hypoxic-ischaemic insult in the newborn piglet. Anaesthetised piglets were subjected to 40 min of hypoxia (3–5% inspired oxygen) and cerebral ischaemia was assessed using cerebral blood volume. Body temperature was maintained at 39.0 ± 0.5°C in the normothermia group (NT, n = 8) and at 33.5 ± 0.5°C (24 h after the insult) in the therapeutic hypothermia (TH, n = 7) and therapeutic hypothermia plus edaravone (3 mg/kg intravenous every 12 h for 3 days after the insult; TH+EV, n = 6) groups under mechanical ventilation.

Results: Five days after the insult, the mean (standard deviation) neurological scores were 10.9 (5.7) in the NT group, 17.0 (0.4) in the TH group ($p = 0.025$ vs. NT) and 15.0 (3.9) in the TH+EV group. The histopathological score of the TH+EV group showed no significant improvement compared with that of the other groups.

Conclusion: Edaravone plus therapeutic hypothermia had no additive neuroprotective effects after hypoxia-ischaemia in neurological and histopathological assessments.

Introduction

Neonatal hypoxic-ischaemic encephalopathy (HIE) is a clinically significant disorder with long-term morbidity [1, 2]. Therapeutic hypothermia (TH) has been used to limit brain damage in term newborns, although it is unable to fully rescue neurological outcomes [3]. Therefore, other therapies that augment the neuroprotection of TH in neonatal HIE are needed.

Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one) is a synthetic free radical scavenger used as a neuroprotective drug. First, in the reoxygenation period after resuscitation, edaravone has promising activity as an antioxidative radical scavenger, quenching HO• and inhibiting both HO•-dependent and •OH-independent lipid peroxidation [4]. Second, the therapeutic benefit of edaravone may be directly related to its multi-target pharmacology, with edaravone regulating numerous signalling pathways to delay neuronal death [5], reduce cerebral oedema, counteract microglia-induced neurotoxicity during secondary energy failure periods and limit long-term inflammation in the latent phase [6]. Kikuchi et al. [7] reported that edaravone markedly reduced the levels of aquaporin protein 4 (AQP4), which is the major water channel in the central nervous system and is involved in the generation of post-ischaemic oedema as well as increased immunoreactivity and protein levels in the cerebral infarct area in a rat model of transient focal ischaemia. Edaravone can mediate the induction and inhibition of microglial activity and inhibit iNOS activity [8, 9]. Microglia are thought to contribute to neural damage via the release of

excessive proinflammatory cytokines and or cytotoxic factors, such as nitric oxide, tumour necrosis factor-α, interleukin-1β and ROS. Accordingly, many adult animal and human studies have reported the efficacy of edaravone in treating brain injury due to cerebral infarction [10]. Indeed, Radicut®, a brand of edaravone marketed by Mitsubishi Tanabe Pharma Corporation (Tokyo, Japan), has been approved in Japan to treat acute ischaemic stroke patients presenting within 24 h of the attack since 2001.

In newborns, oxidative stress also plays an important role in the biochemical cascade that leads to neuronal cell death after hypoxia-ischaemia (HI) [11]. Neonatal rat studies have revealed that edaravone reduces oxidative stress and improves outcomes after HI [12-15]. Ni et al. [16] had already reported the efficacy of edaravone for neuroprotection in a 3–7-day-old piglet model, but they did not use newborn piglets and those treated with TH. In this regard, assessment of the efficacy of edaravone in a perinatal large-animal model would be useful for preclinical evaluation. In this study, we used a previously developed perinatal asphyxia model of newborn piglets that survive 5 days after the HI insult [17, 18].

Edaravone may become an additional therapy to TH for neonatal HI encephalopathy in humans. To our knowledge, no studies have revealed the efficacy of edaravone combined with TH in a large perinatal animal. We thus compared the neuroprotective effects of edaravone plus TH with those of TH alone after HI insult in our newborn piglet model of perinatal asphyxia using

intravenous edaravone administered for 3 days combined with TH for 24 h after resuscitation.

Materials and Methods

Animals

This study was approved by the Animal Care and Use Committee for Kagawa University (15070-1) and was performed in accordance with the Animal Research: Reporting In Vivo Experiments guidelines. Thirty-eight piglets obtained within 24 h of birth and weighing 1.63–2.10 kg were used in this study.

Twenty-one newborn Camborough piglets (Daiwa Chikusan, Kagawa, Japan) were initially anaesthetised with 1–2% isoflurane. Each piglet was then intubated and mechanically ventilated with an infant ventilator. In brief, after the initial intubation, umbilical arterial and venous catheters were inserted for blood pressure monitoring and blood sampling, respectively. After cannulation, the piglets were anaesthetised with fentanyl citrate at an initial dose of 10 μg/kg, followed by infusion at 5 μg/kg/h, and were paralysed with pancuronium bromide at an initial dose of 100 μg/kg. Each piglet was then placed in a copper mesh-shielded cage under a radiant warmer to maintain a rectal temperature of 39.0 ± 0.5 °C. Inspired gas was prepared by mixing O_2 and N_2 gases to obtain the oxygen concentrations required for the experiment. Ventilation was adjusted to maintain PaO_2 and $PaCO_2$ within their normal ranges.

Near-infrared time-resolved spectroscopy and analysis

Supplemental information [19]. [20].

Amplitude-integrated electroencephalography monitoring

Supplemental information [21],

Hypoxic insult

Because the details were reported in our previous studies [20, 22], only an outline of the HI insult protocol is presented here. Piglets were exposed to a systemic hypoxic insult, induced by decreasing FiO2 to 2-4% over 40 min, under monitoring by Near-infrared time-resolved spectroscopy to measure cerebral blood volume [17]. Hypoxia was terminated by resuscitation with 100% oxygen. After 10 min of 100% FiO_2, the ventilator rate and FiO_2 were gradually reduced to maintain a SpO_2 of 95–98%.

Post-insult treatment

The piglets in all groups received mechanical ventilation for 24 h after resuscitation. Immediately after HI, the piglets in the TH+EV group (n = 6) were given intravenous edaravone (Radicut®; 3 mg/kg) every 12 h for 3 days after the insult [13]. Because Yasuoka et al. [13]

reported that repeated injection of edaravone (3 mg/kg) every 12 h for 7 days after HI insult decreased both apoptosis and necrosis in the neonatal rat, we chose the same dose in this study. The NT (n = 8) and TH (n = 7) groups were given an intravenous injection of saline for 3 days. Piglets in the NT group were maintained after resuscitation at 39.0 ± 0.5 °C under a radiant heater. In the TH and TH+EV groups, whole-body hypothermia was achieved using a cooling blanket (Medicool; MAC8 Inc., Tokyo, Japan) after resuscitation. The piglets were cooled to 33.5 ± 0.5 °C for 24 h and then rewarmed at 1 °C/h using a blanket. Once the piglets were weaned off the anaesthesia and ventilator and extubated, they were allowed to recover and were maintained for 5 days in the incubator.

Neurological score

As reported by Thoresen et al. [23], we determined a nine-item (three-step) neurology score (0, abnormal; 1, mildly abnormal; 2, normal; total score range, 0–18) 5 days after the insult. The nine items examined during the neurological assessment were respiration, consciousness, orientation, ability to walk, forelimb control, hind limb control, limb tone, overall activity, sucking, vocalisation and presence of pathological movements.

Histology

After the 5-day period, the animals were initially anaesthetised with isoflurane, and the brain of each animal was perfused with 4% phosphate-buffered paraformaldehyde. Brain tissue was histologically evaluated and irregularities were graded according to a histopathology grading scale for a piglet model of post-hypoxic encephalopathy [24, 25].

Data analysis

GraphPad Prism 5J (GraphPad Software, La Jolla, CA) was used for all statistical analyses. Blood sample and physiological data were compared among the three groups using two-way analysis of variance (ANOVA), whereas neurological score and histopathological score were compared among the three groups using one-way ANOVA. A p value < 0.05 was considered significant.

Results

Physiological results

In our animal model, heart rate (HR) and MABP increased during the approximate 40-min period of the hypoxic insult before subsequently decreasing, with similar values found in all groups (Fig. 1).

MABP was higher within 3 h after the insult in the TH (1 h, $p < 0.05$ vs NT) and TH+EV

groups compared with the NT group, whereas HR was significantly lower in the TH (1 h) and TH+EV (1, 3, 6 and 12 h) groups than in the NT group after the insult (Fig. 2).

Neurological assessment

At 5 days of recovery from HI, the mean (SD) neurological scores were 10.9 (5.7) in the HI-NT group, 17.9 (0.4) in the TH group ($p < 0.05$ vs. NT) and 15.0 (3.9) in the TH+EV group (Fig. 6).

Histopathological results

The histological scores are shown in Fig. 7. Results are expressed as mean (SD). Histological images of the brain damage obtained using H&E staining are shown in Fig. 8. The TH+EV group had the lowest H&E scores in the GM, WM and HIPP. However, there were no significant differences in H&E scores among the three groups (Fig 7a–d). In the GM, pyramidal neurons were affected, showing inflammatory cell infiltration and a considerable number of necrotic neurons. In some animals, necrotic neurons with a pyknotic nucleus and eosinophilic cytoplasm were seen, with the neuropil showing severe vacuolar degeneration, especially in the NT group. H&E staining in the GM of the TH+EV group revealed fewer red neurons compared with the NT and TH groups. However, no significant difference was seen among the three groups

(Fig 8a–c). In our model, the damage was more severe in the WM. H&E staining of WM revealed more oedematous and vacuolar degeneration in the neuropil in most areas, especially in the NT group. Some animals showed complete disintegration of the tissue. The degree of oedema was particularly evident in the NT group. The TH+EV group demonstrated less oedema (Fig 8d–f). There were no regional differences in each area in the respective groups and between the groups. Granular neurons in the dentate gyrus were relatively intact, whereas pyramidal neurons in the CA-1 areas of the HIPP showed necrotic changes in the neuropil with infiltration of inflammatory cells. In some areas, vacuolar degeneration was seen, especially in the NT group (Fig 8G–I). The pyramidal neurons in the TH+EV group were relatively spared compared with those in the other groups. In the CERE, some Purkinje cells showed necrosis. In severe cases, vacuolar degeneration with infiltration of inflammatory cells was evident (Fig 8J–L). Histological improvement was not clear with hypothermia or hypothermia with edaravone.

Discussion

To our knowledge, this is the first report using a perinatal large-animal model to investigate the ability of intravenous administration of edaravone plus TH to protect against brain injury. The TH group showed an improved neurological score on day 5 after the insult. Nevertheless, the findings confirmed the limited neuroprotective ability of edaravone plus TH. However, because

many previous studies have reported the efficacy of edaravone for ameliorating brain injury in adult and neonatal animal models (Table 2), several possibilities need to be discussed to interpret this lack of efficacy.

The first possibility is that optimal efficacy is influenced by the dosage, number, interval and route (e.g., intravenous or intraperitoneal) of drug administration. The method for edaravone administration used in the present study was based on previous reports showing the efficacy of this method in the neonatal rat. In Yasuoka et al. [13], two doses of 3 mg/kg given to neonatal rat were found to be neuroprotective at only 24 h, but not 7 days, after the insult. Thus, we speculated that two doses were insufficient to maintain neuroprotection for long periods. Therefore, we increased the number of doses to six over 3 days. In a previous piglet study, Nii et al. [16] reported that intravenous edaravone 3 mg/kg plus 1.5 mg/kg/h for 5.5 h after an insult (total, 11.5 mg/kg) protected against brain injury. Positive results were obtained in 7-day-old rats given two daily intraperitoneal injections of 9 mg/kg [26] and 9-day-old Harlequin mice given two intraperitoneal injections of 10 mg/kg [27]. However, we administered 3 mg/kg every 12 h for 3 days after the HI insult. Thus, the dose in this study might not be enough to maintain the neuroprotective effects and our discrepant results might be due to this dose difference.

In addition, referring to the administration of edaravone, a few reports have identified adverse effects in patients, such as acute renal failure and fulminate hepatitis. Given these adverse

effects, the use of edaravone at extremely high doses might not be clinically useful. In normal adult humans, edaravone is well tolerated following single or multiple doses. Edaravone shows a short half-life, in the range of 0.15–5.16 h, depending on the administration regimen; otherwise, there is no evidence for the precise half-life of edaravone in the neonate. Future work is required to determine the half-life of edaravone to reveal the optimal administration methods for the newborn piglet.

The second possibility is that edaravone might not be effective under TH conditions. We expected that edaravone plus TH would show more additive neuroprotective effects than TH alone. However, in this study, TH alone clearly improved neurological outcomes compared with edaravone plus TH. This finding is compatible with those of many previous studies. Many previous animal and clinical reports suggested that TH had neuroprotective effects in neonatal HIE [28, 29]. Edaravone has been shown to have neuroprotective properties in animal studies such as those of neonatal rats and young piglets under normothermia. Animal studies have proven that edaravone is mainly excreted into the urine after conjugation to glucuronide or sulfate in the liver [30]. In humans, glucuronide conjugation is the primary pathway and Ma et al. observed that edaravone glucuronidation in human liver and kidney microsomes exhibited biphasic kinetics, as in the animal studies [31, 32]. The half-life of edaravone was found to be 2.5 h under both single- and multiple-dosing regimens, suggesting rapid elimination. Previous work reported that TH

demonstrates a decrease in metabolic activity followed by a return to normal activity after rewarming [33]. Renal tubular secretion and liver conjugation would be suppressed during TH [33, 34]. From the perspective of pharmacokinetics, the edaravone concentration was generally expected to be higher than during normothermia. However, edaravone showed no additive neuroprotection during TH.

We speculate that the distribution of edaravone to the brain might be lower due to decreased cerebral blood flow during TH. The TH and TH+EV groups showed a greater decrease in CBV than the NT group (Fig 3). Therefore, CBF would decrease in both groups. Hence, TH has the potential to reduce the effectiveness of edaravone.

A limitation of this work is that we could not explore the mechanism explaining why edaravone could not improve neurological outcomes using our limited data analysis. Recent mechanistic research has suggested that edaravone suppresses delayed neuronal death [35], counteracts microglia-induced neurotoxicity [36] and reduces long-term inflammation, not only via antioxidant activity. In future work, we will reveal the mechanisms involved by examining cytokine profiles or other histological immunostains. Furthermore, our experimental population was small. This may be a possible reason why edaravone had no additional neuroprotective effect in this study. We intend to increase the size of the population in future studies investigating the efficacy of edaravone.

Conclusion

Intravenous administration of edaravone plus TH for brain injury did not improve neurological outcomes in the newborn piglet. Other methods of drug administration are necessary to address the efficacy of edaravone plus TH for brain injury in newborns.

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Statement of Ethics

Our IACUC is the Animal Care and Use Committee for Kagawa University; the approval number is 15070-1. All piglets were initially anaesthetised with 1–2% isoflurane in air using a facemask. After the umbilical vein was cannulated, we administered fentanyl citrate to ameliorate suffering during the experiment. On day 5, all piglets were initially anaesthetised with enough isoflurane to ameliorate suffering and then the brain of each animal was perfused.

Disclosure Statement

The authors have declared that no competing interests exist.

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Author contributions

S.Y., S.N. and T.K. were involved in the initial study design and wrote the main text. S.Y., S.N., Y.H., M.S., S.K. and T.K obtained the necessary financial support for this project and provided study materials. S.Y., S.N., M.N., W.J., Y.H., T.W., Y.N., T.M. and T.I. carried out the animal experiments and recorded the neurological scores. S.Y., S.N., T.M. and M.U. performed the histopathological experiments and scoring. A.M., M.S., S.K. and S.Y. performed the data and statistical analyses. All members drafted the article and revised it critically.

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Figure legends

Fig 1. Changes over time in the mean arterial blood pressure (MABP) in the three groups (NT, n = 8; TH, n = 7; TH+EV, n = 6) at baseline, end of insult (0 h) and 3, 6, 12 and 24 h after the insult.

MABP was higher within 3 h after the insult in the TH (1 h, $p < 0.05$ vs NT) and TH+EV groups compared with the NT group. Values are shown as the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. NT.

Fig 2. Changes over time in heart rate (HR) in the three groups (NT, n = 8; TH, n = 7; TH+EV, n = 6) at baseline, end of insult (0 h) and 3, 6, 12 and 24 h after the insult.

HR was significantly lower in the TH (1 h) and TH+EV (1, 3, 6 and 12 h) groups than in the NT group after the insult. Values are shown as the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. NT.

Fig 3. Changes in cerebral blood volume (CBV) (ml/100g brain) in the three groups (NT, n = 7; TH, n = 7; TH+EV, n = 5) at the end of insult (0 h) and 3, 6, 12 and 24 h after the insult. (Supplementary fig)

Values are shown as the mean ± SEM. One piglet in the NT group and one in the TH+EV group were missing data.

Fig. 4. Changes in cerebral Hb oxygen saturation (ScO_2) (%) in the three groups (NT, n = 7; TH, n = 7; TH+EV, n = 5) at the end of insult (0 h) and 3, 6, 12 and 24 h after the insult. (Supplementary fig)

Values are shown as the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ vs the NT group. One piglet in the NT group and one in the TH+EV group had missing data.

Fig. 5. Time course of the aEEG score in the three groups (NT, n = 8; TH, n = 6; TH+EV, n = 6) at the end of insult (0 h) and 3, 6, 12 and 24 h after the insult. (Supplementary fig)

Values are shown as the mean ± SEM. One piglet in the TH group was missing data.

Fig. 6. Neurological scores on day 5 after the insult in the three groups (NT, n = 8; TH, n = 7; TH+EV, n = 6).

The neurological score on day 5 was significantly higher in the TH group than in the NT group ($p < 0.05$). Values are shown as the mean ± SD.

Fig. 7. Histological scores on day 5 after the insult among the NT, TH and TH+EV groups in cortical grey matter (GM) (a), subcortical white matter (WM) (b), CA1 of the hippocampus (HIPP) (c) and the cerebellum (CERE) (d) (mean ± SD).

In the GM and WM, the TH and TH+EV groups showed no significantly less damage in the cortex than the NT group (a, b). The TH+EV group showed less damage compared with the other groups, although there was no significant difference in the hippocampus (c). In the cerebellum, there were no differences in damage among the three groups (d).

Fig. 8. Representative images of haematoxylin and eosin (H&E) staining in the GM (a–c), WM (d–f), HIPP (g–i) and CERE (j–l) of the NT (a, d, g, j), TH (b, e, h, k) and TH+EV (c, f, i, l) groups. (Supplementary fig) Scale bars indicate 100 μm.

Supplemental information

Methods

Near-infrared time-resolved spectroscopy and analysis

We used a portable three-wavelength near-infrared TRS system (TRS-21; Hamamatsu Photonics K.K., Hamamatsu, Japan) and attached a probe to the head of each piglet. The light emitter and detector optodes were positioned in the parietal region at an interoptode distance of 30 mm. The TRS system at our institution uses a time-correlated single-photon-counting technique for detection and has been described in detail elsewhere [19]. Oxyhaemoglobin and deoxyhaemoglobin concentrations were calculated from their absorption coefficients using equations that assume background absorption is due to only 85% (by volume) water. Total cerebral haemoglobin concentration, ScO_2 and CBV were calculated as described previously [20].

Amplitude-integrated electroencephalography monitoring

For amplitude-integrated electroencephalography (aEEG), we used the Nicolet One (Cardinal Health, Inc., Dublin, OH, USA). With this device, the signal is displayed on a semi-logarithmic scale at low speed (6 cm/h). In this study, measurements were recorded at 1-s intervals. The gold-plated electrode discs were placed at the P3 and P4 positions (corresponding to the left and right parietal areas on the head). LAEEG was defined as the maximum amplitude < 5 μV; the

aEEG pattern was evaluated using the aEEG scoring system developed by Peeters-Scholte et al. [21], which ranges from 4 (normal) to 0 (worst) and integrates the aEEG background pattern with seizure activity. Five distinct patterns can be discriminated in the aEEG backgrounds of human term neonates: flat trace, burst suppression, continuous low voltage, discontinuous normal voltage and continuous normal voltage. Seizure activity was categorised as follows: no seizures present; an irregular, spiky aEEG (confirmed as multifocal epilepsy on a standard EEG recording); single seizures (less than 3 seizures/h, with a maximal duration of 10 min each); repetitive seizures (more than 3 seizures/h); and status epilepticus (saw-tooth pattern).

Hypoxic insult

Hypoxia was induced by reducing the inspired oxygen concentration of the ventilator to 4% after at least 120 min of stabilisation from the initial anaesthetic induction. If required to obtain a low-amplitude aEEG pattern (LAEEG; maximum amplitude < 5 μV) using a Nicolet One (Cardinal Health, Inc.), the inspired oxygen concentration was further reduced to 2%. From the beginning of the LAEEG, the insult was continued for 30 min. For the final 10 min of the 30-min insult, if mean arterial blood pressure (MABP) exceeded 70% of baseline, hypotension was induced by decreasing the FiO_2 until MABP declined to below 70% of baseline. During the HI insult, CBV was continuously monitored using TRS; we calculated the change in CBV as described previously [17]. When CBV fell to 30% of the height between the peak and baseline during the final 10 min

of the insult, resuscitation was started. Otherwise, if the change in CBV remained above it, the HI lasted for 10 min.

Histology

Coronal blocks of cortical grey matter (GM), subcortical white matter (WM), the CA-1 area of the hippocampus (HIPP) and the cerebellum (CERE) were embedded in paraffin and cut with a microtome at 4 μm. The sections were stained with haematoxylin and eosin (H&E) and three areas of the cortical GM and subcortical WM were studied: the dorsal cortex, sensorimotor cortex and mid-temporal cortex [24, 25]. In each area, three regions of interest (ROIs) were randomly sampled. In the HIPP, three adjacent ROIs were selected from the CA-1 area. In the CERE, three adjacent ROIs were selected from the Purkinje layers. Samples were examined by investigators (M.U., S.N., S.Y. and Y.H.) who were blinded to all clinical information.

The extent of damage in each of the five regions was graded in 0.5-unit intervals on a nine-step scale that ranged from 0.0 to 4.0. Grade 0 indicated no damage; grade 1 indicated that ≤ 10% of the area was affected with morphological changes that included individual necrotic neurons and small patchy, complete or incomplete infarcts; grade 2 indicated that 20–30% of the area was affected with partly confluent incomplete or complete infarcts; grade 3 indicated that 40–60% of the area was affected with large confluent and complete infarcts; and grade 4 indicated that > 75% of the area was affected with neuronal necrosis in the hippocampus and total disintegration of the

cortex.

Results

After resuscitation, arterial blood pH (pHa), partial pressure of arterial blood O_2 (PaO_2) and partial pressure of arterial blood CO_2 ($PaCO_2$) recovered to baseline values within 1 h in each group (Table 1). Some significant differences in base excess were detected among the groups at 6 and 12 h after resuscitation, but these differences were not considered physiologically significant. Hypothermia produced a transient but highly variable hyperglycaemic response at 6, 12 and 24 h after the insult. In addition, the TH-EV group showed more hyperglycaemia than the TH group.

We confirmed the protocol using TRS and monitored and recorded CBV and ScO_2 during and after the HI insult. However, one animal from each of the NT and TH+EV groups had missing data after the HI insult. As in our previous study [22], we calculated the change in CBV and ScO_2 from the end of the insult to evaluate how much the parameters changed after the insult. The TH and TH+EV groups showed more of a decrease in CBV than the NT group after the insult (Fig. 3). In ScO_2, the change in ScO_2 from the end of insult was significantly smaller in the TH+EV group ($p < 0.05$, at 6 h) and TH group ($p < 0.05$ at 12 h, $p < 0.01$ at 24 h) than in the NT group after the insult (Fig. 4). Hence, there was no significant difference in the change in CBV and ScO_2 after the insult between the TH and TH+EV groups. For the aEEG score, we did not find

significant differences among the three groups (Fig. 5).

Histopathological score [mean (SD), NT, TH, TH+EV group (Fig.7)]

GM: 2.7 (1.4), 2.0 (1.2), 1.9 (1.7) (Fig 7a); WM: 3.1 (1.2), 2.4 (1.8),2.2 (1.7) (Fig 7b); HIPP: 2.4 (1.8), 2.2 (1.6),1.1 (1.7) (Fig 7c); CERE: 1.1 (1.7) 1.5 (1.7), 1.4 (1.8) (Fig 7d)

Table 1. Mean (SD) values of arterial pHa, $PaCO_2$, PaO_2, BE, blood glucose and lactate before, at the end of (0 h) and 1, 3, 6, 12 and 24 h after hypoxic-ischaemic insult in the three groups
BE, base excess; TH, therapeutic hypothermia; TH+EV, TH plus edaravone; NT, normothermia. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. NT, $^{\#\#}p < 0.01$ vs. TH+EV

Parameters	Group	Baseline	0	1	3	6	12	24
pHa	NT	7.42 (0.05)	6.85 (0.10)	7.30 (0.06)	7.48 (0.03)	7.46 (0.05)	7.46 (0.05)	7.50 (0.06)
	TH	7.42 (0.11)	6.79 (0.11)	7.22 (0.12)	7.42 (0.10)	7.44 (0.05)	7.44 (0.06)	7.42 (0.03)
	TH+EV	7.45 (0.07)	6.85 (0.12)	7.20 (0.06)*	7.40 (0.08)	7.39 (0.04)	7.45 (0.08)	7.44 (0.06)
$PaCO_2$ (mmHg)	NT	46.9 (4.9)	34.5 (11.3)	42.2 (6.6)	42.5 (4.14)	47.5 (6.4)	45.1 (5.0)	39.3 (3.3)
	TH	43.3 (12.5)	42.5 (12.9)##	42.2 (8.7)	41.5 (8.0)	42.2 (8.0)	41.1 (7.7)	37.4 (5.5)
	TH+EV	40.1 (6.4)	29.5 (8.6)	44.2 (5.3)	41.0 (8.0)	42.2 (7.4)	40.0 (5.8)	37.6 (6.0)
PaO_2 (mmHg)	NT	89.6 (11.5)	21.0 (11.9)	94.5 (28.4)	88.0 (18.1)	89.2 (10.0)	85.8 (15.2)	89.7 (15.1)
	TH	99.4 (15.4)	19.3 (7.2)	110.3 (20.5)	81.4 (21.2)	81.7 (25.3)	81.2 (25.7)	78.6 (22.0)
	TH+EV	96.8 (22.8)	20.2 (5.8)	108.1 (22.8)	86.0 (14.8)	81.5 (13.4)	99.4 (20.7)	110.5 (34.4) **
BE (mmol/mL)	NT	5.8 (2.4)	−26.0 (5.0)	−5.5 (3.9)	7.8 (1.6)	8.6 (1.6)	7.2 (3.4)	6.8 (2.7)
	TH	2.9 (3.5)	−27.6 (3.5)	−10.0 (4.9)*	1.8 (4.2)**	4.2 (3.2)*	3.2 (3.4)	0.7 (3.9)***
	TH+EV	4.2 (3.1)	−26.0(2.4)	−10.1 (2.9)*	2.6 (3.1)*	4.6 (2.5)*	3.5 (3.3)	1.0 (4.2)*
Blood glucose (mg/dL)	NT	146.0 (19.4)	253.5 (73.2)	237.0 (57.0)	218.1 (65.7)	183.9 (56.8)	195.5 (72.6)	115.6 (40.1)
	TH	152.4 (42.3)	226.6 (112.1)	259.4 (75.1)	261.0(64.9)	229.0 (71.4)	236.4 (66.2)	196.3 (53.4)
	TH+EV	159.3 (34.7)	296.8 (82.0)	294.5 (76.9)	311.8 (85.7)*	298.0 (55.4)**	293.3 (85.9)*	186.2 (58.3)
Lactate (mg/dL)	NT	18.0 (4.4)	227.1 (28.0)	122.5 (30.3)	37.5 (11.6)	27.8 (9.9)	36.9 (11.8)	34.4 ± 10.3
	TH	19.7 (9.5)	218.7 (22.3)	124.9 (28.9)	58.9 (36.5)	34.1 (16.4)	41.6(15.2)	48.1 (12.5)
	TH+EV	21.7 (10.9)	224.8 (17.9)	136.3 (16.5)	61.7 (18.7)	39.7 (18.2)	45.3 (20.5)	45.1 (12.2)

Table 2. Summary of edaravone therapies in neonatal animal models after HI insult
i.p., intraperitoneally; i.v., intravenously

	Animal	Route	Dosage and duration	Outcomes
Ikeda et al., 2002[12]	7-day-old rat	i.p.	3, 6 and 9 mg/kg, 2 doses (second given 30 min after the first one)	Dose-dependent effectiveness (most effective with 9 mg/kg)
Yasuoka et al., 2004[10]	7-day-old rat	i.p.	3 mg/kg every 12 h until animal sacrifice (24 h, 48 h and 7 days)	Repeated injection reduced both necrosis and apoptosis
Noor et al., 2007[11]	7-day-old rat	i.p	9 mg/kg every 24 h until day 2, 5 or 10	Two-day treatment with EV showed the best effect
Takizawa et al., 2009[9]	7-day-old rat	i.p	9 mg/kg every 24 h until animal sacrifice	Brain damage reduced 48 h after insult
Ni et al., 2011[13]	3–7-day-old piglet	i.v.	3 mg/kg followed by 1.5 mg/kg/h for 5.5 h	Partial neuroprotection in striatum

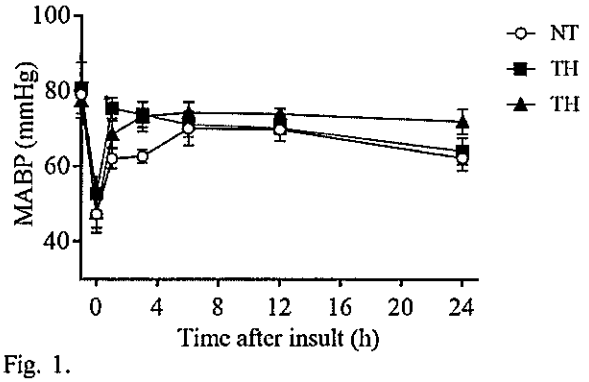
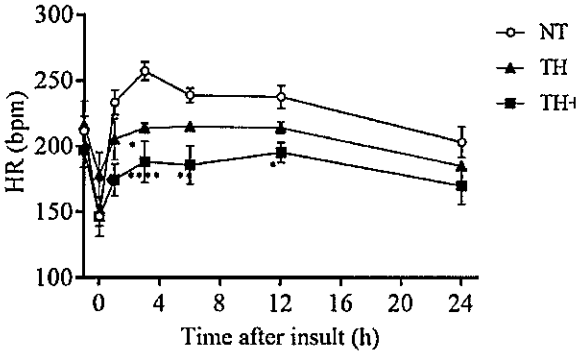


Fig. 1.



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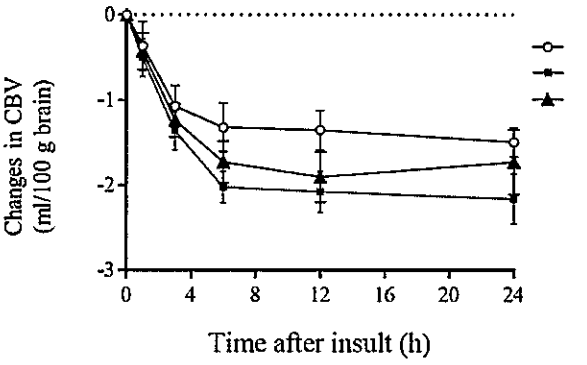
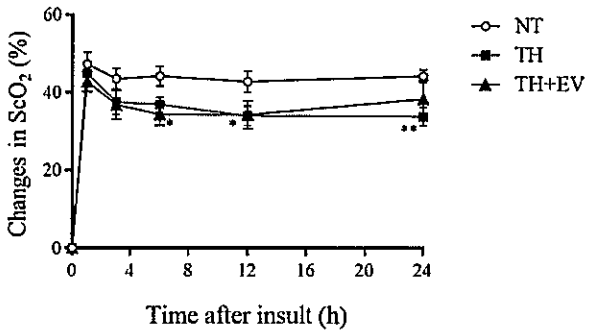


Fig. 3.



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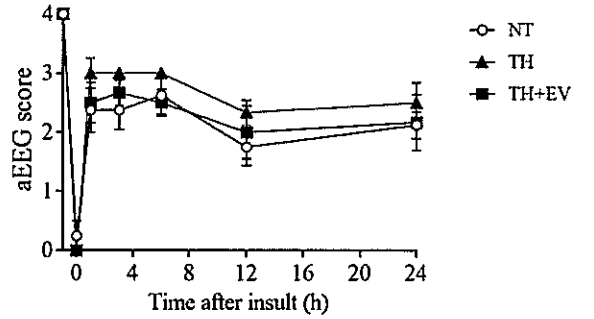

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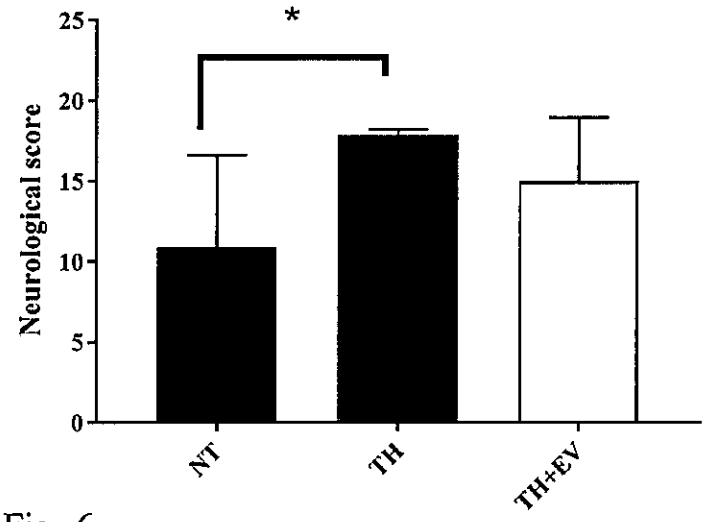
*
25
20
15
10
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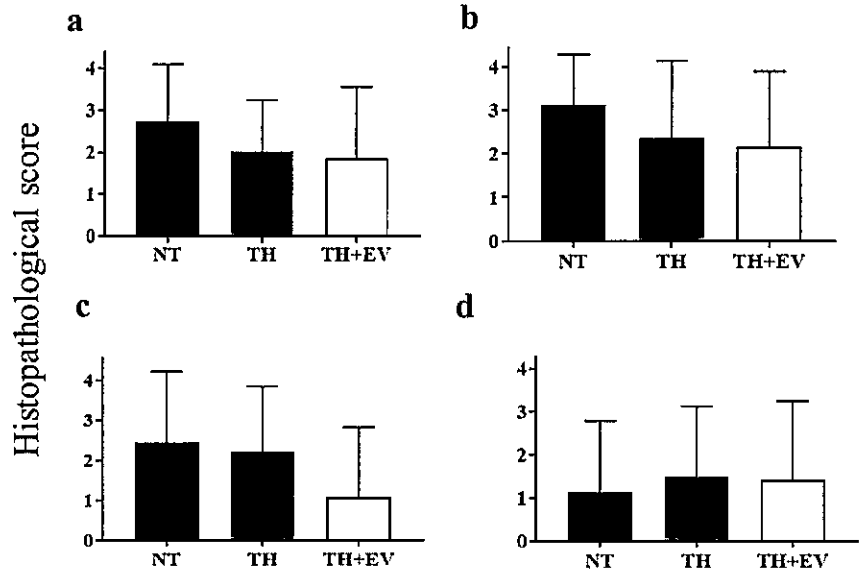
Neurological score
NT
TH
TH+EV

Fig. 6.


a
b
c
d
Histopathological score
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3
2
1
0
NT
TH
TH+EV

Fig. 7.

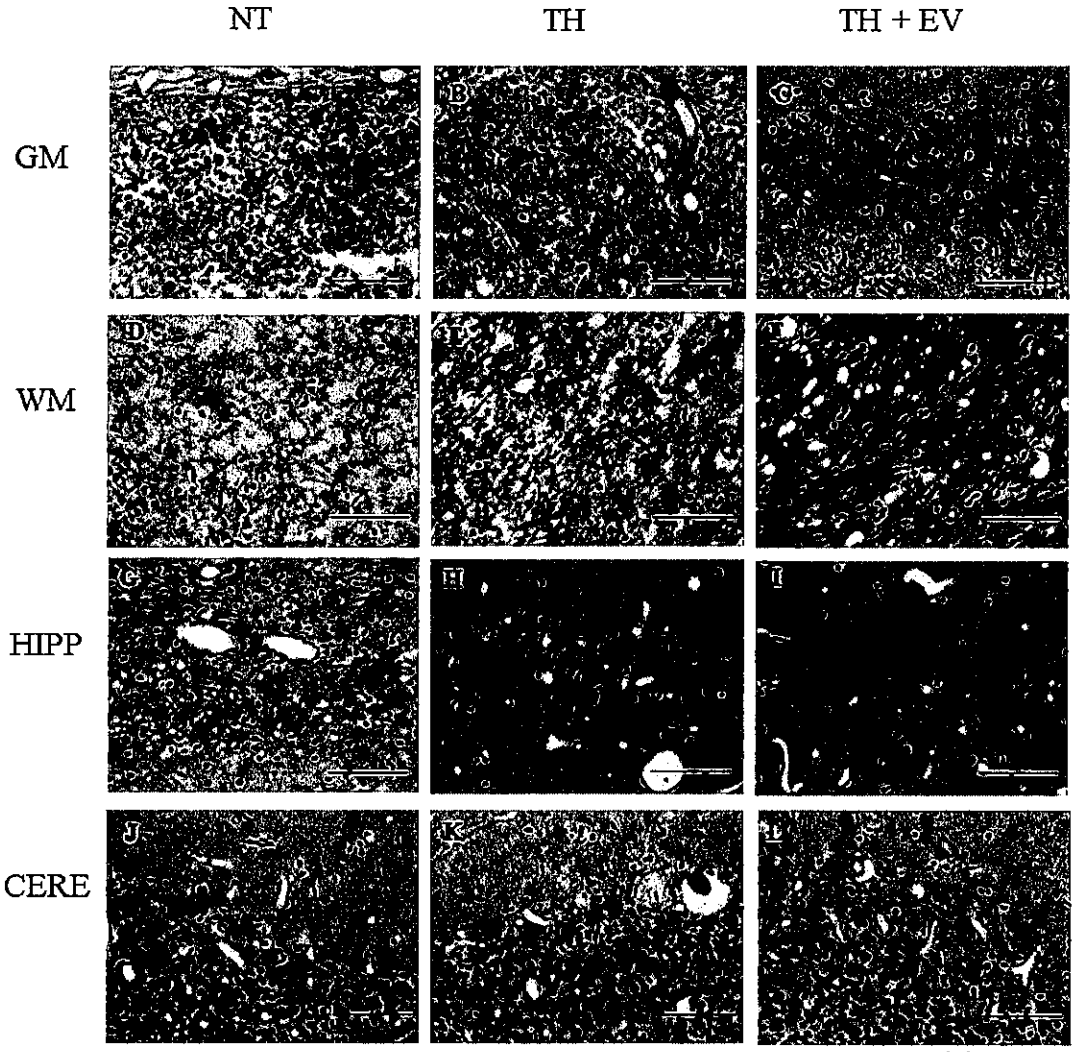

Fig.8.