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Research article

A new Hypoxic Ischemic Encephalopathy model in neonatal rats

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ABSTRACT

Background: Hypoxic-Ischemic Encephalopathy (HIE) occurs when an infant's brain does not receive adequate blood and oxygen supply, resulting in ischemic and hypoxic brain damage during delivery. Currently, supportive care and hypothermia have been the standard treatment for HIE. However, there are still a 20% mortality and most of the survivors are associated with significant neurodevelopmental disability. HIE animal model was first established by Vannucci et al., in 1981, and has been used extensively to explore the mechanisms of brain damage and its potential treatment. The Vannucci model involves the unilateral common carotid artery occlusion followed by 90 min hypoxia (8% oxygen). The purpose of this study is to define and validate a modified HIE model which mimics closely that of the human neonatal HIE.

Method: The classic Vannucci HIE model occludes one common carotid artery followed by 90 min hypoxia. In the new model, common carotid arteries were occluded bilaterally followed by breathing 8% oxygen in a hypoxic chamber for 90, 60 and 30 min, followed by the release of the common carotid artery ligatures, mimicking a reperfusion.

Result: We studied 110 neonatal rats in detail, following the modified in comparison with the classical Vannucci models. The classical Vannucci model has a consistent surgical mortality of 18% and the new modified models have a 20%–46%. While mortality depended on the duration of hypoxia, fifty-two animals survived for behavioral assessments and standard histology. The modified HIE model with 60 min of transient carotid occlusion is associated with a moderate brain damage, and has a 30% surgical mortality. This modified experimental model is regarded closer to the human situation than the classical Vannucci model.

1. Introduction

Hypoxic-Ischemic Encephalopathy (HIE) is a leading cause of neonatal neurologic disabilities, including cerebral palsy, mental retardation, cognitive disorders and epilepsy. The incidence of HIE is about 4 per 1000 live birth (Kurinczuk et al., 2010; Evans et al., 2001; Badawi et al., 1998). The mortality of neonates with HIE is nearly 30% and at least 25% of survivors will live with permanent neurological deficits (Selway, 2010). The current standard treatment for moderate to severe HIE is maximum medical support and hypothermia, This has been shown to reduce mortality and disability as well as to improve neurocognitive function of HIE survivors (Jacobs et al., 2007; Shankaran et al., 2005; Guillet et al., 2012). However, despite the benefits of hypothermia treatment, nearly 20% of HIE babies die, and 20% of the survivors live with long-term mental and physical function impairments. Therefore, it is critical to identify and establish effective therapeutic strategies to minimize brain injury in HIE neonates. In order to explore the mechanisms and the potential treatment of this disease, an appropriate animal model is needed to mimic the pathological conditions of the human HIE. In 1981, Rice and co-workers developed the Vannucci HIE model that permanently ligates one common carotid artery followed by 8% hypoxia at 37 °C for 3.5 h. This model caused moderate to severe brain damage at 50 h after the surgery (Hamdy et al., 2020; Rice et al., 1981). Many researchers used this model to assess mechanisms of injury and therapies

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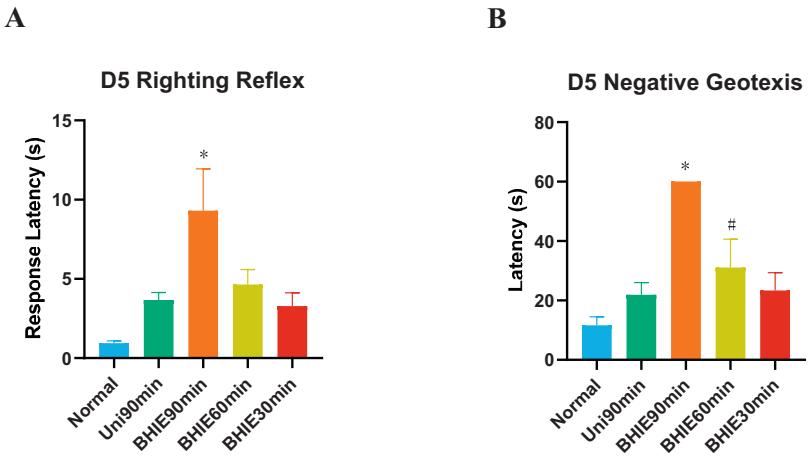


Tukey's multiple comparisons test	Adjusted P Value
Normal vs. Uni90min	0.0008
Normal vs. BHIE90min	<0.0001
Normal vs. BHIE60min	<0.0001
Normal vs. BHIE30min	0.0051
Uni90min vs. BHIE90min	<0.0001
Uni90min vs. BHIE60min	0.5319
Uni90min vs. BHIE30min	0.9711
BHIE90min vs. BHIE60min	<0.0001
BHIE90min vs. BHIE30min	<0.0001
BHIE60min vs. BHIE30min	0.2088

Tukey's multiple comparisons test	Adjusted P Value
Normal vs. Uni90min	0.0004
Normal vs. BHIE90min	<0.0001
Normal vs. BHIE60min	<0.0001
Normal vs. BHIE30min	<0.0001
Uni90min vs. BHIE90min	<0.0001
Uni90min vs. BHIE60min	0.0052
Uni90min vs. BHIE30min	0.9776
BHIE90min vs. BHIE60min	<0.0001
BHIE90min vs. BHIE30min	<0.0001
BHIE60min vs. BHIE30min	0.0253

Figure 1. Day 5 Response latency of (A) righting reflex and (B) negative geotaxis behavior tests. *$p < 0.0001$, BHIE90min vs other groups. #$p = 0.0253$, BHIE60min vs BHIE30min. n = 7, Mean ± SD.

but the model does not mimic three important features of human neonatal HIE. First, the model occluded one carotid artery whereas most human HIE involved occlusion of both carotids. Second, because only one carotid is occluded resulting in only mild ischemia, 3.5 h of hypoxia are required to cause moderate to severe brain damage, longer than most hypoxic situations. Third, the Vannucci model does not have the reperfusion component, which usually occurs in most clinical HIE situations and contributes to further brain damage (Lai and Yang, 2011). We therefore modified this animal model to better mimic the clinical situation of HIE.

2. Materials and methods

2.1. Animal preparation and HIE surgery

The Animal Care and Use Committee of the Chinese University of Hong Kong (CUHK) approved the ethics protocol for the experiments. A total of 110 post-natal Day 7 unsexed Sprague-Dawley rats (10–12g) were used. All the animals were housed under 12h light/12h dark cycle with free access to food and water. Rats were fully anesthetized with isoflurane (3–4% for induction and 1–2% for maintenance). A small incision was made in the middle of the neck. For classical Vannuci HIE model, one side of common carotid artery was doubly occluded by 6–0 silk suture and transected with micro-scissors. For the bilateral temporary occlusion model, both sides of the common carotid artery (CCA) were ligated with 6–0 silk suture. After the incision was closed with sutures, we placed the pups in a hypoxic chamber with 8% oxygen balanced with 92% nitrogen for 90, 60 and 30 min. A heating pad was used to maintain body temperature. After the hypoxic period, the pups anesthetized by isoflurane. We reopen the incision and release the ligatures on both CCA, closed the incision with 6–0 suture, and moved the pups to a recovery box.

2.2. Behavioral tests

On Day 5 after HIE surgery, righting reflex and negative geotaxis behavior were assessed in the animals. To assess righting reflexes, we placed the rats in the supine position and recorded the time required to turn over and place all four paws on the ground; For negative geotaxis, pups were placed on an inclined board (40°) with head down. The time required to turn 180° was recorded with a cut-off time of 60 s. Failure to turn 180° were recorded as 60s (Naik et al., 2015; Lubics et al., 2005).

2.3. Histological analysis

Day 1 and Day 5 after HIE, rats were over anesthetized and perfused through the heart with 4% paraformaldehyde (PFA). We removed the brains and further stored in 4% PFA for 24 h, then embedded in paraffin wax, cut 5μm-thick sections and slides on 0.5mm anterior to the bregma (based on Paxinos and Watson's Atlas) and stained with hematoxylin and eosin (H&E) and TUNEL assay (Roche, Cat#No 11684795910) to study cell death (using Image J). For each animal, 3 slides were chosen and on each side of hemisphere, we captured and analyzed images of the frontal motor-sensory cortex from 2 squares (340 μm × 340 μm) with a fluorescence microscope (Leica Dm2500 fluorescence microscope).

2.4. Statistical analysis

We used GraphPad Prism 8.3.0 for statistical analysis and graphical display of the data. All sets of data were checked in normal distribution. To assess the results of behavioral tests and quantitative analyses of apoptotic cells, we used one-way ANOVA with Tukey's multiple comparisons test. A probability of <0.05 for rejection of the null hypothesis was considered significant.

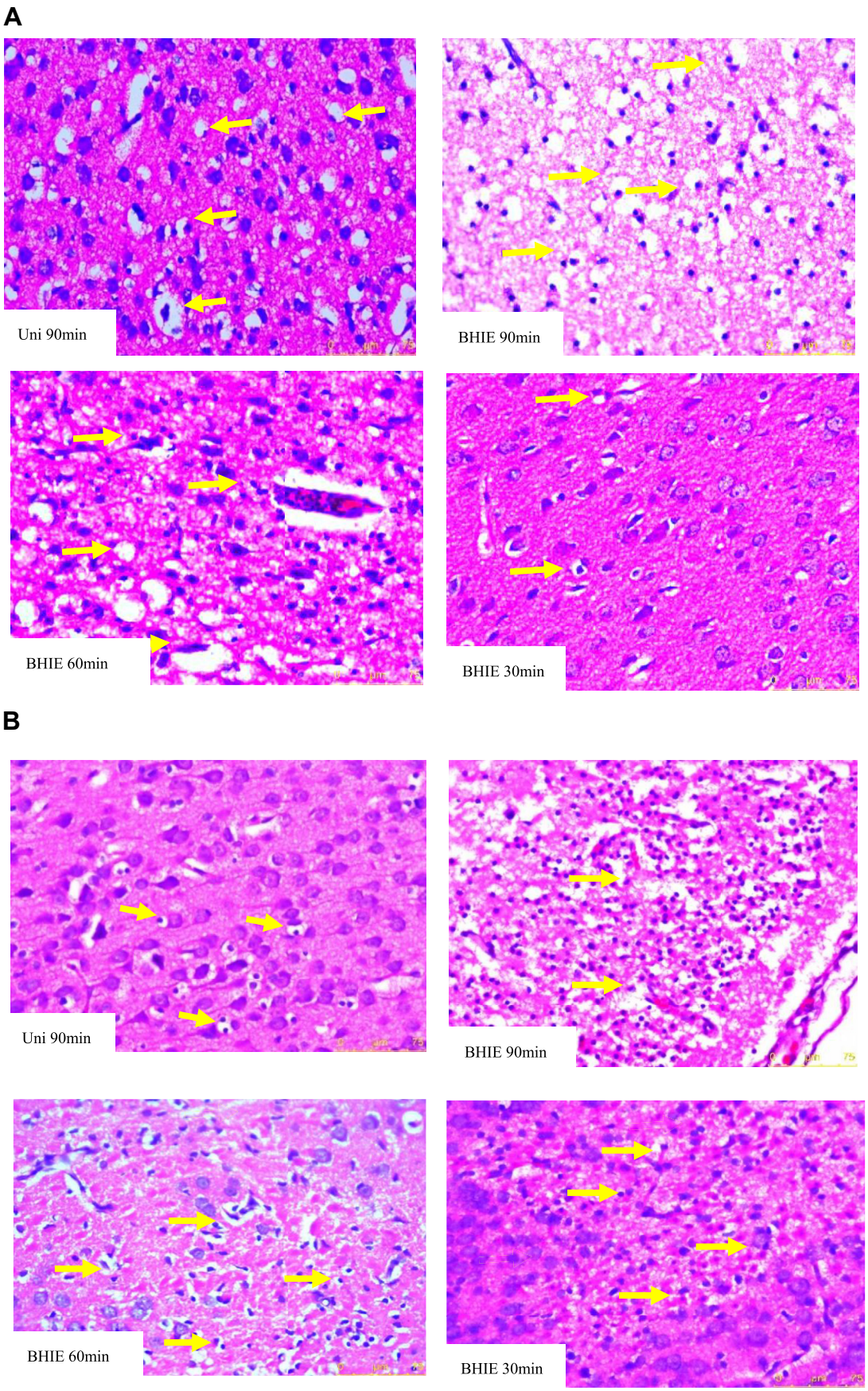
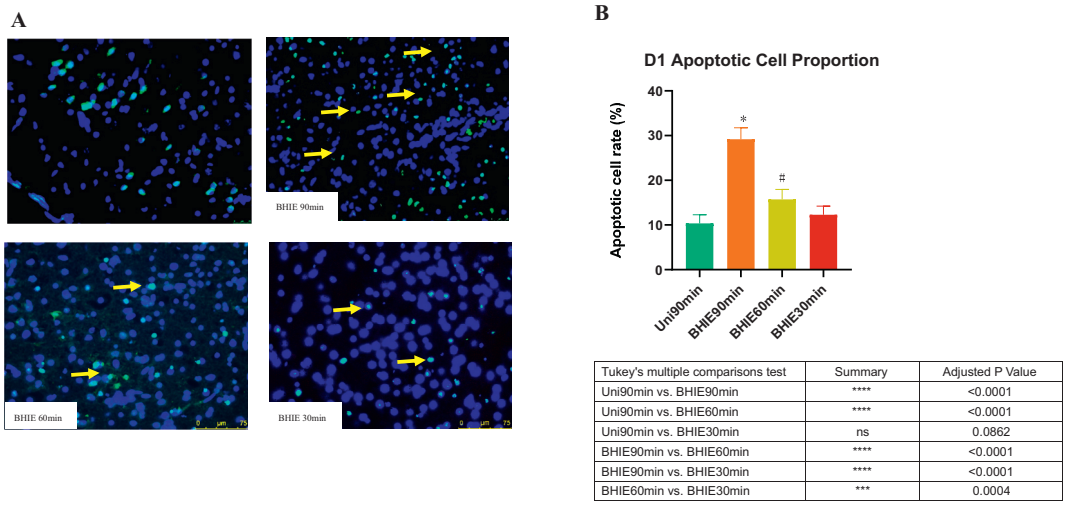
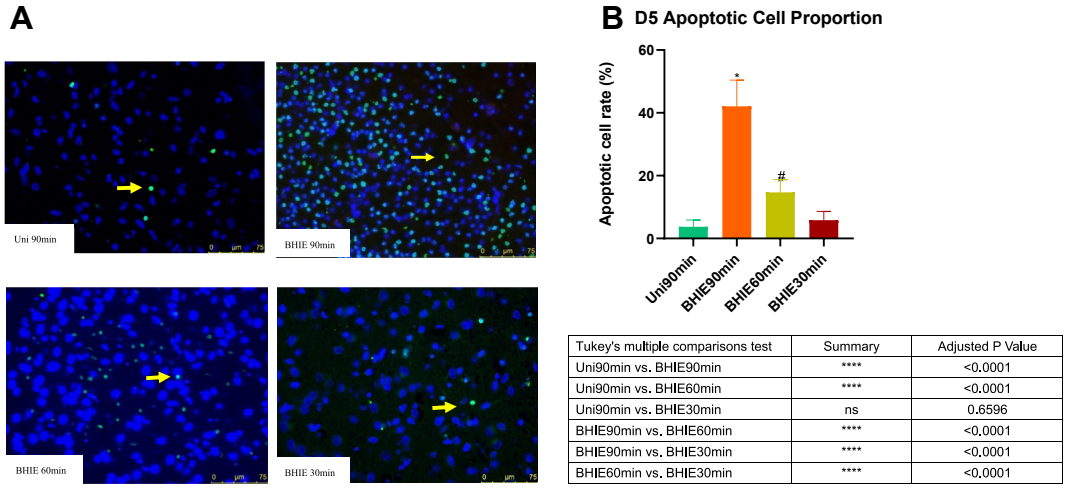


Figure 2. Motor cortex H&E stain on Day 1 (A) and Day (B) after HIE. The yellow arrows illustrate neuronal cell death and brain tissue loss.



Tukey's multiple comparisons test	Summary	Adjusted P Value
Uni90min vs. BHIE90min	****	<0.0001
Uni90min vs. BHIE60min	****	<0.0001
Uni90min vs. BHIE30min	ns	0.0862
BHIE90min vs. BHIE60min	****	<0.0001
BHIE90min vs. BHIE30min	****	<0.0001
BHIE60min vs. BHIE30min	***	0.0004

Figure 3. (A) Apoptotic cells in cortex of unilateral and bilateral 90, 60 and 30 min HIE model on Day 1. The yellow arrows illustrate apoptotic cells. (B) The proportion of apoptotic cell/total cell. *p < 0.0001, BHIE90min vs other groups. #p = 0.0004, BHIE60min vs BHIE30min. n = 7, Mean ± SD.



Tukey's multiple comparisons test	Summary	Adjusted P Value
Uni90min vs. BHIE90min	****	<0.0001
Uni90min vs. BHIE60min	****	<0.0001
Uni90min vs. BHIE30min	ns	0.6596
BHIE90min vs. BHIE60min	****	<0.0001
BHIE90min vs. BHIE30min	****	<0.0001
BHIE60min vs. BHIE30min	****	<0.0001

Figure 4. (A) Apoptotic cells in cortex of unilateral and bilateral 90-, 60- and 30-minute HIE models on Day 5. The yellow arrows illustrate apoptotic cells. (B) The proportion of apoptotic cell/total cell. *p < 0.0001, BHIE90min vs other groups. #p < 0.0001, BHIE60min vs BHIE30min. n = 7, Mean ± SD.

3. Results

3.1. Animal survival rate

A total of 110 unsexed rat pups were assigned to either unilateral carotid occlusion and 90 min of 8% hypoxia (Vannucci model, n = 20) or to our bilateral carotid occlusion HIE model ($n_{90\ minutes} = 13$ and 35 for Day 1 and 5 study; $n_{60\ minutes} = 22$; $n_{30\ minutes} = 20$). Day one mortality after permanent unilateral common carotid occlusion and 90 min of 8% hypoxia was 18%, whereas the Day one mortality of the bilateral HIE model 46%, 27% and 20% at 90, 60 and 30 min of 8% hypoxia. The Day 5 mortality of bilateral HIE model with 90 and 60 min increased to 80% and 33%.

3.2. Behavioral tests

Figure 1A showed in righting reflex tests, the respond latency was significant increase in HIE groups compared with normal rats and in bilateral 90-minute HIE compared with the other groups (ANOVA P value <0.0001; p < 0.0001, one-way ANOVA with Tukey's multiple comparisons test); No significant difference was found between the unilateral HIE rats and the bilateral 60-, 30-minute HIE rats. Figure 1B illustrated that the latency in negative geotaxis test was significant increase in HIE groups compared with normal rats; in bilateral 90-minute HIE differ from all other groups (ANOVA P value < 0.0001; p < 0.0001, one-way ANOVA with Tukey's multiple comparisons test).

3.3. Histological evaluation

On Day 1 and Day 5 after HIE, dead neurons and tissue loss were documented on H and E stained sections after both unilateral permanent carotid occlusion followed by 90 min 8% hypoxia (the Vannucci model) and bilateral temporary carotid occlusion followed by 90, 60 and 30 min 8% hypoxia in the motor-sensory cortices (Figure 2). Apoptotic cells stained by TUNEL/DAPI were present in both unilateral and bilateral HIE models. We used Image J software (https://imagej.nih.gov/ij/) to count apoptotic cells. On Day 1 (Figure 3) and Day 5 (Figure 4) after HIE, bilateral 90-minute HIE rats apoptotic cells were significantly increase compared with other three groups in motor-sensory cortex (ANOVA P value < 0.0001; p < 0.0001, one-way ANOVA with Tukey's multiple comparisons test) and increased in bilateral 60-minute compared with 30-minute HIE rats on Day 5 (ANOVA P value < 0.0001; p < 0.0001, one-way ANOVA with Tukey's multiple comparisons test).

4. Discussion

The postnatal HIE model simulates cerebral hypoxia and ischemia in newborn babies. HIE researchers use rodents, piglets and sheep but rats are by far the most popular HIE model (Roohey et al., 1997). Postnatal Day 7 (P7) rat pups have brain development that is similar to human brain at 32–34 weeks of gestation (Mu et al., 2017; Sengupta 2013).Since Rice and Vannucci established the unilateral carotid artery occlusion HIE model in 1981, many laboratories have used this model to study HIE (Mu et al., 2017). Unfortunately, the Rice-Vanucci model produces variable infarct sizes, ranging from mild and moderate to severe (Cuccione et al., 2016; Liu et al., 2009; Noh et al., 2006; Okusa et al., 2014; Ota et al., 1997), depending on the methods of anesthesia (Chen et al., 2011), body weights (Oakden et al., 2002) and diet (Barks et al., 2017).

In 1992, Schwartz et al. developed a bilateral permanent carotid occlusion plus hypoxia model to produce hippocampus CA1 damage (Schwartz et al., 1992). In 2000, Tomoaki et al. made minor modification of this model to test short-term and long-term learning, memory impairment and sensorimotor function after HIE (Ikeda et al., 2001). In 2019, the Tayla's laboratory gave a single intraperitoneal dose of human umbilical cord blood cells at 24 h after unilateral HIE to assess the long-term behavioral outcomes of this cell therapy (Penny et al., 2019). HIE kills neurons through two mechanisms: necrosis and apoptosis (Northington et al., 2011). Necrosis results from calcium ions entering damaged neurons. Intracellular calcium activity is normally very low (~70 nM) and extracellular calcium is over 10,000 times higher (~1.2 mM). Calcium ions rush into the ischemic and hypoxic cells, binding mitochondrial electron transport mechanisms to disrupt metabolism and generate free radicals. At concentrations exceeding 1 μM, calcium ions activate cytosolic enzymes, including phospholipase, phosphatases, and phosphokinases that break down cell components, causing necrosis. Necrotic cells tend to be swollen and have indistinct intracellular features.

Apoptosis is genetically programmed cell death. During development, apoptosis is the predominant mechanism of cell death in the brain. In neonatal HIE, delayed apoptotic cell death is abundant in the thalamus (Blomgren et al., 2001; Blomgren and Hagberg, 2006; Graham et al., 2004; Northington et al., 2001a, b, c; Zhu et al., 2007) and tends to be delayed by several days and usually peaks at a week, scattered all around the brain and occur most frequently in adult brain.

Reperfusion injury occurs when blood flow is restored to the tissue (Alvarez-Díaz et al., 2007; Katz et al., 2004). Hypoxia and ischemia deprive the brain of oxygen and blood flow, causing loss of energy metabolism, subsequent biochemical changes that damage the cells, causing cell dysfunction and finally cell death (Perlman, 2006). Due to the huge gradient between extracellular and intracellular calcium ions, they rush into cells, with dying cells releasing calcium binding proteins and phosphates, resulting in falling extracellular calcium ionic activity. With reperfusion, extracellular calcium returns to the tissue and initiate additional cellular damage, called reperfusion injury. Reperfusion restores extracellular calcium ions that rush into cells, binding mitochondrial electron transport mechanisms, disrupting brain metabolism, generating free radicals, releasing glutamate, and activating phospholipases, proteases, and phosphatases to cause necrosis of neurons (Young, 1985, 1986). Glutamate release by ischemic neurons (Gücüyener et al., 1999; Johnston, 2001) activates glutamate receptors in surrounding tissues (Zhang et al., 2020), allowing calcium entry to increase concentration of cytosolic calcium and the release of free radicals to damage surviving neurons (Alvarez-Díaz et al., 2007) (Lai and Yang, 2011).

In this study, we did bilateral temporary occlusion of both common carotids and observed behavioral tests impairment of righting reflex and negative geotaxis on Days 1 and 5. Comparing with the unilateral common carotid artery occlusion HIE model, the 90- minute bilateral HIE model had the most severe brain injury and the highest mortality of 80% on Day 5. The apoptotic cell count also showed that there was a significant difference between bilateral 90-minute HIE with the other groups both on Day 1 and Day 5 after HIE. The 60-minute bilateral HIE model had similar mortality as the human HIE babies (30%) as well as a moderate brain damage among three bilateral HIE groups. The apoptotic cell count showed a significant difference between 60 and 30-minute group on Day 5 after HIE. In future studies, we prefer choose the 60-minute bilateral HIE model to study cellular changes and molecular mechanisms of HIE, as well as treatment strategies, targeting on decreasing brain injury, therapeutic window confirmation and improvement of neurological outcomes of HIE.

5. Conclusion

The 60-minute bilateral HIE turns out to be the model of choice for future studies on mechanisms of brain injuries and treatment strategies because:

1. It carries a similar mortality to the human HIE babies of 30%;
2. The negative geotaxis behavioral test impairment at a moderate level better than the 90-minute bilateral and more than the 30-minute bilateral and the classical Vannucci model;
3. The TUNEL assay and apoptosis cell counts of the cerebral sensorimotor cortices indicate that they suffer significantly less brain damage than the 90-minute bilateral but more in comparison with the rat pups with the 30-minute HIE and the classical Vannucci model.

Declarations

Author contribution statement

Hao Lyu: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Dong Ming Sun, Hadi Askarifirouzjaei: Contributed reagents, materials, analysis tools or data.

Chi Ping Ng: Contributed reagents, materials, analysis tools or data; Wrote the paper.

Jun Fan Chen, Yu Zhong He, Sin Yu Lam, Zhi Yuan Zheng: Performed the experiments.

Chi Chiu Wang: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Wise Young, Wai Sang Poon: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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