European Review for Medical and Pharmacological Sciences 2016; 20: 4972-4979

Remote ischemic preconditioning provides neuroprotection: impact on ketamine-induced neuroapoptosis in the developing rat brain

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Abstract. – OBJECTIVE: Previous studies have demonstrated that the commonly used anesthetic ketamine can acutely increase apoptosis and have long-lasting detrimental effects on cognitive function as the animal matures. Remote ischemic preconditioning (RIPC) has been confirmed to have a cerebral protective role in animal models of brain damage. The aim of this study was to investigate whether RIPC can protect the developing brain from anesthetic-induced neurotoxicity.

MATERIALS AND METHODS: To investigate the protective properties of RIPC, 60 new-born Sprague-Dawley (SD) rats were randomly allocated into four groups: ketamine (20 mg/kg was diluted in saline, six times at an interval of 2 hours); RIPC (left hind row ischemia 5 min, reperfusion 5 min, a total of four cycles); ketamine + RIPC: RIPC was induced at postnatal day 5 and rats underwent the same treatment with the ketamine group after 48 hours; and saline (group vehicle). Neuronal apoptosis in the frontal cortex and hippocampal CA1 region was measured 24 h after treatment using immunohistochemistry of cleaved caspase-3. Learning and memory abilities were tested at the age of 60 days by Morris water maze test.

RESULTS: The percentage of cleaved caspase-3 immunohistochemical staining positive cells in the ketamine + RIPC group showed a more marked decline in neuronal apoptosis of the CA1 region than that in the ketamine group ($p < 0.05$) but not in the CA1 region ($p > 0.05$). The mice exposed to RIPC alone showed no difference from the saline-treated mice. Moreover, RIPC significantly reversed the learning and memory deficits observed at 60 days of age.

CONCLUSIONS: Our data indicate that RIPC treatment provides protection against ketamine-induced neuroapoptosis in the frontal cerebral cortex but not in the hippocampal CA1 region in developing rats and attenuates long-term behavioural deficits as the animals mature, suggesting a new possible strategy for neuroprotection.

Key Words:
Remote ischemic preconditioning, Ketamine, Neuroprotection, Apoptosis.

Introduction

Ketamine, a noncompetitive N-methyl-D-aspartate (NMDA) receptor blocker, is an anesthetic commonly used in pediatric patients. Recent studies have demonstrated that prolonged exposure to ketamine causes widespread apoptotic neurodegeneration in the developing animal brain and persistent cognitive deficits as the animal matures[1-4]. Ketamine induces neuroapoptosis in both developing animal brains[1,3,4] and primary cultured neurons[5,6], and results in persistent cognitive deficits[1,4]. Clinical studies also indicate that some learning disabilities and behavioural disturbances in children correlate with anesthetic exposure during surgery before 4 years of age, even in children experiencing only a single exposure to anesthesia[7,8]. Adjuvant neuroprotective strategies that inhibit ketamine-induced neuroapoptosis and persistent cognitive impairments are urgently needed.

Remote ischemic preconditioning (RIPC), a phenomenon in which brief sub lethal ischemic stimuli of one organ protect another organ against a prolonged injurious ischemic insult, has been demonstrated to protect the myocardium and adult brain in animal models[9]. A variety of strategies, in-

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cluding RIPC and administration of vitamin C, lithium, erythropoietin, carnitine, dexmedetomidine, and melatonin have been previously found to have a protective effect on hypoxic-ischemic brain in reperfusion injury model animals. In recent years, research has shown that vitamin C, lithium, erythropoietin, carnitine, dexmedetomidine, and melatonin have potentially damage-mitigating or neuroprotective effects[10]. However, the protective effects of RIPC against ketamine-induced neuroapoptosis in the developing brain and long-term behavioural deficits as animals mature have not been fully explored. RIPC using the hind limb has been demonstrated to be neuroprotective in rodent models of stroke[11]. RIPC may be a new way to prevent and relieve developmental neurotoxicity caused by anesthetics.

Thus, the aim of this study was to evaluate whether treatment with RIPC after injection of ketamine prevented ketamine-induced adverse effects.

Materials and Methods

Animals and Drug Treatment

Animals (Sprague-Dawley P7 rats with a roughly equal number of males and females, weighing 14.6 ± 2.9 g) were obtained from the Laboratory Animal Center of Ningxia Medical University, China. All experiments were approved by the Institutional Animal Care and Use Committee of Ningxia Medical University. The rats were placed in a room with a controlled environment with a 12-/12-h light/dark cycle and allowed access ad libitum to standard rodent chow and tap water. All efforts were made to minimize animal number and suffering. The room temperature was maintained at 21-23°C with 60% relative humidity.

The 60 new-born SD rats were randomly allocated into four groups (n=12): normal control group (group vehicle) and injected intraperitoneally with saline; ketamine: ketamine was diluted in saline (20 mg/kg, six times at an interval of 2 hours); RIPC: (injection of the same dose, with the number of saline, left hind row ischemia 5 min, reperfusion 5 min, a total of four cycles) limb ischemic preconditioning was induced alone, an equal volume of normal saline was given intraperitoneally, the tourniquet remained tied for 5 min followed by 5 min of reperfusion. Each preconditioning session consisted of 4 cycles of ischemia/reperfusion; ketamine + RIPC: RIPC was induced at postnatal day 5 and rats underwent the same treatment with the ketamine group after 48 hours.

Ketamine hydrochloride (RenFu Medical Inc., FuJian, China) treatment was performed as described previously[11]. Animals aged postnatal day 7 were used for ketamine pharmacological interventions (20 mg/kg in saline, intraperitoneally, six times at an interval of 2 hours). Throughout the treatments, the rats were housed in a neonatal incubator to maintain their body temperature and were provided with low-flow oxygen to reduce potential stressors. During anesthesia, the respiratory frequency and skin colour were observed in order to detect apnoea and hypoxia. To avoid differences in weight among the groups, all rat pups were separated from their mothers approximately 250 min after the administration of all of the treatments[12]. The ketamine model was selected to ensure significant neuroapoptosis and long-term behavioural deficits in neonatal rats[12].

Non-invasive limb RIPC was performed as described previously[13]. RIPC was conducted with right hind-limb ischemia for five cycles of 5 min by placing an elastic rubber band tourniquet on the proximal part of the limb, followed by an intervening 5 min of reperfusion, during which the tourniquet was untied. A pulse sensor was placed on the dorsal pedal artery. Asphygmia indicated that the femoral artery was blocked, while a sphygmus indicated that the artery had reperfused. The procedure was performed in four cycles with rats aged postnatal day 5. The animals were kept spontaneously breathing during the preconditioning. The RIPC model was selected based on previous reports that have demonstrated its neuroprotective effects in developing rats[14].

All treatments were made to reduce the numbers and suffering of animals used. The rats were decapitated under deep anesthesia 24 h after the last injection, and the frontal cortex and hippocampal CA1 were isolated for detection of neuronal apoptosis by immunohistochemical analysis for cleaved caspase-3 (n=3). Furthermore, to determine whether RIPC could improve the ketamine-induced cognitive deficits, learning and memory abilities were tested using the Morris water maze when the rats were 60 days of age (n=12).

Immunohistochemical Staining for Cleaved Caspase-3

Immunohistochemistry was performed as we have described previously[15]. In brief, the animals were anesthetized after the last injection and per-

fused with 4% paraformaldehyde through the left cardiac ventricle and ascending aorta. Brain sections were incubated at 4 overnight with an anti-cleaved caspase-3 primary antibody (1:200; monoclonal antibody, Cell Signaling Technology, Beverly, MA, USA), followed by incubation with a secondary antibody (1:200, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 40 min and the avidin-biotinylated peroxidase complex (Vectastain ABC-Kit, Vector Lab, Burlingame, CA, USA) for 40 min. Tissue sections were colorized with diaminobenzidine (DAB, Vector Laboratories, Burlingame, CA, USA). Cleaved caspase-3 positive cells in the frontal cortex and hippocampal CA1 were analyzed with Olympus FV1000 confocal microscope imaging processing and analysis software (Olympus Corporation, Tokyo, Japan). Immunohistochemistry quantitative cell counts were performed previously[16]. The density of cleaved caspase-3 positive cells in the frontal cortex and hippocampal CA1 regions was calculated by dividing the number of caspase-3 positive cells by the area of that brain region.

The Morris Water Maze Test

At 60 days of age, the rats (n=12 in each group) were tested to evaluate their spatial memory and learning abilities using the Morris water maze test. The rats were weighed after the test. The Morris water maze apparatus consists of a hidden platform trial and the spatial probe trial. The rats were trained over two trials each day for six consecutive days to detect the hidden platform. Each rat was released in the water facing the wall of the water maze at one starting position. The time to reach the platform and the length of the swim path were recorded in each trial, with a maximal time limit of 90 s. At the end of the training period, the rats were tested on a spatial probe trial in which the platform was removed, and they were allowed to swim freely for 90 s. Time spent to reach the platform, number of attempts to cross the platform, the ratio of time spent in the target quadrant, and the number of times that the rats crossed the previous location of the platform were recorded. A computerized tracking system was used to record all measurements. The average data from the daily tests were subjected to statistical analysis.

Statistical Analysis

Data analysis was performed using SPSS software version 11.0 (SPSS Inc., Chicago, IL, USA). The data were expressed as the mean ± SD. The mean escape latency and swim distance to find the platform assessed by the Morris water maze test during the training days were analyzed by repeated-measures analysis of variance (ANOVA). The other data were statistically analyzed using one-way ANOVA followed by the Least Significant Test (LSD) for between-group comparisons. The alpha levels were set at 0.05.

Results

RIPC attenuates Ketamine-Induced Apoptosis of Frontal Cortex and Hippocampal Neurons at CA1 Region

To investigate whether RIPC treatment can ameliorate apoptosis of neurons at the hippocampal CA1 region and the frontal cortex in the developing rat brain, we administered RIPC and/or ketamine to P7 rats and neuroapoptosis was determined by cleaved caspase-3 immunohistochemistry. Compared to the vehicle group, a robust degenerative reaction was detected in the frontal cortex of the ketamine-treated group ($F = 66.57$, $p < 0.05$). The administration of RIPC had no influence on the amount of on-going physiological apoptosis ($p > 0.05$). However, the co-administration of RIPC with ketamine significantly ameliorated the neuroapoptosis induced by ketamine exposure ($p < 0.05$) (Figure 1A). A statistically significant difference was noted in the percentage of cleaved caspase-3 immunohistochemical staining positive cells in the hippocampal CA1 region among the four groups ($F = 24.11$, $p < 0.05$). The percentage of cleaved caspase-3 positive cells in the ketamine group (CA1, 39.0 ± 4.95) was not higher than that in the ketamine + RIPC group (CA1, 35.1 ± 3.08) (Figure 1B).

RIPC Mitigates Ketamine-Induced Decline in Learning and Memory Abilities of Rat

At 60 days of age, all of the rats were trained in the Morris water maze to test their learning and memory abilities. The escape latency and swim distance of the rats to find the platform are shown in Figure 2. All animals showed a progressive decline in escape latency and swim distance with training, and the main effects of group ($F = 2.89$, $p < 0.05$, and $F = 2.30$, $p < 0.05$, respectively) and day ($F = 1165.84$, $p < 0.001$, and $F = 869.10$, $p < 0.05$, respectively) were significant. Further analysis showed that rats in the ketamine group took significantly longer to find the

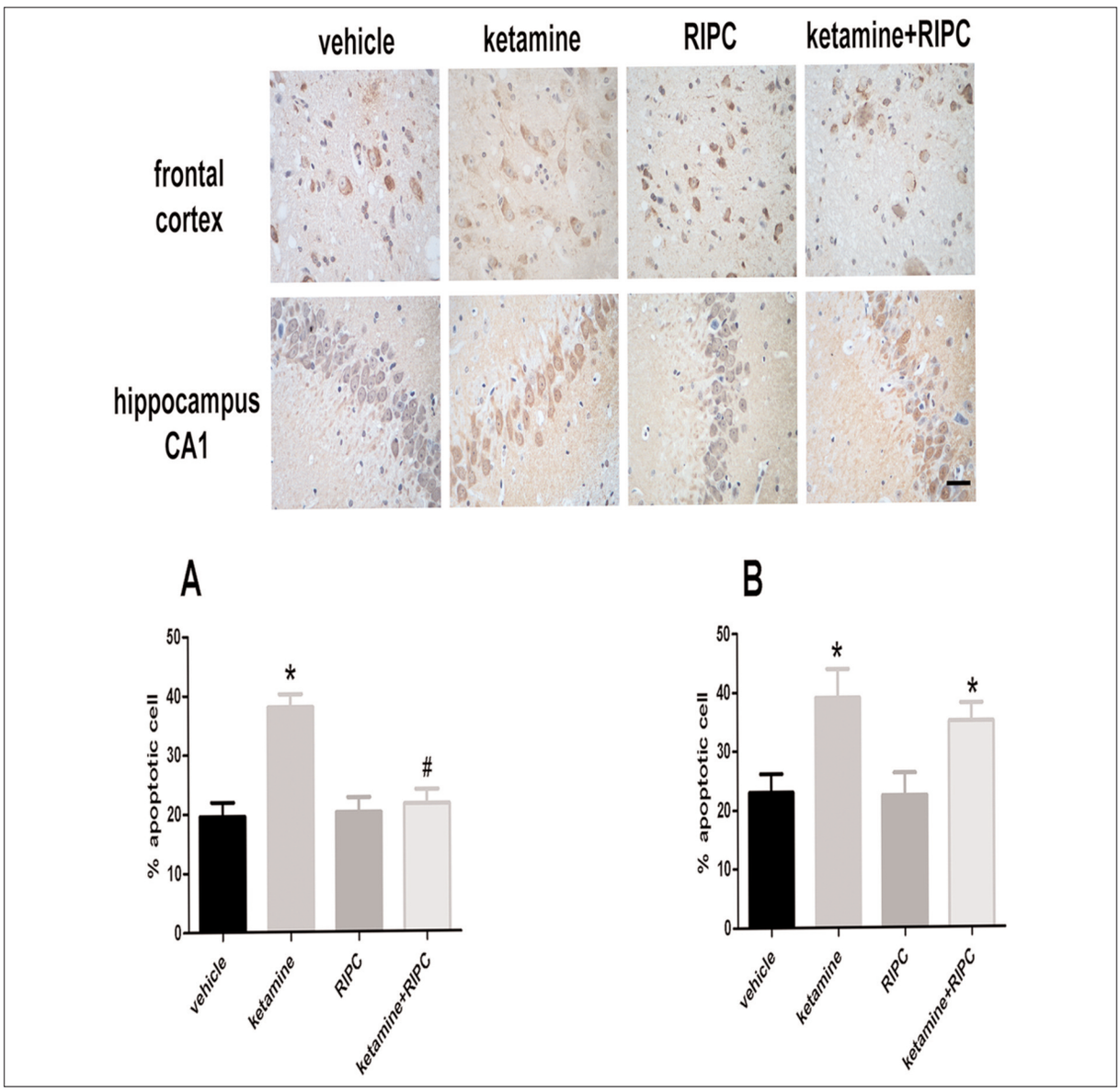

Figure 1. RIPC attenuates ketamine-induced neuroapoptosis. The cleaved caspase-3 immunohistochemical staining assay was performed to visualize the extent of neuroapoptosis. Bar = 50 μm. Cleaved caspase-3 immunohistochemical staining positive cells percentage in frontal cortex ***(A)*** and hippocampal CA1 ***(B)*** region of rats in different groups. The results are shown as the mean ± SD from three independent experiments. $^{*}p < 0.05$ *vs*. the vehicle group. $^{\#}p < 0.05$ *vs*. the group treated with ketamine alone.

hidden platform on the fourth, fifth, and sixth training days compared with the ketamine + RIPC group ($p < 0.05$). No significant difference was observed among the vehicle, RIPC, and ketamine + RIPC groups ($p > 0.05$) (Figure 2A). The main effects of day ($F = 25.90$, $p < 0.001$) and group ($F = 5.90$, $p < 0.05$) were also significant in swim distance. Further analysis showed that rats in the ketamine + RIPC group had an increased swimming distance to find the hidden platform on the fourth, fifth, and sixth training days compared with the other three groups ($p < 0.05$) (Figure 2B).

The rats were subjected to the 90-s probe trial as shown in Figure 2. There were significant differences in time spent to reach the platform initially, the number platform crossings, the ratio of time spent in the target quadrant, and the ratio of swimming distance in the target quadrant among all groups ($F = 9.57$, $p < 0.05$; F =

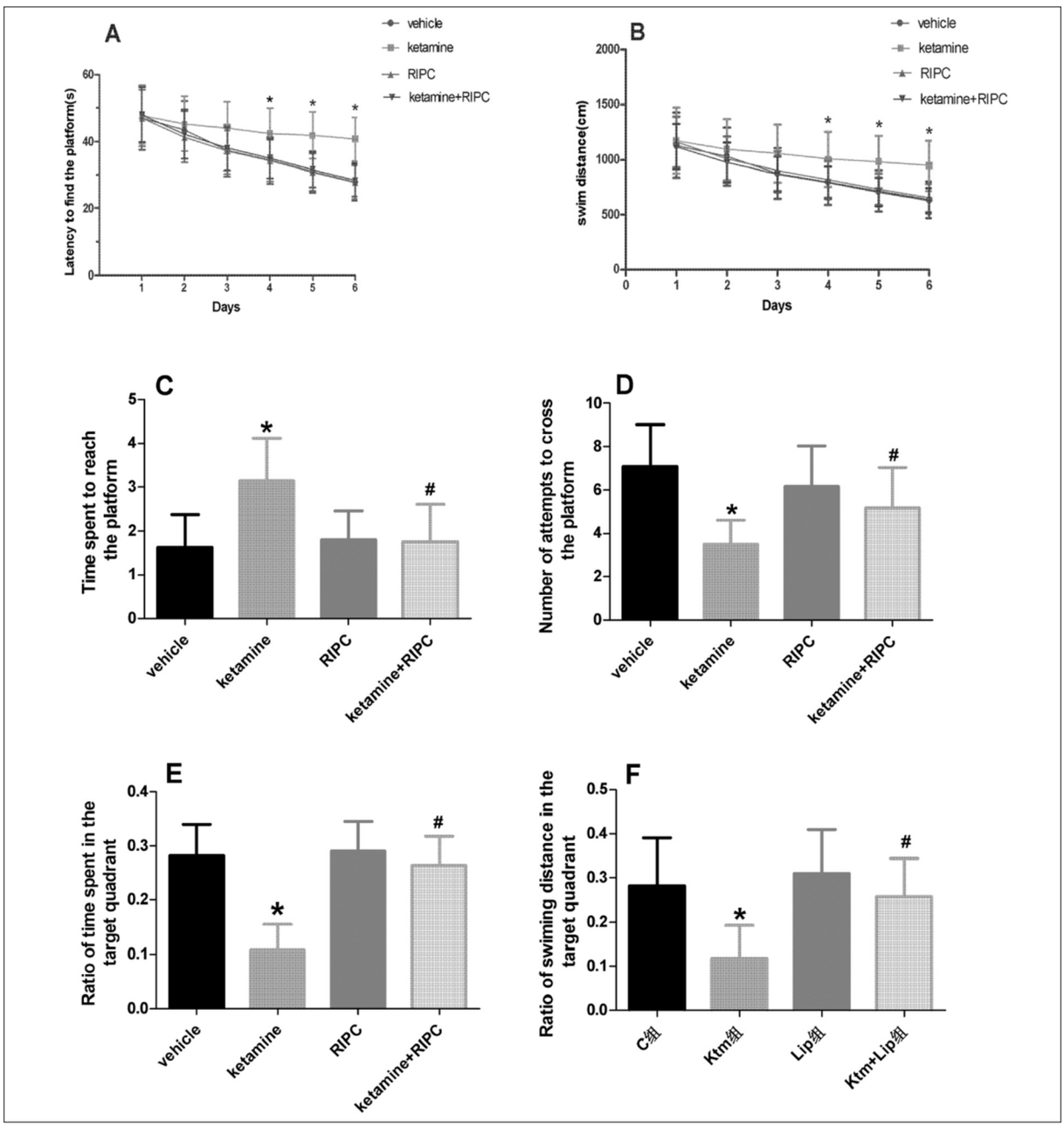

Figure 2. RIPC mitigates the ketamine-induced decline in learning and memory parameters of rats in the Morris water maze test. The values shown are the mean ± SD (n = 12 from each group). Line graph of latency ***(A)*** and swim distances ***(B)*** to find the platform for each trial during the 6 days. Time spent to reach the platform (C) and number of attempts to cross the platform ***(D)*** of rats in different groups. Bar chart of the ratio of time spent ***(E)*** and ratio of swimming distance ***(F)*** in the target quadrant of rats in different groups. $^{*}p < 0.05$, vehicle *vs*. ketamine-treated group. $^{\#}p < 0.05$, ketamine+RIPC *vs*. ketamine-treated group.

9.26, $p < 0.05$; F = 30.67, $p < 0.05$, F = 9.81, $p < 0.05$; respectively). Further analysis showed that the rats treated with ketamine took longer to reach the platform initially than rats in the other three groups ($p < 0.05$). Rats in the ketamine+RIPC group were faster than those in the ketamine-treated group ($p < 0.05$, Figure 2C) in reaching the platform. The number of attempts to cross the platform in the ketamine-treated group was significantly lesser than that of the other three groups ($p < 0.05$, Figure 2D). The results indicated that the number of attempts to cross the platform, the ratio of time spent in the target quadrant, and the ratio of swimming dis-

tance in the target quadrant were significantly less than those of the other three groups ($p < 0.05$, Figure 2D, 2E and 2F, respectively), Moreover, rats in the ketamine+RIPC group spent a considerably longer time than the ketamine-treated group ($p < 0.05$, Figure 2D, 2E, and 2F, respectively).

Discussion

Our data indicate that RIPC treatment provides protection against ketamine-induced neuroapoptosis in the frontal cerebral cortex but not the hippocampal CA1 region in developing rats and attenuates long-term behavioural deficits as the animals mature.

Animals undergoing RIPC have smaller infarcts than animals not exposed to this intervention before stroke[17]. In the brain, RIPC has been shown to provide neuroprotection in contexts of both focal and global ischemia[18,19]. RIPC after hypoxia ischemia in piglets protects cerebral white matter, suggesting that RIPC may have therapeutic potential as a safe neuroprotective strategy for protecting from brain injury and improving outcomes in babies with birth asphyxia[20]. These findings strongly indicate a neuroprotective role of the RIPC. Here, we showed that RIPC did not increase neuronal apoptosis in the frontal cortex and hippocampal CA1 region of neonatal rats, indicating that the RIPC is not neurotoxic to the developing brain. When RIPC was co-administered with ketamine, we observed a markedly reduced rate of neuronal apoptosis in the frontal cortex regions compared with rats given ketamine alone, suggesting that RIPC noticeably attenuates ketamine-induced neuroapoptosis. However, the proportion of cleaved caspase-3 positive cells in the ketamine + RIPC group showed a small but statistically insignificant decline in neuronal apoptosis of the CA1 region of neonatal rats compared with that in the ketamine alone group. This result might be related to the existence of multiple windows of susceptibility for anesthesia-induced neuroapoptosis, with different brain regions being vulnerable at distinct developmental times[21]. In the area of remote preconditioning against brain ischemia, there are only a few studies demonstrating that RIPC reduces delayed neuronal death in the hippocampal CA1 region[22,23]. In another study, Shu et al[24] assessed anesthesia-induced neuroapoptosis caused by xenon in different brain regions with different peak times of synaptogenesis and consequently different vulnerabilities to xenon.

Some studies[25-27] have confirmed the cerebroprotective roles of RIPC in animal models of transient focal cerebral ischemia or whole cerebral ischemia. Recently, it was found that RIPC could improve the ability of spatial learning and memory after focal cerebral ischemia-reperfusion injury[14]. Thus, we further determined whether this neuroprotection offered by RIPC was translatable into functional benefits. Our findings showed that RIPC improved long-term behavioural deficits in maturing rats.

In the current work, we demonstrated that ketamine caused significant apoptosis of neurons in the frontal cortex and hippocampal CA1 region of neonatal rats, which are in the period of brain growth spurt[28]. Consistently, ketamine at clinically relevant doses triggered widespread apoptotic neurodegeneration in the developing rat brain[12] and repeated injections of the ketamine were found to cause neurodegeneration and persistent learning and memory impairment[12]. Thus, we strictly controlled the animal models. However, our methods are also subject to several limitations[29]. Painful stimuli induce a variety of physiological changes, which may or may not affect anesthesia-induced neuroapoptosis[30].

There are no data available on whether RIPC has neuroprotective properties that mitigate anesthetic-induced neurotoxicity in the developing brain. Our team was the first to observe whether RIPC can protect against ketamine-induced neurotoxicity. The current study did not address the mechanism underlying the neuroprotective effects of RIPC on ketamine-induced neurotoxicity in neonatal rats. The underlying mechanisms of RIPC are very complex and not yet fully defined. It has been hypothesized that RIPC predominantly involves systemic multifactorial anti-inflammatory, neuronal, and humoral signalling pathways, which may differ in response to various ischemic stimuli and are likely to interact with each other. Nevertheless, the question remains important and should be addressed by further studies.

Conclusions

Our present data suggest that RIPC might be a new possible strategy for neuroprotection. This finding may have important clinical implications.

The method described by us provides a simple, reliable, and non-invasive protocol for inducing neuroapoptosis. RIPC has great clinical advantages, and may be a potential prevention and treatment strategy for anesthesia-induced cognitive impairment, which may ultimately lead to safer anesthesia care and better postoperative outcomes for children.

Acknowledgements

This research was supported by grants from the National Natural Science Foundation of China (grant 81560225 to Jinhai Meng), the Ningxia Natural Science Foundation (NZ15143 to Jinhai Meng and grant NZ16147 to Wan MA) and the Ningxia Medical University Science Research Fundation (XZ2016008 to Wan MA).

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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