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Original Article

The protective effect of the Rho-kinase inhibitor hydroxyfasudil on propofol-induced hippocampal neuron apoptosis in neonatal rats

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Abstract: Propofol is widely applied for anesthesia induction in pediatric patients. However, accumulating evidence has proved that propofol is neurotoxic to the immature or developing brain. In the present study, we found that hydroxyfasudil, a specific inhibitor of Rho kinase, alleviated the apoptotic neurodegeneration induced by propofol in the developing rat brain. A spatial probe test and Morris water maze test revealed that hydroxyfasudil showed a potential improvement of the tendency towards cognitive impairments induced by propofol. Mechanistically, hydroxyfasudil markedly ameliorated the activation of RhoA and the expression of Rock1, Rock2, Bak, Bax, and Bad induced by propofol and rescued the expression of Bcl2 suppressed by propofol. Our findings suggest that hydroxyfasudil may serve as an effective agent to reduce the propofol-induced neurotoxic effects in pediatric medical procedures.

Keywords: Hydroxyfasudil, anesthesia, propofol, hippocampal neuron, apoptosis

Introduction

Millions of children and pregnant women receive anesthesia for surgery or other medical procedures each year worldwide. Although conventional anesthetics have been proven safe for surgery in adults, more and more studies suggest that some commonly-used anesthetics are neurotoxic to the immature or developing brain in mammals such as rodents and primates [1-3]. Children who have undergone general anesthesia during their early years have a higher incidence of learning impairment [4]. This compelling evidence questions the safety of anesthetics used for pediatric patients.

Propofol, an alkyl phenol derivative, is widely used in pediatric and obstetric anesthesia due to its rapid onset and subsequent recovery, though the use in certain age groups might be off-label [5-9]. Unfortunately, an increasing number of studies suggest that propofol could be neurotoxic to young, developing brains [10-14]. *In vitro*, propofol induces neuroapoptosis, influences neuronal differentiation, and impairs dendritic development [10-14]. *In vivo*, propofol potentiates neuroapoptosis in developing animal brains and even results in long-term cognitive deficits [10-14]. However, the mechanisms underlying propofol-induced neurotoxicity on the developing brain remain largely unknown.

The Rho family GTPases, which play an essential role in neuronal survival and death, are important regulatory molecules that mediate many diverse critical cellular processes in neural development, including dendritic arborization, spine morphogenesis, growth cone development, and axon guidance [15-17]. Previous studies have shown that the Rho-kinase (ROCK) inhibitor could protect against excitotoxicity- and ischemia-induced neuronal death [16]. Fasudil hydrochloride (hydrofasudil), a potent rho kinase (ROCK) inhibitor, can significantly improve the learning and memory performance in aged rats and rats with chronic cerebral ischemia [18-21]. In clinical practice, hydrofasudil could markedly improve the clinical outcomes of subarachnoid hemorrhage patients by reducing the occurrence of cerebral vasospasm and cerebral infarction in patients [22, 23]. However, the protective effects of fasudil against propo-

fol-induced neuroapoptosis *in vivo* and long-term behavioral deficits as animals mature have not fully been explored.

In this study, we found that hydroxyfasudil, a specific inhibitor of Rho kinase, alleviated apoptotic neurodegeneration induced by propofol in the developing rat brain. Mechanistically, hydroxyfasudil markedly ameliorated the activation of RhoA and the expression of Rock1, Rock2, Bak, Bax, and Bad induced by propofol and rescued the expression of Bcl2 suppressed by propofol. Our findings suggest that hydroxyfasudil may serve as an effective agent to reduce the propofol-induced neurotoxic effects in pediatric medical procedures.

Materials and methods

Primary cell culture of hippocampal neurons and drug treatment

Primary hippocampal neurons were isolated from the hippocampi of 7-day old SD rat embryos. After washing with a papain-free solution, the hippocampal cells were carefully dissociated by trituration through a flame-narrowed Pasteur pipette. After being trypsinized with 0.05% trypsin for 30 minutes in basic Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA, USA), the cells were then collected and re-suspended in DMEM without glutamine, supplemented with 10% FBS fetal bovine serum (FBS; Gibco), 2% (v/v) B27 (Gibco), 0.5 mmol/L glutamine, 100 U/mL penicillin, and 100 U/mL streptomycin. The cultures were maintained at 37°C with 5% CO_2. At the end of incubation, the morphology of the cells was monitored under an inverted light microscope (Olympus, Tokyo, Japan). The cells were randomly divided into four different groups: the control group (C group); the treated with propofol group (P group; 50 μM for 24 h); the hydroxylfasudil administration group (F group; 10 μM for 24 h); and the propofol and hydroxylfasudil co-administration group (PF group).

Animals and experimental design

All experimental procedures were approved by the Ethics Review Committee for Animal Experimentation of Hebei Medical University. Eighty female SD rats (postnatal day 7) were provided by the Laboratory Animal Center of Hebei Medical University. Pups were bred and maintained under a standard housing temperature (24°C) with a 12 h light-dark cycle and had access to food and water *ad libitum*. The rats were randomly divided into four different groups: the blank control group (C group); the propofol administration group (P group); the hydroxylfasudil administration group (F group); and the propofol and hydroxylfasudil co-administration group (PF group). The P group rats were injected intraperitoneally (i.p.) with propofol; the PF group pups were dosed i.p. with propofol and hydroxylfasudil; the pups in the C group were injected with an equal volume of saline. All drugs were given once a day at regular 24 h intervals for 7 days. Throughout all the procedure, the pups were maintained in a neonatal incubator to maintain their body temperature and were provided with low-flow oxygen to avoid potential hypoxia and stressors until they could successfully perform the righting reflex. The propofol dosage of 50 mg/kg once a day for seven consecutive days was selected to ensure significant neurotoxicity and avoid respiratory depression in neonatal rats. The hydroxylfasudil dose of 10 mg/kg was chosen in accordance with a previous study that demonstrated its neuroprotective effects in neonatal rats. The hydroxylfasudil was administered i.p. 30 min after the propofol in order to reduce pain stimulation and avoid possible drug incompatibility. During the anesthesia procedure, the respiratory frequency and skin color were observed to detect hypoxia and apnea. If apnea was detected, the pups received a tail pinch pain stimulus. To avoid differences in weight among the groups, all of the rat pups were separated from their mothers approximately 300 min after the administration of all of the treatments.

The rats were sacrificed by decapitation under deep anesthesia 24 h after the last injection, and the hippocampi were isolated immediately for the detection of neuronal apoptosis using a TUNEL assay (n = 5) and the determination of RhoA, Rock1, Rock2, Bcl2, Bax, and Bad using Western blot (n = 5). Moreover, to determine whether fasudil could improve the propofol-induced cognitive deficits, the rats' learning and memory abilities were tested using the Morris water maze at 60 d of age (n = 10).

Terminal transferase dUTP nick end labeling (TUNEL) fluorescent assay

The TUNEL staining was performed to detect the hippocampal apoptosis using an *in situ* cell

death detection kit (Roche Applied Science, Mannheim, Germany), following the manufacturer's manuals. The apoptotic cells (per 0.01 mm^2) in the hippocampal CA1 regions were counted under an Olympus light microscope (400×). The apoptotic cells were identified according to the following criteria: a condensed chromatin, a smaller size, and a dark brown nucleus. For TUNEL staining, one slide from each pup was randomly selected. Two fields at each slide were selected, and a total of 10 fields were used for analysis. The apoptotic cell numbers from the 10 fields were assessed.

Western blot analysis

Twenty-four hours after the last injection, five pups of each group were killed by decapitation, and the hippocampus was removed and placed on ice while the caudate/putamen was dissected. Hippocampal tissues were homogenized in a RIPA buffer (BestBio, Shanghai, China) containing a protease inhibitor. The homogenate was centrifuged at 10,000 rpm for 30 min at 4°C. The supernatants were separated for the Western blot analysis. The protein concentration was measured by the ultraviolet absorption method. The protein content of the supernatants was separated using SDS-PAGE on 10% acrylamide gels. They were then transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore) and blocked with 5% nonfat dry milk in a Tris-buffered saline Tween-20 (TBST) buffer (for 1 h at room temperature. The membranes were then incubated with the primary antibodies of total RhoA (Abcam; ab187027), Rock1 (Abcam; ab134181), Rock2 (Abcam; ab66320), Bcl-2 (Abcam; ab59348), Bak (Abcam; ab134181), Bax (Abcam, ab32503), Bad (Abcam; ab62465), and β-actin (Abcam; ab6276) antibodies overnight at 4°C. Then the signals were detected using an anti-mouse or rabbit horseradish peroxidase-conjugated secondary anti-body and a dual-color infrared laser imaging system (Odyssey9120, Li-COR, USA). The quantification of Western blot signals was performed using densitometric measurements.

Quantitative RT-PCR

Quantitative RT-PCR was performed as previously described [24]. The primers were as follows: RhoA-forward: GCCTCTCTCCTACCCAGACA; RhoA-reverse: TTTTGGCTAACTCCCGCCTT; Rock1-forward: CGGGTTTGCCTACGGTAGTT; Rock1-reverse: TCCTTATCCAAAGAGCCACCT; Rock2-forward: TCCAGCTCCAGACCCTTTTG; Rock2-reverse: AGAAGGCAGTTAGCTTGGTT; Bcl2-forward: CACGGTGGTGGAGGAACTCTT; Bcl2-reverse: GGTGACATCTCCCTGTTGACG; Bak-forward: GAGCCCTTCCGGATCTTTGTC; Bak-reverse: TTGTCACCTGCCTGACTGCTC; Bax-forward: TGGAAGAAGATGGGCTGAGGC; Bax-reverse: CATTCCCACCCCTCCCAATAAT; Bad-forward: CCGAAGAATGAGCGATGAAT; Bad-reverse: GATAATGCGCGTCCAACTG; β-actin-forward: AGGGAAATCGTGCGTGACAT; β-actin-reverse: CCTCGGGGCATCGGAA.

Morris water maze test and spatial probe trial

At 60 days of age, ten pups from each group underwent a spatial learning and memory abilities evaluation using the Morris water maze test. The Morris water maze (Zhenghua Bioinstrumentation Ltd., Anhui, China) apparatus is a black circular pool (120 cm diameter and 50 cm height) with four quadrants. The water temperature was maintained at 23 ± 1°C. The pool was located in a dimly lit, soundproof test room with a number of visual cues, including a white-black-colored poster on the wall, a halogen lamp, a camera and the experimenter. A white, 10 cm diameter platform was placed in the center of quadrant 4 and submerged 2 cm below water's surface. The position of the platform was unchanged during the training trials. A digital camera was suspended above the pool and connected to a video recorder and tracking device, which permitted automated tracking of the path taken by the rats. Two training trials per day were conducted for five consecutive days before the experimental Morris water maze test for each group. Each rat was released in the water facing a wall of the water maze at one starting position. The length of time to reach the platform was recorded in each trial, with a maximal time limit of 120 s. If the rat failed to find the platform within the set time, the computer stopped tracking and recorded the time as 120 s. And if the rat found the platform within 120 s, it was allowed to stay on it for 30 s. Otherwise, the rat was gently guided to find the platform by the experimenter and allowed to remain on the platform for 30 s, and the latency was recorded at 2 min. The time to reach the platform (latency to find the platform) was measured with a computerized tracking system. The average data from

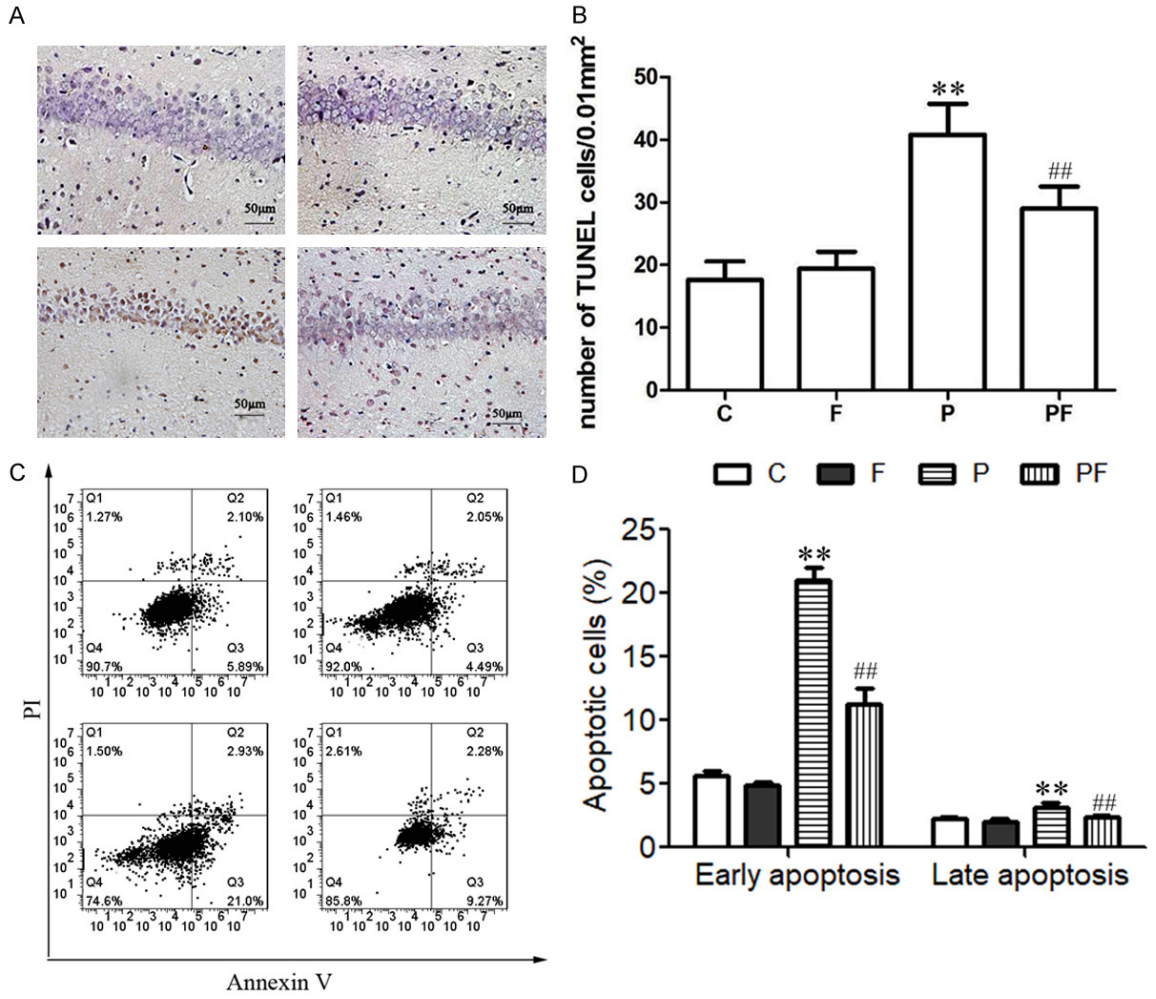

Figure 1. Hydroxyfasudil protects against propofol-induced neuroapoptosis in the hippocampal neurons both *in vivo* and *in vitro*. (A) Morphological changes in apoptotic cells in the hippocampus of neonatal rats with indicated treatment. The TUNEL assay was used to detect apoptotic cells in the hippocampus. C: Control group; F: Rats with hydroxyfasudil treatment; P: Rats with propofol treatment. PF: Rats administrated with propofol and hydrofasudil. Scale bar: 50 μm. (B) Quantification of TUNEL-positive cells. (C and D) Flow cytometry pictographs (C) and statistical analysis (D) of the apoptotic rate of primary pprimary hippocampal neurons with indicated treatment. Data were shown as the mean ± SD, $^{**}P < 0.01$ *vs.* the control group; $^{\#\#}P < 0.01$ *vs.* the "P" group.

the daily tests were used for statistical analysis. At the end of the training period, the mice were tested on a spatial probe trial in which the platform was removed, and they were allowed to swim freely for 2 min. The time spent to reach the platform initially and the times of crossing the platform were recorded.

RhoA activation assay

The RhoA activation assay was performed to detect the endogenous active form of RhoA (RhoA-GTP) using a RhoA activation assay kit (Abcam; ab211164) following the manufacturer's manuals.

Statistical analysis

Data were expressed and graphed as the mean ± standard error of the mean and analyzed using SPSS version 16.0 (SPSS, Inc., Chicago, IL, USA) and Graphpad Prism version 5.01 (Graphpad Software, Inc., CA, USA). The presented values are expressed as the mean ± SD. The data in the quantitative RT-PCR, the Western blot analysis, and the TUNEL assay were analyzed by a one-way ANOVA. For the Morris water maze test, latencies to find the platform were analyzed using repeated-measures analysis of variance (ANOVA) during the training period. For the probe test, these data

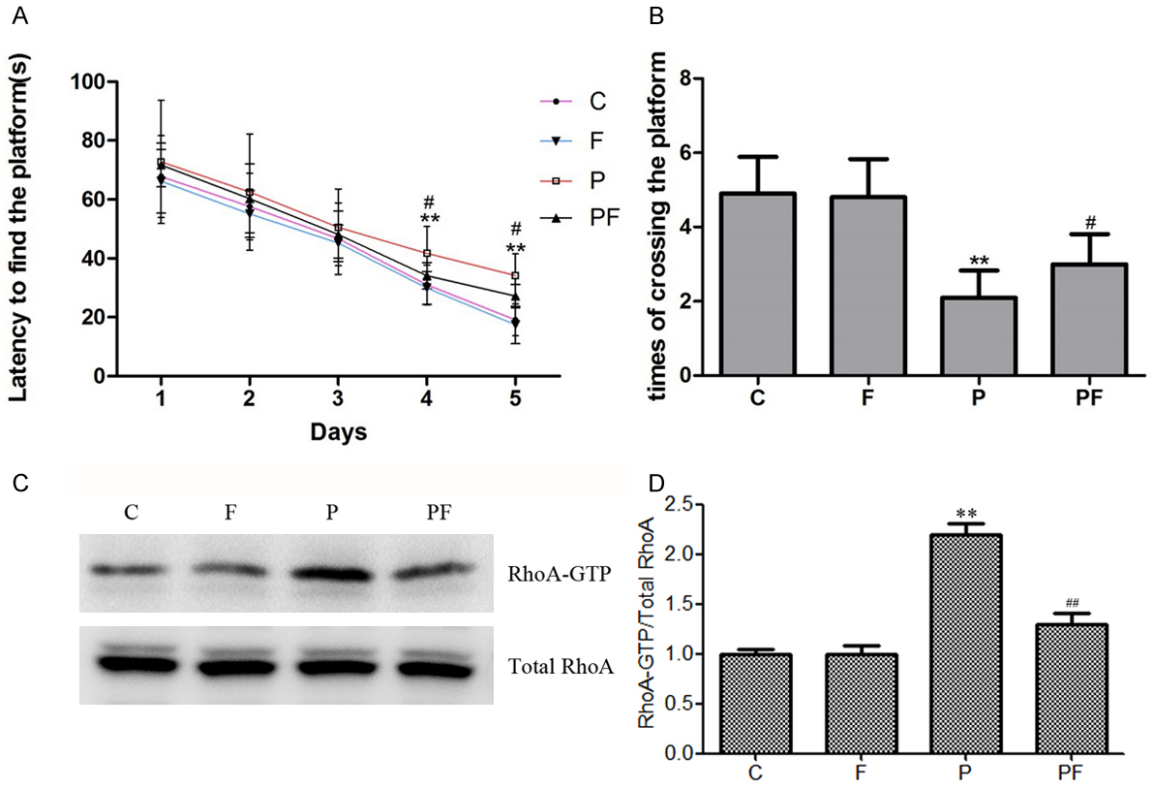

Figure 2. Hydroxyfasudil improves the tendency towards cognitive impairments induced by propofol. Evaluation of rat learning and memory in the Morris water maze. A. Mean escape latency to the platform in the Morris water maze. B. Time spent in platform quadrant among the different groups. C and D. Pull-down experiments for GTP-bound RhoA in primary culture hippocampal neurons with indicated treatment. Data were shown as the mean ± SD, $^{**}P < 0.01$ *vs.* the control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* the "P" group.

were assessed by a one-way ANOVA. Post-hoc individual means comparisons were conducted by a Dunnett's test. All statistical assessments used a significance level of $P < 0.05$.

Results

Hydroxyfasudil exerts neuroprotective effects against propofol-induced neuroapoptosis in the hippocampal neurons

To investigate whether hydroxyfasudil administration can alleviate apoptotic neurodegeneration induced by propofol in the developing rat brain, hydroxyfasudil and/or propofol was/were administered to P7 rats. Neuroapoptosis was determined by the TUNEL assay at the CA1 region. Compares with the control group (C group), the propofol induced significant hippocampal neuroapoptosis ($P < 0.01$). Meanwhile, the administration of hydroxyfasudil alone had no influence on the apoptosis of hippocampal neuron (**Figure 1A**, **1B**). However, the co-administration of hydroxyfasudil with propofol significantly ameliorated the neuroapoptosis induced by propofol exposure (**Figure 1A**, **1B**). Moreover, we also determined the effect of hydroxyfasudil and propofol on the primary cultures of the hippocampal neurons. Consistent with the *in vivo* findings, treatment with hydroxyfasudil alone had no effect on cellular apoptosis in primary hippocampal neuronal cells (**Figure 1C**, **1D**). However, propofol significantly induced neuronal cell apoptosis, which can be rescued by hydroxyfasudil (**Figure 1C**, **1D**). Taken together, these results suggest that hydroxyfasudil can be a protective chemical to reduce the toxicity of propofol in the nervous system.

Hydroxyfasudil showed potential improvement of the tendency towards cognitive impairments induced by propofol

Next, a spatial probe test and the Morris water maze test were performed to evaluate the protective effect of hydroxyfasudil on the memory impairment and neuronal injury induced by propofol. As shown in **Figure 2A**, the propofol-

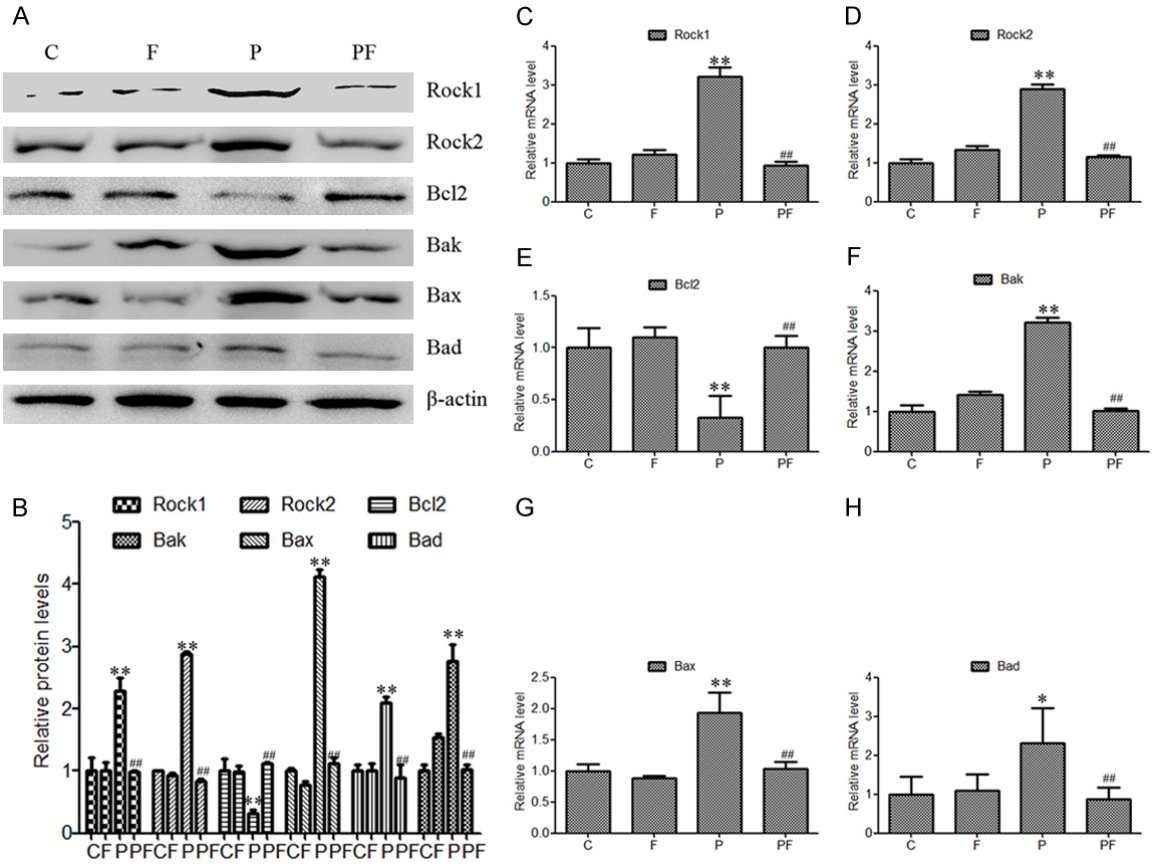

Figure 3. Hydroxyfasudil markedly ameliorated the activation of Rho kinase and the expression of Bad induced by propofol and rescued the expression of Bcl2 suppressed by propofol. (A and B) Representative western blots (A) and quantification results (B) of protein band intensities of Rock1, Rock2, Bcl2, Bak, Bax, and Bad for sample mixture of synaptic fractions of the hippocampus from each group. (C-G) Quantitative RT-PCR to determine the expression of Rock1 (C), Rock2 (D), Bcl2 (E), Bak (F), Bax (G), and Bad (H) in the samples mentioned above. Data were shown as mean ± SD, $^{**}P < 0.01$ vs. the control group; $^{\#\#}P < 0.01$ vs. the "P" group.

treated rats exhibited longer escape latencies compared with the control group and the rats administrated with hydroxyfasudil alone. Importantly, the prolonged latency of the propofol-treated group was significantly shortened by treatment with hydroxyfasudil. A similar observation was seen in the spatial probe test whereby the propofol-treated rats spent less time swimming in the target quadrant compared with the untreated rats, which could be reversed by hydroxyfasudil treatment (**Figure 2B**).

Hydroxyfasudil markedly ameliorated the activation of RhoA and the expression of Rock1, Rock2, Bak, Bax, and Bad induced by propofol and rescued the expression of Bcl2 suppressed by propofol

Next, the activity of the small GTPase RhoA was also determined using a pulldown assay. As shown in **Figure 2C**, **2D**, after 24 h treatment with propofol, RhoA activity was elevated compared to the control group and the administration of hydrofasudil reversed the RhoA-GTP level. To elucidate the underlying mechanisms how hydroxyfasudil protects hippocampal neurons against propofol, we measured the expression of Rock1, Rock2, Bak, Bcl2, Bax, and Bad in the indicated groups in vivo mentioned above. The results showed that propofol significantly increased Rock1, Rock2, Bak, Bax, and Bad expression in hippocampal tissues (**Figure 3A-D**, **3F-H**), whereas hydroxyfasudil markedly ameliorated this increase induced by propofol (**Figure 3A-D**, **3F-H**). Meanwhile, propofol significantly decreased the expression of the anti-apoptotic gene Bcl2, which could be re-elevated by hydroxyfasudil (**Figure 3A**, **3B**, **3E**). Moreover, hydroxyfasudil attenuated the ex-

pression of the pro-apoptotic gene Bad induced by propofol in the hippocampal tissues (**Figure 3A**, **3B**, **3E**).

Discussion

Propofol is an intravenous agent commonly used for achieving anesthesia and sedation in children [25-27]. The mechanisms underlying propofol involve its interactions with the central nervous system at various neurotransmitter receptors, especially the GABAA receptors and NMDA glutamate receptors [28, 29]. The use of propofol for anesthesia induction in children less than 3 years of age still remains off-label [30]. It has been reported that propofol could trigger significant neuroapoptosis at a dose of 50 mg/kg, while the dosage required to induce a surgical plane of anesthesia is 200 mg/kg [30]. Clinical concerns of neuroapoptosis in developing brains induced by propofol continue to be a focus of research. In this study, we confirmed that propofol induced significant hippocampal neuroapoptosis in neonatal rats and induced the tendency towards cognitive impairments.

RhoA/Rock is a well-documented signaling cascade involved in regulating cell movement, adhesion, proliferation, differentiation, and migration [17]. The inhibition of RhoA/Rock could promote neuron growth and alleviate nerve injury. Rock inhibitors Y-39983 and Y-27632 have been also proven to exhibit a protective effect on axonal regeneration [17]. Yu et al. have reported that fasudil treatment blocks the RhoA/Rock pathway and significantly reduces the apoptosis of primary retinal ganglion cells and relieves retinal nerve injury [12]. In the present study, we demonstrated that hydrofasudil reversed the hippocampal neuron apoptosis induced by propofol. Functionally, hydroxyfasudil ameliorated the tendency towards cognitive impairments induced by propofol. Mechanistically, propofol activated Rho kinase in hippocampal tissues, which could be reduced by hydroxyfasudil. These results showed a series of pathological changes in hippocampal tissues with propofol treatment with an increasing activity of Rho kinase, and importantly, our data showed that hydrofasudil treatment blocked the RhoA/Rock pathway and significantly reduced propofol-induced apoptosis of hippocampal tissues and relieved nerve injury. It is known that Rho is involved in the activation of transcription factors in the nucleus that control the synthesis of members of the apoptotic cascades [15, 31, 32]. Therefore, we speculated and confirmed that treatment with hydrofasudil to block Rho activation after propofol administration suppresses apoptosis by preventing the synthesis of pro-apoptotic proteins such as Bcl2, and by rescuing the expression of anti-apoptotic genes including Bak, Bax, and Bad.

Hydrofasudil is a selective Rock inhibitor in clinical applications, which has therapeutic benefits in brain disorders by reducing inflammatory cytokines, improving local blood circulation, preventing axonal injury, and promoting injured axonal regeneration [33-35]. In a future study, we plan to explore the role and underlying mechanisms of hyrdrofasudil and propofol in neuroimmunology, cerebral blood flow, and axonal injury and regeneration.

In conclusion, we established a rat model of propofol-induced hippocampus injury. Hydrofasudil treatment significantly reduced the apoptosis of hippocampal tissues and relieved neuronal injury both *in vitro* and *in vivo*.

Acknowledgements

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Disclosure of conflict of interest

None.

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