Erythropoietin protects propofol induced neuronal injury in developing rats by regulating TLR4/NF-κB signaling pathway

Abstract

Chunyan Zhang, Yuxia Wang, Jin Jin, Kezhong Li

PII: S0304-3940(19)30620-2
DOI: https://doi.org/10.1016/j.neulet.2019.134517
Reference: NSL 134517
To appear in: Neuroscience Letters
Received Date: 26 August 2019
Revised Date: 18 September 2019
Accepted Date: 23 September 2019

Please cite this article as: Zhang C, Wang Y, Jin J, Li K, Erythropoietin protects propofol induced neuronal injury in developing rats by regulating TLR4/NF-κB signaling pathway, Neuroscience Letters (2019), doi: https://doi.org/10.1016/j.neulet.2019.134517

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

© 2019 Published by Elsevier.
Erythropoietin protects propofol induced neuronal injury in developing rats by regulating TLR4/NF-κB signaling pathway

Abstract

Objective: To explore the protective effect of erythropoietin on propofol induced neuronal injury in developing rats by regulating the TLR4/NF-κB signaling pathway.

-running title: Erythropoietin protects propofol induced neuronal injury

Chunyan Zhang1,2 #, Yuxia Wang1 #, Jin Jin1, Kezhong Li1 *

1 Department of Anesthesiology, the Affiliated Yantai Yuhuangding Hospital of Qingdao University, Shandong, P.R. China 264000

2 Department of Anesthesiology, School of Medicine, Shandong University, Jinan, Shandong, P.R. China 250000

# The two authors contributed equally in the study.

* Corresponding author,
Kezhong Li, Department of Anesthesiology, the Affiliated Yantai Yuhuangding Hospital of Qingdao University. No.20, Yudong Road, Yantai, 264000, Shandong, P.R. China.
Tel: 86-0535-6691999-83935; 86-18561000699
Email: lunnen7053448@126.com

Highlights

- Erythropoietin has a protective effect on neuropathic injury induced by propofol in rats
- Erythropoietin mechanism is relevant to the regulation of TLR4/NF-κB signaling pathway.

Abstract

Objective: To explore the protective effect of erythropoietin on propofol induced neuronal injury in developing rats by regulating the TLR4/NF-κB signaling pathway.
neuronal injury in developing rats by regulating TLR4/NF-κB signaling pathway

**Method:** Rats were divided into normal control group (Control), propofol group (PPF), erythropoietin group (EPO), propofol + erythropoietin group (P+E), propofol+TAK-242 group (P+T), and propofol+EPO+LPS group (P+E+L) (n=12). The pathology of hippocampal neurons was observed. The inflammatory factors were detected by ELISA. The expression of TLR4/NF-κB pathway-related proteins were detected by Western blot.

**Results:** Compared with the PPF group, the percentage of apoptotic cells in the hippocampal CA1 area of the erythropoietin treatment groups were greatly decreased while the percentage of positive cells in the hippocampus were remarkably increased (p<0.05). The production of inflammatory factors and the expressions of TLR4/NF-κB pathway-related proteins were greatly improved in treatment groups (p<0.05). Compared with P+E group, the percentage of apoptotic cells in CA1 area of the P+E+L group was significantly increased and the percentage of positive cells was remarkably reduced (p<0.05), the levels of interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8) and tumor necrosis factor-α (TNF-α) were significantly increased and interleukin-4 (IL-4) and interleukin-10 (IL-10) was notably reduced (p<0.05). The protein expression of TLR4 and p-P65 was significantly increased, while the protein expression of p-IκBα was significantly decreased (p<0.05).

**Conclusion:** Erythropoietin has a protective effect on propofol induced neuropathic injury propofol in rats, and its mechanism is relevant to the regulation of TLR4/NF-κB signaling pathway.

**Keywords:** Erythropoietin, propofol, developmental rats, TLR4, NF-κB

**Introduction**

Propofol (2,6-diisopropylphenol) has great sedative-hypnotic effect that is widely used in the process of surgery or in ICU to keep anesthesia and sedation. An increasing number of studies have proved that propofol can lead to cell death in cortical cells[1], developing hippocampal neurons[2] and neural progenitor cell or stem
cells\textsuperscript{[3]} performed on cell lines. In addition, many in vivo studies have shown that propofol can damage neuronal cell in the young or adult brain of different animals\textsuperscript{[4,5]}. Therefore, it is significantly to explore potent measures against the underlying pernicious effects of propofol in the developing brain.

Toll-like receptors (TLRs) could recognise pathogen-associated molecular patterns and allow the host to detect microbial infection. TLRs also can regulate innate and adaptive immune response\textsuperscript{15}, which are associated with noninfectious inflammatory diseases. TLR4 is identified as a kind of transmembrane proteins, which has important effects in inflammatory reaction\textsuperscript{[6]}. More and more works have proved that the expression of TLR4 is elevated in many nerve lesion, including ischemic, autoimmune disease, hemorrhagic brain injuries and Parkinson’s disease \textsuperscript{[7,8]}. When the TLR4 is activated, the downstream of the NF-κB can also be activated, which is an important pivot participated in the inflammatory signaling pathway. NF-κB is a transcription factor that regulates the inflammation-related genes, including interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β)\textsuperscript{[9]}. Neuronal injury induced by propofol in brain is closely related to the inflammatory responses, thus it can be seen that the inhabitation of the inflammatory responses is an important strategy for propofol injury.

Erythropoietin (EPO) is originally as a cytokine, which has an important effect in erythropoiesis. Previous studies have shown that EPO has great protective effect on hypoxic-ischemic and inflammatory damages in different tissues and organs\textsuperscript{[10,11]}. Furthermore, EPO decreases the levels of inflammatory factors\textsuperscript{[12,13]}, and more and more studies have confirmed that EPO has a potential agent for the therapy of diseases relevant with neuronal damage, including mechanical brain damage, Parkinson’s disease, stroke and spinal cord damage\textsuperscript{[14]}. The previous researches\textsuperscript{[15,16]} have noted that EPO has an outstanding neuroprotective activity against CO-induced neuronal injury. Nevertheless, whether EPO can defend the propofol-induced damage and decrease the level of TLR/NF-κB signaling pathway in developing rats after propofol injury remains unknown.

In this study, we investigated the remarkable effects of EPO on the TLR4/NF-κB
signaling pathway and the inflammatory factors in-1β, interleukin-6, interleukin-8 and tumor necrosis factor-α in developing rat hippocampus, which is the most pregnable area with the brain damage induced by propofol.

Materials and Methods

1.1 Experimental animals

Healthy SPF grade 96 7-day-old Sprague-Dawley rats, weighing (16±4g), male or female, were purchased from Jinan Pengyue Experimental Animal Breeding Co., Ltd., animal production license number: SCXK (Lu) 2014-0007. All animals were housed in a controlled environment at 22-24 °C and a relative humidity of 50-60% with free access to food and water. Animal experiments follow the NIH guidelines (NIH Pub. No. 85-23, revised 1996) and have been reviewed and approved by the Affiliated Yantai Yuhuangding Hospital of Qingdao University Animal Protection and Use Committee.

1.2 The groups and administration of animals

Rats were randomly divided into control group (Control) without being treated, propofol (Propofol, Beijing Fresenius Kabi Pharmaceutical Co., Ltd.) group (PPF), erythropoietin (Harbin Pharmaceutical Group Bioengineering Ltd.) group (EPO), propofol + erythropoietin group (P+E), according to the random number table method (n=12). PPF group was received intraperitoneal injection of propofol 80mg/kg[17,18]. The control group was injected the same amount of saline. EPO group was received intraperitoneal injection of EPO 5000U/kg[19,20]. PPF+EPO group was received intraperitoneal injection of propofol 80mg/Kg, EPO 5000U/kg, once daily, for 7 consecutive days. To further verify the effect of erythropoietin on the pathway, TLR4 activator (LPS, Beijing Solabao Technology Co., Ltd.) and TLR4 inhibitor (TAK-242, purchased from Invivogen) were used to treat the rats. The test groups are as follows: control group (Control), propofol group (PPF), propofol + EPO group (P+E), propofol + TAK-242 group (P+T), propofol + EPO + LPS group (P+E+L), propofol + TAK-242 + EPO (P+E+T); propofol + LPS (P+L) (n=12). Rats in the P+T group were intraperitoneally injected with PPF 80 mg/kg, TAK-242 2 mg/kg[21], and P+E+L rats were intraperitoneally injected with PPF 80 mg/kg, EPO 5000 U/kg,
LPS 40 mg/kg, once daily, continuous injection for 7 days. After the injection, the righting reflex disappeared. The young rats were placed in a constant temperature incubator and the oxygen flow was continued for a low flow. After the righting reflex was restored, it was returned to the mother, and the separation time from the mother was no more than 5 hours.

1.3 Hematoxylin-Eosin(HE) staining to observe morphological changes of hippocampal neurons

Six rats in each group were anesthetized by ether inhalation, then were fixed in supine position. And the abdominal cavity was quickly opened. The left ventricle was inserted from the apex, and the pre-cooled saline was quickly input inward. At the same time, the right atrial appendage was broken with an ophthalmology scissors. After the effluent was cleared, it was replaced with a pre-cooled 4% paraformaldehyde fixative for rapid systemic perfusion fixation. Rat hippocampus tissue was taken and fixed in 4% paraformaldehyde for 24 hours, and the tissue was sliced and routinely dehydrated. Then they were embedded in paraffin. The paraffin tissue was serially sliced to a thickness of 5 μm. The sections were conventionally dewaxed with xylene and hydrated by various stages of ethanol. The section was stained in hematoxylin (Solarbio, Beijing, China) for 5 min, and then rinsed with tap water. The section was differentiated in hydrochloric acid ethanol for 30 s, and soaked in tap water for 15 min, then placed in Eosin stain (Solarbio, Beijing, China) for 2 min. Lastly, the section was routinely dehydrated, transparent, and sealed. It was observed under a ×400 optical microscope (Olympus Model BX51, Olympus, Japan).

1.4 Nissl staining to observe the damage of hippocampal neurons

The slices were dewaxed, and the gradient ethanol solution was dehydrated. After washed with distilled water, the slices were stained in the tar purple dye solution for 30 min (stained in a 37 °C incubator for 10 min), and separated by a 95% ethanol solution. The Nissl was purple, the nucleus was lavender, and the glial cells were lavender. The section was routinely dehydrated, transparent, sealed and placed under a light microscope. The hippocampal neurons were observed under a light microscope. 3 discrete sections counted per rat and the average was taken. The number of neurons
in the hippocampal CA1 region was counted (×400).

1.5 TUNEL staining to observe the apoptosis of hippocampal neurons

The sections of the apoptosis of hippocampal neurons were detected by TUNEL method using the apoptosis detection kit (batch number: ZK-8005, Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., China). Five fields of view were randomly selected under a 400-fold optical microscope. The normal cell nucleus was blue, and the apoptosis-positive cells were brown-yellow. Apoptotic index was calculated (apoptotic Index, AI), which reflects the degree of apoptosis. AI = (number of apoptotic positive cells /total cells) × 100%.

1.6 Immunohistochemistry to observe hippocampal neuronal cell proliferation (BrdU)

The dewaxed sections (n=3) were inactivated by adding 3% H2O2 methanol solution for 20 min, and was heat-fixed for 10 min via the high temperature antigen in the buffer solution (pH 6.0), and the slices were blocked with BSA for 20 min. Rabbit anti-rat BrdU (1:500, orb109973, Biorbyt, Cambridge, UK) was added dropwise and allowed to react overnight at 4 °C. After rewarming, the sections were incubation with secondary antibody. DAB was developed at room temperature for 5-10 min, hematoxylin counterstained for 10 min, gradient alcohol dehydration for 5 min, xylene treatment for 2 times each for 10 min, and the gum was sealed. The results were observed under a ×400 optical microscope (Olympus, Japan) and counted using AperioImagescope 11.1 software, and the ratio (%) of positive cells were counted as the results.

1.7 The proinflammatory factors (TNF-α, IL-1β, IL-6, IL-8) and anti-inflammatory factors (IL-4, IL-10) in hippocampus were detected by ELISA

The remaining rats were anesthetized and then dislocated and sacrificed. The left hippocampus tissue was quickly placed in a -80 °C refrigerator for use, and the right hippocampus was used for ELISA. The hippocampus tissue was ground and homogenized, then centrifuged at 3000 r/min for 15 min at 4°C. The content of TNF-α (orb79138, Biorbyt, Cambridge, UK), IL-1β (orb79117, Biorbyt, Cambridge, UK), IL-6 (orb79123, Biorbyt, Cambridge, UK), IL-8 (orb312288, Biorbyt, Cambridge,
UK), IL-4 (orb219830, Biorbyt, Cambridge, UK), IL-10 (orb79114, Biorbyt, Cambridge, UK) were detected at 450 nm from a microplate reader (RT-6100, Lei Du).

1.8 The expression of TLR4/NF-κB pathway-related proteins (TLR4, p-P65, P65, p-IκBα, IκBα) in hippocampus were detected by Western blot

After the hippocampus tissues were weighed and selected in equal amounts, then boiled and loaded with SDS-PAGE electrophoresis, The PVDF membrane (Merck, Darmstadt, Germany) was rotated at 80 V for 30 min, blocked with 5% skim milk powder in TBST for 1 h at 4 °C, and rabbit anti-rat TLR4 was diluted with 3% bovine serum albumin in TBST (1:500, orb11489, Biorbyt, Cambridge, UK), P65 (1:500, orb229138, Biorbyt, Cambridge, UK), p-P65 (1:500, orb304662, Biorbyt, Cambridge, UK), IκBα (1:500, orb223182, Biorbyt, Cambridge, UK), p-IκBα (1:500, orb223035, Biorbyt, Cambridge, UK), β-actin (1:2000, orb178392, Biorbyt, Cambridge, UK) polyclonal antibody, reacted at 4°C overnight. They were incubated with horseradish peroxidase-labeled goat anti-rabbit IgG (1:1000, ABIN101988, antibodies-online, Aachen, Germany) for 1 h, and washed with ECL luminescent substrate for 3-5 min. Protein expression levels were normalized by β-actin and the protein levels of p-P65 normalized by P65, p-IκBα normalized by IκBα. Grayscale scanning and quantification were performed by Image J (NIH) software.

1.9 statistical methods

Data processing was performed using SPSS 19.0 statistical analysis software, and the results were expressed as mean ± standard deviation (mean ± SD). Data analysis between groups was performed by one-way analysis of variance (ANOVA) and subsequent analysis using LSD test. The difference was statistically significant at p < 0.05.

Results

2.1 The effect of Erythropoietin on morphological changes and nerve cells in hippocampal CA1 area

HE staining results (Figure 1) showed that the neurons of the control group and the EPO group were conical, uniform in size, neatly arranged, and clearly defined. In
the PPF group, the hippocampal neurons were disorderly arranged and the structure was unclear. Additionally, the cell body was shrunk as well as the nucleus was constricted into a triangle or a polygon. The nucleolus disappeared and the cytoplasm was strongly eosinophilic. However, the hippocampal nerve cells were arranged neatly and some of the nerve cell bodies were swollen and the neurons were degenerated in P+E rats. The results of Nissl staining also showed that the tissue structure of hippocampal CA1 area in control group and EPO group was normal and no pathological changes. The hippocampal pyramidal cells were arranged neatly and tightly, cytoplasmic staining was clear and Nissl was abundant. It was also shown that neuronal degeneration and necrosis occurred in the CA1 region and the number of hippocampal pyramidal cells was decreased in the PPF group. Beyond that the structure was blurred and the cell body was swollen. And the arrangement was scattered and the cytoplasmic Nissl bodies were largely lost. In the P+E group, the lesions in the hippocampal CA1 area were alleviated, the pyramidal cells were arranged neatly and tightly, and the cytoplasmic Nissl was richer and clearer.

2.2 The effect of Erythropoietin on neuronal apoptosis and BrdU expression in hippocampal CA1 region

Compared with control group, the percentage of apoptotic cells of the PPF and P+E groups were significantly increased (p<0.05) (figure 2A). The percentage of apoptotic cells was significantly decreased (p<0.05) in the hippocampal CA1 area of the P+E group versus the PPF group.

As shown in the figure 2B, the positive BrdU is mainly located in the nucleus of the neuron, which is brown-yellow granular. The expression of positive cells of Control group and EPO group was less. The positive cell count of P+E group was remarkably raised (p<0.05) than PPF group.

2.3 The effect of Erythropoietin on the level of inflammatory factors in hippocampus

There was no significant difference in the expression of inflammatory factors within the control group and the EPO group (Figure 3) (p>0.05). But the expression of proinflammatory factors (TNF-α, IL-1β, IL-6, IL-8) in the hippocampus of the PPF
and P+E groups was obviously raised, and the level of anti-inflammatory factors (IL-4, IL-10) was remarkably reduced (p<0.05) than those in the control group. Compared with the PPF group, the expression of proinflammatory factors (TNF-α, IL-1β, IL-6, IL-8) of the P+E group was obviously decreased, and the expression of IL-4 and IL-10 was significantly increased (p<0.05).

2.4 The impact of Erythropoietin on the TLR4/NF-κB-related protein expression in hippocampus

Western blot analysis indicated that there was no outstanding difference in the expression of pathway-related proteins in the hippocampus between control group and EPO group (p>0.05) (Figure 4). The level of TLR4 and p-P65 protein of PPF and P+E groups were obviously up-regulated, while the expression of p-IκBα protein was significantly decreased than the control group (p<0.05). Furthermore, the expression of TLR4 and p-P65 protein in the hippocampus of the P+E group were significantly decreased than the PPF group, while the expression of p-IκBα protein was significantly increased (p<0.05).

2.5 Effects of TLR4/NF-κB pathway on morphological changes and injury of hippocampal CA1 neurons

As shown in figure 5, the neurons of the Control group were conical, uniform in size, neatly arranged and clearly defined. The hippocampal neurons in the PPF and P+E+L groups were disorderly arranged. The structure was unclear and the cell bodies were shrunk. It was condensed into a triangle or a polygon. The nucleolus disappears. The cytoplasm was also strongly eosinophilic. The hippocampal neurons in the P+E group and the P+T group were arranged neatly and some of the nerve cell bodies were swollen and the neurons were degenerated. The results of Nissl staining showed that the tissue structure of hippocampal CA1 area in control group was normal and no pathological changes. The hippocampal pyramidal cells were arranged neatly and tightly. The cytoplasmic staining was clear and Nissl was abundant. PPF and P+E+L group rats CA1 Neuronal degeneration and necrosis occurred in the area. The number of hippocampal pyramidal cells decreased and the structure was blurred. The cell body was swollen. The arrangement was scattered and the cytoplasmic Nissl bodies
were largely lost. It was also shown that lesions in the hippocampal CA1 area of P+E group and P+T group were alleviated. The somatic cells were arranged more closely and tightly, and the cytoplasmic Nissl was richer and clearer.

2.6 Effect of TLR4/NF-κB pathway on neuronal apoptosis and BrdU expression in hippocampal CA1 region

The percentage of apoptotic cells of the other groups was remarkably increased than that of the control group (p<0.05) (figure 6A). Comparing with the PPF group, the percentage of apoptotic cells of the P+E, P+T groups was significantly decreased, and the difference was statistically significant (P<0.05). The percentage of apoptotic cells of the P+E+L group was significantly increased than that in the P+E group (P<0.05).

The positive expression of BrdU was mainly located in the nucleus of the neuron, which was brown-yellow granular. As shown in the figure 6B, the positive cells of the control group were less expressed. The positive cells in the P+E and P+T groups were significantly increased when compared with control group (p<0.05). The positive cell counts in the P+E+L group were notably reduced compared to the P+E group (p<0.05).

2.7 Effect of regulation of TLR4/NF-κB pathway on expression of inflammatory factors in hippocampus

As shown in figure 7, the expression of proinflammatory factors (TNF-α, IL-1β, IL-6, IL-8) in the hippocampus of the other groups was significantly increased and anti-inflammatory factors (IL-4, IL-10) were significantly decreased than the control group (p<0.05). Compared with the PPF group, the inflammatory factors were also significantly improved (p<0.05). In addition, in comparison with the P+E group, the expression of the inflammatory factors were also significantly improved (p<0.05) in the P+E+L group.

2.8 Effect of regulation of TLR4/NF-κB pathway on expression of related proteins in hippocampus

When comparing to the Control group, the expression of TLR4 and p-P65 protein in hippocampus of the other groups were remarkably up-regulated, while the level of
p-IκBα protein was notably decreased (p<0.05) (Figure 8). Compared with the PPF group, the levels of TLR4 and p-P65 protein in hippocampus of P+E and P+T groups were remarkably down-regulated, but the expression of p-IκBα protein was significantly up-regulated (p<0.05). In addition, the protein expression of TLR4 and p-P65 in hippocampus of P+E+L group was obviously up-regulated than the P+E group, while the protein expression of p-IκBα was greatly down-regulated (p<0.05).

**Discussion**

Many studies have shown that the anesthetics, such as propofol and isoflurane can induce neuronal death in developing animal brains\(^\text{[22,23]}\). But the underlying mechanisms of propofol injury in the neurons still need to be explored. Therefore, the search for the protective substances against the injury was critical important. In this study, we verified the protective effect of erythropoietin on propofol induced neuronal injury young rats. The present research demonstrated that EPO could affect the TLR4/NF-κB signaling pathway in rat hippocampal tissues injured by propofol. In addition, the results showed that EPO decreased the levels of inflammatory factors, such as interleukin-1β(IL-1β), interleukin-6(IL-6), interleukin-8(IL-8) and tumor necrosis factor-α(TNF-α) increased the levels of interleukin-4(IL-4) and interleukin-10(IL-10) in hippocampal tissues. Hence, the neuroprotective role and anti-inflammatory activities against propofol damage maybe closely related to the down-regulation of the TLR4/NF-κB signaling pathway.

Toll-like receptors (TLRs) play important effects in inflammatory responses\(^\text{[24]}\). TLR4 as a member of TLRs family has been proved to play an important role in inducing the inflammatory response in brain damage\(^\text{[25]}\). The NF-κB pathway can be facilitated by the activation of TLR4, then resulting in the generating of proinflammatory factors and accelerating the inflammatory reactions\(^\text{[26]}\). In addition, many studies have demonstrated that the downstream signaling pathway regulated by TLR4 can exacerbate the brain injury\(^\text{[27]}\). In the present work, the TLR4 and NF-κB levels were up-regulated in propofol group, which implies that TLR4/NF-κB pathway promoted the inflammatory reactions after brain being damaged by propofol.
Therefore, the expression of TLR4/NF-κB pathway may be an underlying treatment target for propofol damage in brain.

EPO was an important regulator of ischemia reperfusion injury (IRI)\(^2\) and exerted great neuronal protection while exposure extensively to animals suffering from severe ischemia\(^2\). EPO had great effect against IRI in the brain injury\(^3\). Many studies showed that EPO had great safety and efficacy in curing acute ischemic stroke\(^4\) and hypoxic-ischemic encephalopathy\(^5\). Previous studies proved that when the patients with CO injury were given EPO timely, neurological results and neurological sequelae could be alleviated in great extent\(^6\). Nevertheless, propofol may cause neuronal death in newborn rat brains, which is prevented by low-dose rEPO but not high-dose rEPO, the protective effect of EPO on the neuronal injuries induced by propofol are dose dependent\(^7\). Some studies have shown that EPO protects IRI neurons and induces TLR-induced responses by activating NF-κB, PI3K and Akt\(^8\). EPO could impact many downstream factors to regulate inflammation, oxidative stress and apoptosis \(^9,10\). In the present research, we proved that the pathology of neuronal necrosis and apoptosis were improved by EPO. Furthermore, EPO also inhibited the levels of TLR4 and NF-κB and further reduced the levels of proinflammatory factors (TNF-α, IL-1β, IL-6, IL-8) and increased anti-inflammatory factors (IL-4, IL-10). All of the above demonstrated that EPO prevented the damage induced by TLR4, exerting a great neuroprotective effect against neurological damage caused by propofol.

**Conclusions**

In summary, the present work indicated that erythropoietin has a protective effect on propofol induced neuropathic injury in rats, and its mechanism is relevant to the TLR4/NF-κB caused inflammatory reaction. Nevertheless, the indicators might not be comprehensive. In addition, we only limited the study on hippocampus tissue and the study findings may not be applicable to other areas.

**Funding:**

This work was supported by the Natural Science Foundation of Shandong Province,
China (Grant No. ZR2019MH124 and ZR2019PH119).
References
[1] C. Buechler, R. Pohl, C. Aslanidis, Pro-resolving molecules-new approaches to treat sepsis? Int. J. Mol. Sci. 18 (2017) 476.
[2] S. Kahraman, S.L. Zup, M.M. McCarthy, G. Fiskum, GABAergic mechanism of propofol toxicity in immature neurons. J Neurosurg Anesthesiol. 2008, 20: 233-240.
[3] A.M. Drewry, E.A. Ablordeppey, E.T. Murray, C.R.T. Stoll, S.R. Izadi, C.M. Dalton, et al., Antipyretic therapy in critically ill septic patients: a systematic review and meta-analysis, Crit. Care Med. 45 (2017) 806–813.
[4] D. Cattano, C. Young, M.M. Straiko, J.W. Olney, Subanesthetic doses of propofol induce neuroapoptosis in the infant mouse brain. Anesth Analg. 2008, 106: 1712-1714.
[5] V. Pesić, D. Milanović, N. Tanić, J. Popić, S. Kanazir, V. Jevtović-Todorović, S. Ruzdijić, Potential mechanism of cell death in the developing rat brain induced by propofol anesthesia. Int J Dev Neurosci. 2009, 27: 279-287.
[6] F. Hua, J. Ma, T. Ha, Y. Xia, J. Kelley, D.L. Williams, et al., Activation of Toll-like receptor 4 signaling contributes to hippocampal neuronal death following global cerebral ischemia/reperfusion. Journal of Neuroimmunology. 2007, 190: 101-111.
[7] W. Fan, Q. Liu, X. Zhu, Z. Wu, D. Li, F. Huang, et al., Regulatory effects of anesthetics on nitric oxide, Life Sci. 151 (2016) 76–85.
[8] C.X. Ma, W.N. Yin, B.W. Cai, J. Wu, J.Y. Wang, M. He, et al., Toll-like receptor 4/nuclear factor-kappa B signaling detected in brain after early subarachnoid hemorrhage. Chinese Medical Journal. 2009, 122: 1575-1581.
[9] M. Neri, I. Riezzo, C. Pomara, S. Schiavone, E. Turillazzi, Oxidative-nitrosative stress and myocardial dysfunctions in sepsis: evidence from the literature and postmortem observations, Mediat. Inflamm. 2019, 3423-450.
[10] W.N. Barakat, N.N. Safwet, Candesartan and glycyrrhizin ameliorate ischemic brain damage through downregulation of the TLR signaling cascade. European Journal of Pharmacology. 2018, 724: 43-50.
[11] M. Gassmann, K. Heinicke, J. Soliz, O.O. Ogunshola, Non-erythroid functions of
erythropoietin. Hypoxia: Through the Lifecycle. 2003, 543: 323-330.

[12] W. Jelkmann, K. Wagner, Beneficial and ominous aspects of the pleiotropic action of erythropoietin. Annals of Hematology. 2004, 83: 673-686.

[13] D.P. Agnello, P. Bigini, T. Villa, A. Mennini, M.L. Cerami, Erythropoietin exerts an anti-inflammatory effect on the CNS in a model of experimental autoimmune encephalomyelitis. Brain Research. 2002, 952: 128-134.

[14] J.G. Travers, F.A. Kamal, J. Robbins, K.E. Yutzey, B.C. Blaxall, Cardiac fibrosis: The fibroblast awakens. Circ Res. 2016, 118:1021-1040.

[15] L. Pang, M. Bian, Y. Zang, D.H. Wu, N. Xu, Neuroprotective effects of erythropoietin in patients with carbon monoxide poisoning. Journal of Biochemical and Molecular Toxicology. 2013, 27: 266-271.

[16] S. Shahsavand, A.H. Mohammadpour, R. Rezaee, E. Behravan, R. Sakhtianchi, S.A. Moallem, Effect of erythropoietin on serum brain-derived biomarkers after carbon monoxide poisoning in rats. Iranian Journal of Basic Medical Sciences. 2012, 15: 752-758.

[17] P. Li, X.C. Hao, J. Luo, F. Lv, K. Wei, S. Min, Propofol Mitigates Learning and Memory Impairment After Electroconvulsive Shock in Depressed Rats by Inhibiting Autophagy in the Hippocampus. Med Sci Monit. 2016, 22: 1702-1708.

[18] Y.T. Tai, Y.L. Lin, C.C. Chang, Y.G. Cherng, M.J. Don, R.M. Chen, Ring-oxidative biotransformation and drug interactions of propofol in the livers of rats. Biomed Res Int. 2015, 2015: 658-928.

[19] K.M. Lan, L.T. Tien, Z. Cai, S. Lin, Y. Pang, S. Tanaka, et al., Erythropoietin Ameliorates Neonatal Hypoxia-Ischemia-Induced Neurobehavioral Deficits, Neuroinflammation, and Hippocampal Injury in the Juvenile Rat. Int J Mol Sci. 2016, 17: 289.

[20] M.E. Schober, D.F. Requena, B. Block, L.J. Davis, C. Rodesch, T.C. Casper, et al., Erythropoietin improved cognitive function and decreased hippocampal caspase activity in rat pups after traumatic brain injury. J Neurotrauma. 2014, 31: 358-369.

[21] T. Moore-Morris, N. Guimarães-Camboa, K.E. Yutzey, M. Pucéat, S.M. Evans, Cardiac fibroblasts: From development to heart failure. J Mol Med (Berl). 2015, 93:
823–830.

[22] C. Creeley, K. Dikranian, G. Dissen, L. Martin, J. Olney, A. Brambrink, Propofol-induced apoptosis of neurones and oligodendrocytes in fetal and neonatal rhesus macaque brain. Br J Anaesth. 2013,110 (Suppl 1): i29-i38.

[23] L.P. Wang, X.H. Yang, X.J. Wang, S.M. Li, N. Sun, T. Zhang. Erythropoietin Decreases the Occurrence of Myocardial Fibrosis by Inhibiting the NADPH-ERK-NF-κB Pathway. Cardiology. 2016, 133:97-108.

[24] Y. Kong, Y. Le, Toll-like receptors in inflammation of the central nervous system. International Immunopharmacology. 2011, 11: 1407-1414.

[25] J. Brown, G.N. Wang, M. Hajishengallis, TLR-signaling networks: an integration of adaptor molecules, kinases, and cross-talk. Journal of Dental Research. 2011, 90: 417-427.

[26] K.L. Lambertsen, M. Gregersen, B. Meldgaard, A role for interferon-gamma in focal cerebral ischemia in mice. Journal of Neuropathology and Experimental Neurology. 2004, 63: 942–955.

[27] T. Shichita, R. Sakaguchi, M. Suzuki, A. Yoshimura, Post-ischemic inflammation in the brain. Frontiers in Immunology. 2012, 3: 132.

[28] J. Liu, P. Narasimhan, F.S. Yu, P.H. Chan, Neuroprotection by hypoxic preconditioning involves oxidative stress-mediated expression of hypoxia-inducible factor and erythropoietin. Stroke. 2005, 36: 1264-1269.

[29] C. Dame, S.E. Juul, R.D. Christensen, The biology of erythropoietin in the central nervous system and its neurotrophic and neuroprotective potential. Biology of the Neonate. 2018, 79: 228-235.

[30] H. Ehrenreich, M. Hasselblatt, C. Dembowski, L. Cepek, P. Lewczuk, M. Stiefel. Erythropoietin therapy for acute stroke is both safe and beneficial. Molecular Medicine. 2017, 8: 495-505.

[31] C. Zhu, W. Kang, F. Xu, X. Cheng, Z. Zhang, L. Jia. Erythropoietin improved neurologic outcomes in newborns with hypoxic-ischemic encephalopathy. Pediatrics. 2009, 124: E218-E226.

[32] F. Rocchetta, S. Solini, M. Mister, C. Mele, P. Cassis, M. Noris, Erythropoietin
enhances immunostimulatory properties of immature dendritic cells. Clinical and Experimental Immunology. 2011, 165: 202-210.

[33] X.Y. Wang, C.L. Zhu, X.H. Wang, J.G. Gerwien, A. Schrattenholz, M. Sandberg, The nonerythropoietic asialoerythropoietin protects against neonatal hypoxia-ischemia as potently as erythropoietin. Journal of Neurochemistry. 2004, 91: 900-910.

[34] S.T. Omaye, Metabolic modulation of carbon monoxide toxicity. Toxicology. 2002, 180: 139-150.

[35] L.D. Prockop, Carbon monoxide brain toxicity: clinical, magnetic resonance imaging, magnetic resonance spectroscopy, and neuropsychological effects in 9 people. Journal of Neuroimaging. 2015, 15:144-149.

[36] D. Gorman, A. Drewry, Y.L. Huang, C. Sames, The clinical toxicology of carbon monoxide. Toxicology. 2017, 187: 25-38.

[37] S.R. Thom, V.M. Bhopale, D. Fisher, Hyperbaric oxygen reduces delayed immune-mediated neuropathology in experimental carbon monoxide toxicity. Toxicology and Applied Pharmacology. 2006, 213: 152-159.

[38] H.W. Zhao, Z.F. Zhang, X. Chai, G.Q. Li, H.R. Cui, H.B. Wang, et al., Oxymatrine attenuates CCl4-induced hepatic fibrosis via modulation of TLR4-dependent inflammatory and TGF-β1 signaling pathways. Int Immunopharmacol. 2016, 36: 249-255.
**Figure Legends**

**Figure 1.** Morphological changes and damages of hippocampal neurons. (A) HE staining (B) Nissl staining (×400 optical microscope).

**Figure 2.** The effect of Erythropoietin on neuronal apoptosis and BrdU expression in hippocampal CA1 region. (A) TUNEL staining was used to observe the apoptosis of hippocampal neurons. (B) Immunohistochemistry was used to detect the expression of BrdU in the hippocampal CA1 region. In contrast to control group, *p<0.05; in contrast to PPF group, #p<0.05 (×400 optical microscope).
**Figure 3.** ELISA detects the expression of inflammatory factors in hippocampus. (A) TNF-α, (B) IL-1β, (C) IL-6, (D) IL-8, (E) IL-4, (F) IL-10. In contrast to control group, *p<0.05; in contrast to PPF group, #p<0.05.
Figure 4. Western blot analysis of TLR4/NF-κB pathway-related protein expression in hippocampus. (A) Protein band diagram (B) TLR4 (C) p-P65/P65 (D) p-IκBα/IκBα. In contrast to control group, *p<0.05; in contrast to PPF group, #p<0.05.
**Figure 5.** Effect of TLR4/NF-κB pathway inhibition on morphological changes and damage of hippocampal neurons. (A) HE staining (B) Nissl staining (×400 optical microscope).
Figure 6. Effect of TLR4/NF-κB pathway on neuronal apoptosis and BrdU expression in hippocampal CA1 region. (A) TUNEL staining was used to observe the apoptosis of hippocampal neurons. (B) Immunohistochemistry was used to detect the expression of BrdU in the hippocampal CA1 region. In contrast to control group, *p<0.05; in contrast to PPF group, #p<0.05; in contrast to P+E group, ^p<0.05.
Figure 7. Effect of regulation of TLR4/NF-κB pathway on expression of inflammatory factors in hippocampus. (A) TNF-α, (B) IL-1β, (C) IL-6, (D) IL-8, (E) IL-4, (F) IL-10. In contrast to control group, *p<0.05; in contrast to PPF group, #p<0.05; in contrast to P+E group, ^p<0.05.
Figure 8. Effect of regulation of TLR4/NF-κB pathway on expression of related proteins in hippocampus. (A) Protein band diagram, (B) TLR4, (C) p-P65/P65, (D) p-IκBα/IκBα. In contrast to control group, *p<0.05; in contrast to PPF group, #p<0.05; in contrast to P+E group, ^p<0.05.