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FANGCHINOLINE AMELIORATES THE EXPRESSIONS OF ANGIOGENIC MOLECULE IN CEREBRAL ISCHEMIA INDUCED NEURONAL DEGENERATION IN NEONATAL RATS

Han Daicheng,
Xia Shiwen*,
Zhu Huaping,
Liu Yong,
Zhou Qianqian,
Hu Changxia

Department of Neonatology, Hubei Maternal and Child Health Hospital, Wuhan, Hubei 430070, China

Abstract

Background: Present investigation evaluates the beneficial effect of fangchinoline on cerebral ischemia induced neuronal degeneration in neonatal rats and also postulates the possible mechanism of its action. Methodology: Cerebral ischemia was produced by the ligation of right common carotid artery in neonatal rats on postnatal day 5 (P5) and further pups were treated with fangchinoline 3, 10 and 30 mg/kg, i.p. for the period of 3 days. Effect of fangchinoline was estimated by determining the brain injury and enzyme linked immunosorbent assay (ELISA) method was used for the estimation of pro-inflammatory mediators and markers of oxidative stress in the cerebral tissues of neonatal rats. Moreover western blot assay and histopathology study was also performed on the brain tissue. Results: Result of this investigation reveals that the percentage of brain injury significantly reduces and enhancement of myelin basic protein in the cerebral tissues of fangchinoline than ischemic group. Treatment with fangchinoline attenuates the altered level of proinflammatory mediators and markers of oxidative stress in the cerebral tissue of cerebral ischemia induced neuronal injury neonatal rats. Moreover expressions of inducible nitric oxide synthtase (iNOS), vascular endothelial growth factor (VEGF), p53 and nuclear receptor factor-2 (Nrf2) in the brain tissue attenuated by fangchinoline treated group. Conclusion: In conclusion, fangchinoline ameliorates the cerebral ischemia induced neuronal injury in neonatal rats by enhancing angiogenesis molecules.

Keywords

• Fangchinoline • cerebral ischemia • Neurodegeneration • Angiogenesis

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Introduction

Prenatal hypoxia-ischemia is one of the major causes of morbidity and mortality throughout the world in the neonates [1]. Infants suffer from prenatal hypoxia suffer from neurological manifestations such as learning deficits, motor disturbance, alteration in behavior and neurological disability due to brain injury and inflammation [2]. Moreover a study also reports the cerebral dysmaturation disorder may occur in the neonatal rats that suffer from cerebral ischemia/ hypoxia during prenatal time [3].

Brain function of infants suffering from prenatal hypoxia-ischemia could be recovered by neurogenesis and angiogenesis [4]. Literature reveals that vascular endothelial growth factor (VGEF) enhances the angiogenesis and thereby protects the cerebral injury [5]. Moreover p53 expression reported to be enhanced in the brain tissues of cerebral ischemic rats and p53 regulates the level of VEGF [6]. In addition angiogenesis factors report to protect the cerebral ischemia induced injury by reducing the expressions of inflammatory cytokines [7]. Inflammatory cytokines level was found to be enhanced in the hypoxic brain tissues and thereby promoting brain injury [8].

In the past few decades alternative medicine has shown the potential role in the management of several disorders including neuronal disorders. Fangchinoline is chemically an alkaloid isolated from *Stephania tetrandra* S. and reported for its anti-inflammatory activity [9-10]. Literature reveals that fangchinoline attenuates the production of tumor necrosis factor-α (TNF-α), interlukin-6 (IL-6) and IL-1 and inhibit the activity of cyclooxygenase [11]. Fangchinoline has also posse's strong radical scavenging, hypotensive, anti cancer, antioxidant and antithrombotic activity [12-14]. Thus present study evaluates the beneficial effect of fangchinoline on cerebral ischemia induced neuronal injury in neonatal rats.

Material and methods

Animals

Female Wistar rats were procured from Changzhou Cavens Laboratory Animal Co. Ltd. on the 19th day of gestation. Animals were kept under the standard environment as per the guideline such as Humidity maximum 60% and 22 ± 2^{0}C of temperature. The day of birth was defined as postnatal day 0 (P0). All the protocol of this investigation was approved by institutional animal ethical committee of Hubei Maternal and Child Health Hospital, China (HMCHH/IAEC/2017/02).

Experimental

All the animals on postnatal day 5 (P5) was used to perform the surgery. The surgery was performed as per the previously reported method. All the animals were anesthetized with isoflurane and ligation of right common carotid artery was done using 8-0 suture and later

* *E-mail: ScottqPerezlb@yahoo.com*

for recovery from anesthesia rats were placed on a heating pad at 37 °C for the period of 90 min. During the recovery period all the animals were exposed to the hypoxic condition. All the animals were separated in to five different groups such as Sham group in which pups were operated without ligation and receive saline solution. Pups of ischemic group were operated for the induction of ischemia and receive saline solution. Fangchinoline was procured Sigma Aldrich, USA. Fangchinoline (3, 10 and 30 mg/kg) receives fangchinoline 3, 10 and 30 mg/kg, i.p. for the duration of 3 days from the day of surgery. All the pups (P21) were sacrificed after the sixteen days of operation by cervical dislocation.

Estimation of brain damage

Brain was isolated from all the pups and brain section of 10 µm thickness was cut after embedded in to paraffin. Staining of Myelin basic protein (MBP) was done and Micro Image version 4.0 was used for the quantitative estimation of white and gray matter injury.

Histopathology

Isolated brain was fixed by deeping it into formalin solution for the period of one day at room temperature. Brain tissue of 4 µm thickness was section and hematoxylin and eosin (H&E) was used to stain the tissue sample. Confocal microscope was used for observing the tissue samples.

Estimation of oxidative stress parameters

ELISA kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) and the level of malonaldehyde (MDA) and activity of superoxide dismutase (SOD) was determined in the brain tissue of ischemia induced brain injured rats by using these kits. Absorbance of the sample was estimated at a wavelength of 450 nm using UV-spectrophotometer.

Estimation of pro-inflammatory mediators

Brain tissue homogenate was used for the determination of level of IL-1β and TNF-α by ELISA assay. Enzyme-linked immunosorbent assay kits were used for the determination of pro-inflammatory mediators and method was performed as per the instruction given by the manufacturer.

Western blot assay

Isolation of protein sample was achieved by homogenizing samples and thereafter estimating of protein was done by BCA kit. Further electroblotting technique was used for the transfer of protein to nitrocellulose membrane after the separation of protein with 10% SDS PAGE. 5% blocking solution was used to block the membrane and thereafter membrane was incubated for the period of overnight at 4 °C with primary antibody inducible nitric oxide synthase (iNOS), Nrf2, VEGF, p53 and β-actin (Proteintech Group Inc, China). Later membrane was incubated with horseradish peroxidase-conjugated secondary antibody (Proteintech Group Inc, China) and the density of blots was determined by scanning densitometry using the Quantity One Software.

Statistical analysis

All data were expressed as mean ± SEM (n = 10). The statistical analysis was performed using one way ANOVA. Post-hoc comparison of means was carried out by Dunnett's post hoc test (Gradpad prism 6.1., CA, USA) multiple comparisons. The level of statistical significance was set at $P < 0.05$.

Result

Effect of fangchinoline on the brain injury

Fig.1. Shows the effect of fangchinoline on the brain injury in cerebral ischemia induced neuronal degeneration in neonatal rats. Brain injury was found to be significantly enhanced in ischemic group than sham group. Moreover myelin basic protein was also found to be significantly ($p<0.01$) decreases in ischemic group than sham group. It was observed that percentage of brain injury decreases and increase in myelin basic protein in the cerebral tissue of fangchinoline treated group than ischemic group of rats.

Assessment of pathphysiological changes

Effect of fangchinoline on the histopathological changes in the brain tissues of cerebral ischemia induced neuronal degeneration neonatal rats was shown in Fig. 2. H&E staining was done for the examination of histopathological changes. It was observed that the number of neuronal cells were lower in the brain of ischemic group than sham group. Moreover number of neuronal cells was higher in the brain tissues of neuronal cells than ischemic group.

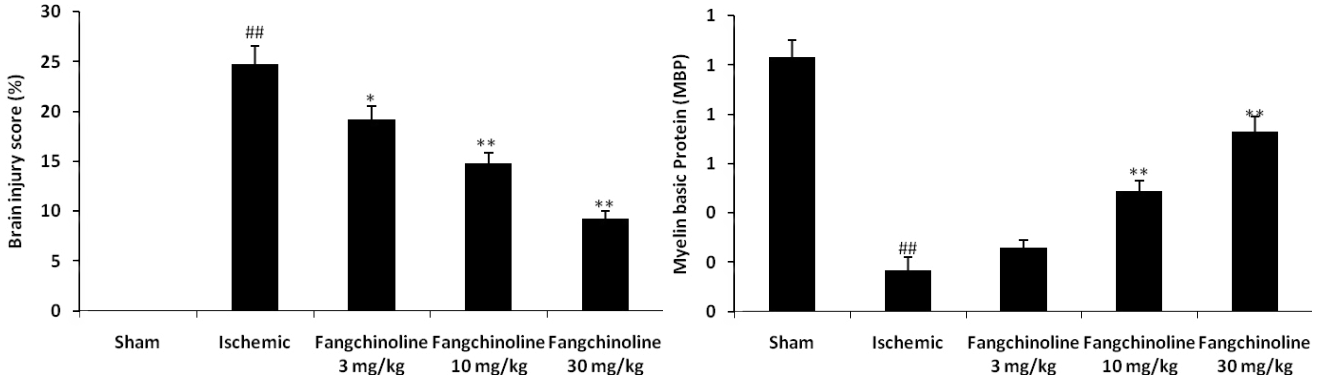

Fig. 1. Effect of fangchinoline on the brain injury in cerebral ischemia induced neuronal degeneration in neonatal rats. Mean±SEM (n=10), ##p<0.01 Vs Sham group; *p<0.05, **p<0.01 Vs Ischemic group

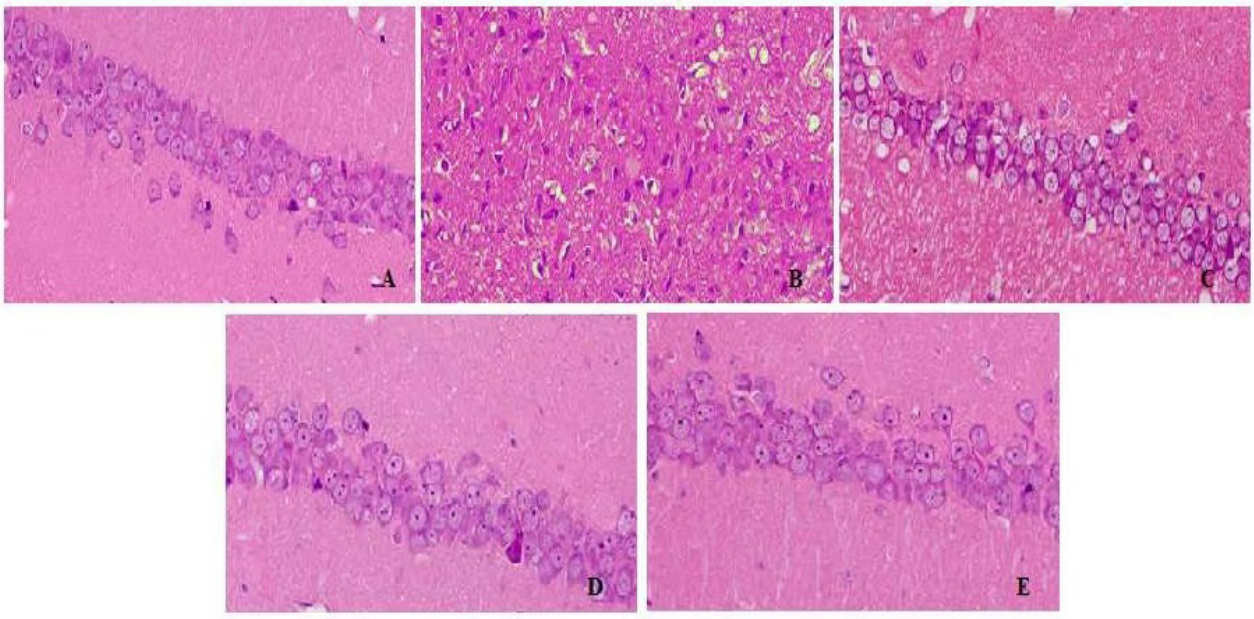

Fig. 2. Effect of fangchinoline on the histopathology of brain of cerebral ischemia induced neuronal degeneration in neonatal rats. A: Sham; B: Ischemic; C: Fangchinoline 3 mg/kg; D: Fangchinoline 10 mg/kg; E: Fangchinoline 30 mg/kg

Effect of fangchinoline on the markers of oxidative stress

Fig 3 shows the effect of fangchinoline on the markers of oxidative stress such as SOD and MDA in the brain tissue of cerebral ischemia induced neuronal degeneration neonatal rats. Level of MDA was found to be significantly enhanced and activity of SOD significantly reduced in ischemic group than sham group of rats. However Level of MDA significantly reduced and activity of SOD significantly enhanced in the brain tissue of fangchinoline treated group compared to ischemic group as per dose dependent manner.

Effect of fangchinoline on the proinflammatory mediators

Effect of fangchinoline on the pro-inflammatory mediators such as IL-1β and TNF-α was shown in the cerebral tissues of cerebral ischemia induced neuronal degeneration neonatal rat model (Fig.4.). It was observed that level of IL-1β and TNF-α significantly enhanced ($p<0.01$) in the brain tissue of ischemic group compared to sham group of rats. However there was significant decrease ($p<0.01$) in the level of IL-1β and TNF-α in the brain tissue of fangchinoline treated group than ischemic group.

Effect of fangchinoline on the expression of iNOS, Nrf2, VEGF and p53

Effect of fangchinoline on the expression of *iNOS, Nrf2, VEGF and p53* was shown in the brain tissues homogenate of cerebral ischemia induced neuronal degeneration in neonatal rat (Fig. 5.). It was observed that expressions of iNOS and VEGF significantly enhanced ($p<0.01$) and expression of p53 significantly ($p<0.01$) reduced in the cerebral tissue of ischemic group than sham group. However treatment with fangchinoline significantly reduced ($p<0.01$) the expression of iNOS and VEGF and

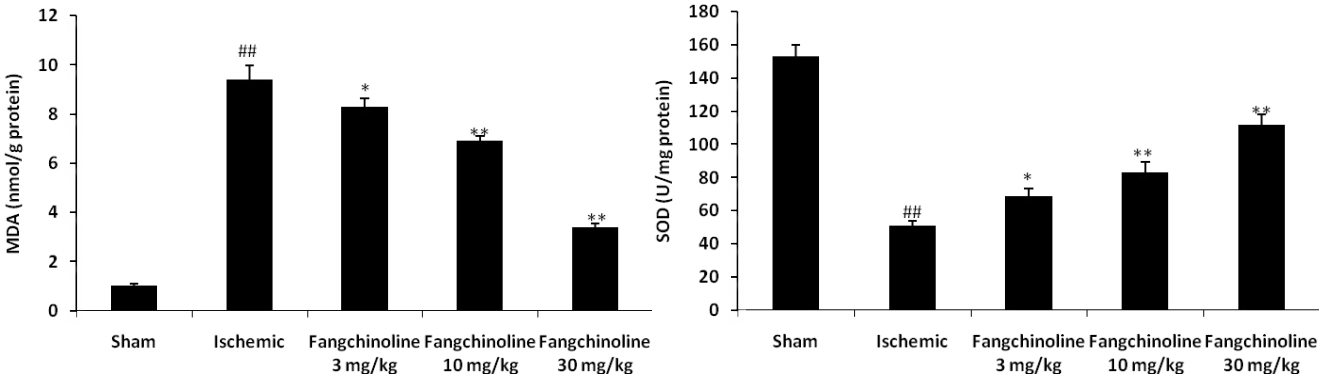

Fig. 3. Effect of fangchinoline on the markers of oxidative stress in the brain tissues homogenate of cerebral ischemia induced neuronal degeneration in neonatal rats. Mean±SEM (n=10), ##p<0.01 Vs Sham group; *p<0.05, **p<0.01 Vs Ischemic group

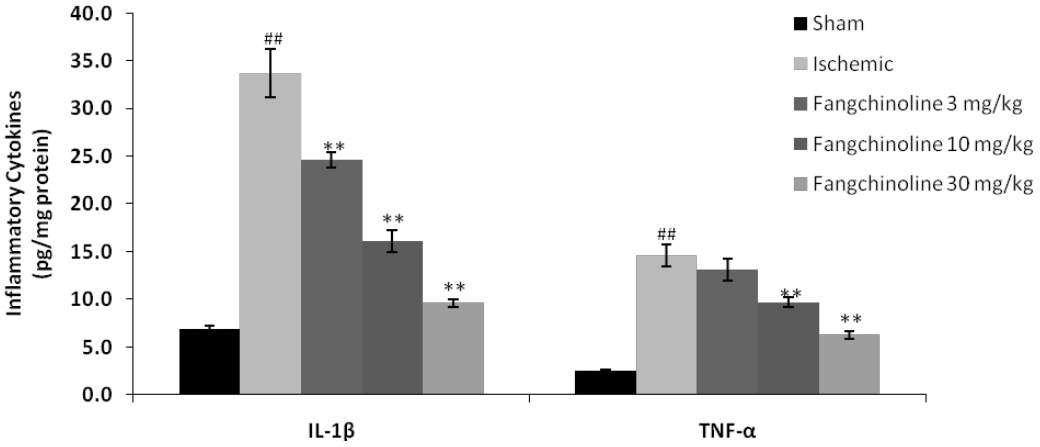

Fig. 4. Effect of fangchinoline on the proinflammatory mediators such as IL-1β and TNF-α in the cerebral tissue of cerebral ischemia induced neuronal degeneration in neonatal rats. Mean±SEM (n=10), ##p<0.01 Vs Sham group; *p<0.05, **p<0.01 Vs Ischemic group

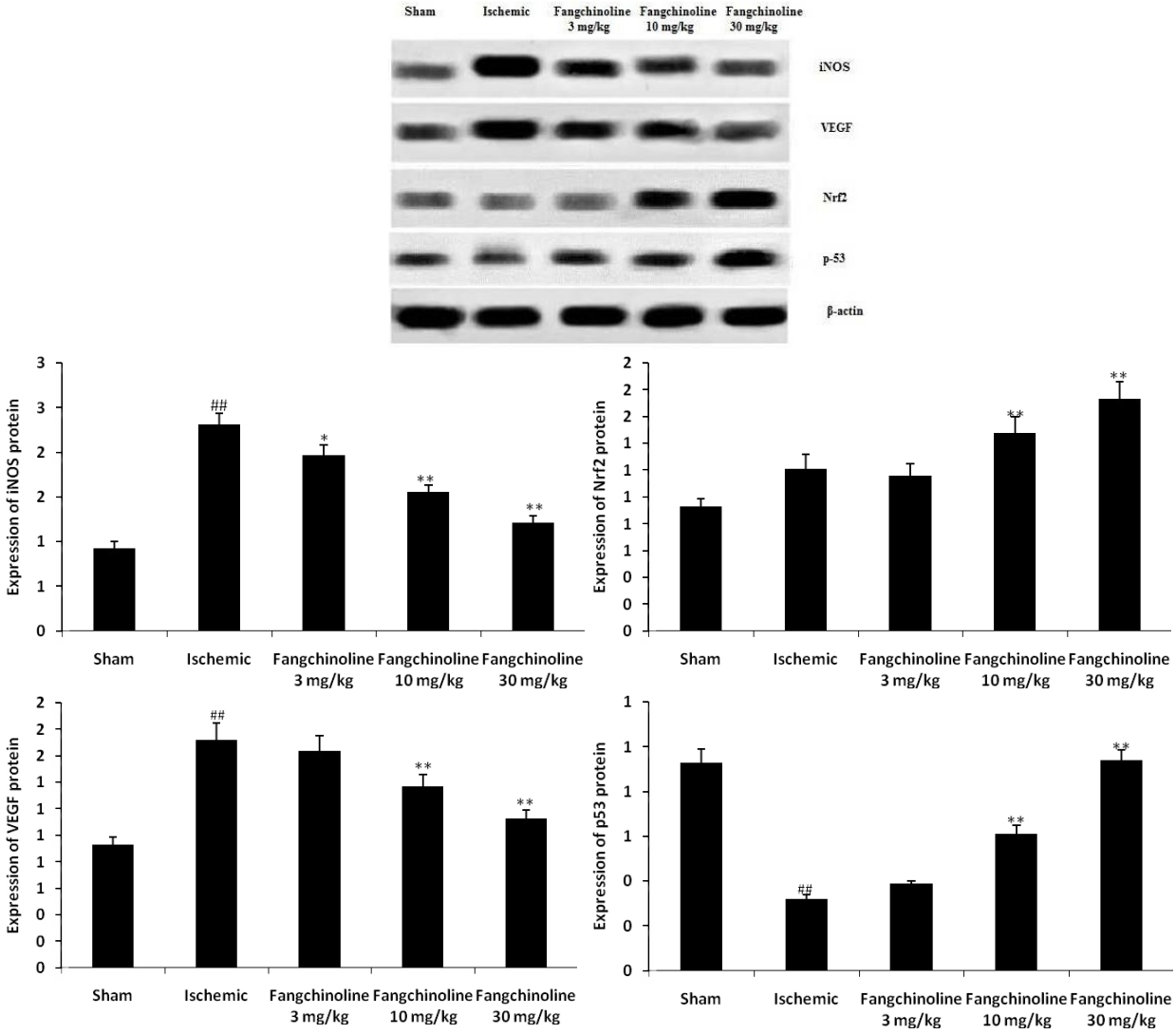

Fig. 5. **Effect of fangchinoline on the expression of** iNOS, Nrf2, VEGF and p53 **in the cerebral tissues of cerebral ischemia induced neuronal degeneration in neonatal rats.** Mean±SEM (n=10), ##p<0.01 Vs Sham group; *p<0.05, **p<0.01 Vs Ischemic group

enhanced the expression of p53 and Nrf2 in the cerebral tissue compared to sham group of rats.

Discussion

Hypoxia-ischemia in neonatal rats causes degeneration of neurons and causes permanent neurological defects [2]. Thus present investigation evaluates the benifical effect of fangchinoline against cerebral ischemia induced brain injury in neonatal rat model. Cerebral ischemia was produced by the ligation of right common carotid artery and effect of fangchinoline was estimated by determining the brain injury and ELISA method was used for the estimation of pro-inflammatory mediators and markers of oxidative stress in the cerebral tissues of neonatal rats. Moreover western blot assay and histopathology study was also performed on the cerebral tissue.

Literature reveals that oxidative stress was produced due to increase in the production of reactive oxygen species (ROS) in the hypoxia ischemia induced brain injured tissues [15]. Moreover balance between antioxidant and oxidant was also disturbed due to hypoxia ischemia [16]. Data of the study reveals that fangchinoline attenuates the enhanced level of oxidative stress in the brain tissue of ischemia induced neuronal injury neonatal rats. Moreover fangchinoline was significantly ($p<0.01$) reduces the expressions of iNOS in the brain tissue compared to ischemic group. Inflammatory mediators play vital role in the development of neuronal injury and induce the neuronal apoptosis in hypoxia ischemia induced brain injury [17]. Nrf2 pathway has reported to reduce the apoptosis of neuronal cell [18]. Result of the study reveals that level of pro-inflammatory mediators significantly decreases and the expression of Nrf2 enhanced in the brain tissue of fangchinoline treated group than ischemic group.

p53 is a marker of neuronal cell death in cerebral ischemia injury and decrease in the expression of p53 protects the neuronal damage in the injured brain tissues [19]. Moreover VEGF enhances angiogenesis in the cerebral ischemic injury and thereby protects cerebral injury. Expression of p53 in the brain tissue was significantly decreases and increases the expression of VEGF in fangchinoline treated group compared to ischemic group. Literature suggested that attenuation of altered expression of p53 and VEGF in the brain tissue of cerebral injury promotes angiogenesis and neurogenesis [20].

Conclusion

In conclusion, data of study suggest that fangchinoline ameliorates the neuronal injury in cerebral ischemia induced brain injury in neonatal rats by enhancing the angiogenesis molecules. Oxidative stress and mediators of inflammation reduces in fangchinoline treated group which further protects the neuronal injury.

Conflict of interest

No

Acknowledgement

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