International Journal of Pharmacology

ISSN 1811-7775

science alert

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Asian Network for Scientific Information

OPEN ACCESS International Journal of Pharmacology

ISSN 1811-7775
DOI: 10.3923/ijp.2019.801.808

Research Article
Barbaloin Ameliorates the Memory in Isoflurane Induced Neuronal Injury by Regulating the BDNF/Bcl-2/PI3K Signaling Pathway

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Abstract

Background and Objective: Clinical application of anesthetics in neonatal or children causes neuronal degeneration. Present study evaluates the neuroprotective effect of barbaloin against anesthetic induced neuronal injured rats and also postulates the possible mechanism of its action. **Materials and Methods:** Animals on P7 were exposed for 6 h with 30% of oxygen that contain 0.75% of isoflurane and rats were treated with barbaloin 10 and 20 mg kg^{-1}, p.o. for the duration of 21 days. Cognitive function was evaluated by using morris water maze (MWM) and level of mediators of inflammation and oxidative stress were estimated in the brain tissue by using enzyme-linked immunosorbent assay (ELISA). Moreover western blot assay was performed for the determination of expression of proteins in the neuronal tissues and apoptosis of neuronal cells were observed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay and Fluro Jade B staining. **Results:** Data of report suggest that barbaloin promotes the cognitive function by MWM and apoptosis of neuronal cells was also found to be reduced in barbaloin treated group than isoflurane (INF) group. There was decrease in the mediators of inflammation and oxidative stress in barbaloin treated group compared to INF group. Moreover western blot assay data reveals that barbaloin ameliorates the altered expression of JNK, ERK-1/2, Bcl-2, PI3K, Akt, BDNF and TrKB proteins in the brain tissues of anesthetic induced neuronal injured rats. **Conclusion:** Data of the report reveals the protective effect of barbaloin against isoflurane induced neuronal injured rats by regulating BDNF/Bcl-2/PI3K signaling pathway.

Key words: Barbaloin, isoflurane, neuronal injury, neonatal rats, inflammation, oxidative stress

Citation: Bingqi Duan, Yi Huang and Yuan Chen, 2019. Barbaloin ameliorates the memory in isoflurane induced neuronal injury by regulating the BDNF/Bcl-2/PI3K signaling pathway. Int. J. Pharmacol., 15: 801-808.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Anesthetics are used for the management of several medical procedures, surgeries and for reliving the pain. Literature reveals that for the purpose of medical care every year millions of children were exposed to anesthesia[1]. Several report suggested that the use of isoflurane leads to injury to kidney, brain, cardiovascular and other organs including degeneration of neurons which further impairs the memory and learning in rodents[2]. Even clinical reports also suggested that children's with the age of less than 4 years exposed to anesthetic have more chances of impairment of memory and cognitive functions[3]. All these investigations focused the concern on the use of anesthetics in children. Growth of neuronal cell in the CNS is depending on the expression of Akt/PI3K pathway, as activation of brain-derived neurotrophic factor occurs by PI3K protein[4]. BDNF activates p75 receptor, TrkB and receptors of cell surface and thereby shows its neuroprotective effect[5]. Moreover Akt protein activates the growth factor which controls the process of apoptosis in neuronal cells. Literature reveals that anesthetics induce toxicity to neuronal cells by regulating the ERK1/2 and JNK signaling[6]. Akt protein involved in JNK signalling induced apoptosis of neuronal cells[7].

Management of neuronal injury still is a challenge for the medical field and thus scientist are focusing on new therapy strategies. Alternative medicines have shown promising effect in the development of drug therapy for the treatment of anesthetics induced neuronal injury[8]. Barbaloin is a chemical constituent isolated from Aloe, which is chemically known as 10- beta -D-glucopyranosyl-1, 8-dihydroxy-3-hydroxymethyl-9(10H)-anthracenone[9]. Barbaloin reported for its anti-inflammatory, antihistaminic, antimicrobial, anticancer and laxative activity[10]. Thus present study evaluates the neuroprotective effect of barbaloin against anesthetic induced neuronal injured rat model.

MATERIALS AND METHODS

Animals: Pups of SD rats of 7 days old age were purchased from Dashuo Laboratory Animal Reproduction Center, China. Standard guidelines (Humidity: 60±5%, Temperature: 24±3°C) were used to store the pups for 12 h light and dark cycle. All the protocols of the study were approved by The Institutional Animal Care and Use Committee of Xiangya Hospital Central South University, China (IACUC/XH-CSU/2017/16).

Experiment: All the animals were separated into four different groups like normal group, IFN group which receives only isoflurane and barbaloin 10 and 20 mg kg^{-1} treated group receives Barbaloin 10 and 20 mg kg^{-1}, p.o. for the duration of 21 days. All the animals on P7 were exposed for the duration of 6 h with 30% of oxygen that contain 0.75% of isoflurane. At the end of treatment protocol rats were sacrificed and the brain was isolated from each animal for the determination of neuronal apoptosis and expression of protein.

Determination of behavioral changes: Behavioral changes in the rat were determined by using Morris water maze apparatus as per previously reported method[11]. Observation of swimming behavior was done for the duration of 6 days continuously and escape latency was determined. Later by escaping the platform spatial memory of each mouse was determined and effect of barbaloin was also estimated in isoflurane induced cognitive dysfunction rats.

Fluoro-jade B staining: The FJB staining was used to determine the degeneration of neuron in the hippocampi tissues. Section of tissue of 30 µm thickness was fixed at room temperature and potassium permanganate solution (0.06%) was used to incubate with the tissue for the duration of 15 min. Further FJB stain was used to stain the tissues. Microscope was used to determine the neuronal apoptosis after incubating it for the duration of half an hour with acetic acid (0.1%).

TUNEL assay: The TUNEL assay was performed for the evaluation of neuronal apoptosis. All the rats were sacrificed by cervical dislocation and the brain was isolated, further hippocampi was separated from it. Hippocampi tissue was dehydrated and further seeded into paraffin. Further microtome was used to section into 6 mm thickness of about 200 µm apart. Promega's TUNEL system kit was used to estimate the neuronal apoptosis as per the instruction given by manufacturer. Estimation of number of apoptotic cell was determined by using NIS-Elements BR imaging processing and analysis Software.

Determination of biochemical parameters: ELISA kits were used to determine the concentration of mediators of inflammation such as NF-κB, IL-1β and TNF-α in the brain tissues of isoflurane induced neuronal injured rats. Moreover parameters of oxidative stress such as level of MDA and GSH and activity of SOD were estimated in the brain tissues by using ELISA kit.

RT-PCR: Separated hippocampus from isolated brain was used to isolate the RNA by Trizol Reagent. RevertAid First Strand cDNA Synthesis Kit was used to reversely transcribe the RNA. Primers mention below was mixed with RT 2 SYBR Green Master to determine the gene expression by Quantitative SYBR Green PCR assays.

Western blot assay: Brain tissues of injured location were separated out and tissue lysis was done using lysis buffer. Supernatant from the lysed tissue was separated out by centrifuging the lysate for the period of 5 min at 10000 rpm. Later sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used to separate the total protein and then separated proteins were filtered on nitrocellulose membrane. Further membrane was incubated with I^{ry} antibodies like p-PI3K, p-Akt Bcl-2, Bad, Bax, ERK-1/2, p-ERK-1/2, BDNF, JNK, GADPH and β-actin for overnight at 4°C. Later horseradish peroxidase-conjugated secondary antibody was used to incubate for the period of 60 min at room temperature with total protein. Image J software was used to estimate the band density.

Assessment of cyclic AMP: Level of cyclic AMP was determined by using ELISA kit in the brain tissues of isoflurane and barbaloin treated groups as per the instruction given by manufacturer. Level of cAMP was expressed as pmol/mg.

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 $p<0.05$.

RESULTS

Barbaloin improves the cognitive functions: Assessment of cognitive function in barbaloin and isoflurane treated group was shown in Fig. 1. Percentage number of crossing and time spent in the target quadrant was found to be less and escape latency was more in INF group than normal group. Off note, barbaloin reported to significantly ($p<0.01$) improve the memory as percentage number of crossing and time spent in the target quadrant was enhanced ($p<0.01$) and escape latency was significantly ($p<0.01$) reduced in barbaloin group than INF group.

Barbaloin ameliorates the neuronal cell apoptosis: Apoptosis of neuronal cell was determined in the different regions of brain by TUNEL and Fluro Jade B staining (Fig. 2). TUNEL positive and Fluro Jade B positive cells were enhanced ($p<0.01$) significantly in the different regions of the brain of INF group than normal group. However apoptosis of neuronal cells was attenuated in the barbaloin treated anesthetic induced neuronal injured rats.

Barbaloin ameliorates biochemical parameters: Several biochemical parameters like mediators of inflammation and oxidative stress were observed in brain tissue of anesthetic induced neuronal injured rats. Mediators of inflammation like NF-κB, TNF-α and IL-1β were effectively ($p<0.01$) enhanced in the brain tissues of INF treated group than normal group. Barbaloin significantly ($p<0.05$; $p<0.01$) reduces the level of NF-κB, TNF-α and IL-1β in the brain tissues than INF group. Level of MDA found to be more and activity of SOD reduced in the brain tissues of INF group than normal group. However, treatment with barbaloin ameliorates the altered level of MDA and activity of SOD in the tissue homogenate of anesthetic induced neuronal injured rats (Fig. 3).

Barbaloin ameliorates the BDNF-TrKB pathway: Expression of TrKB and BDNF protein were estimated in the brain tissues of anesthetic induced neuronal injured rats (Fig. 4). In INF group, expression of TrKB and BDNF protein were reduced significantly ($p<0.01$) than normal group. However, barbaloin attenuates the expression of BDNF and TrKB in anesthetic induced neuronal injured rats.

Barbaloin ameliorates the expression of JNK/ERK-1/2/BDNF signaling proteins: Animals treated with barbaloin and isoflurane were assessed for the expression of JNK, ERK-1/2 and BDNF (Fig. 5). It was observed that the expression of JNK enhanced and expression of ERK-1/2 and BDNF reduced significantly ($p<0.01$) in INF group than normal group. There was reduction in the expression of JNK and expression of ERK-1/2 and BDNF significantly ($p<0.01$) enhanced in the brain tissues of barbaloin treated group than INF group.

Barbaloin ameliorates the apoptotic pathway: Effect of barbaloin on the apoptosis of neuronal cells was determined by estimating the expression of p-PI3K, p-Akt, Bcl-2, Bad and Bax in anesthetic induced neuronal injured rats. It was found that expression of protein that contribute in the apoptosis such as p-PI3K, p-Akt, Bcl-2 and Bax protein reduced

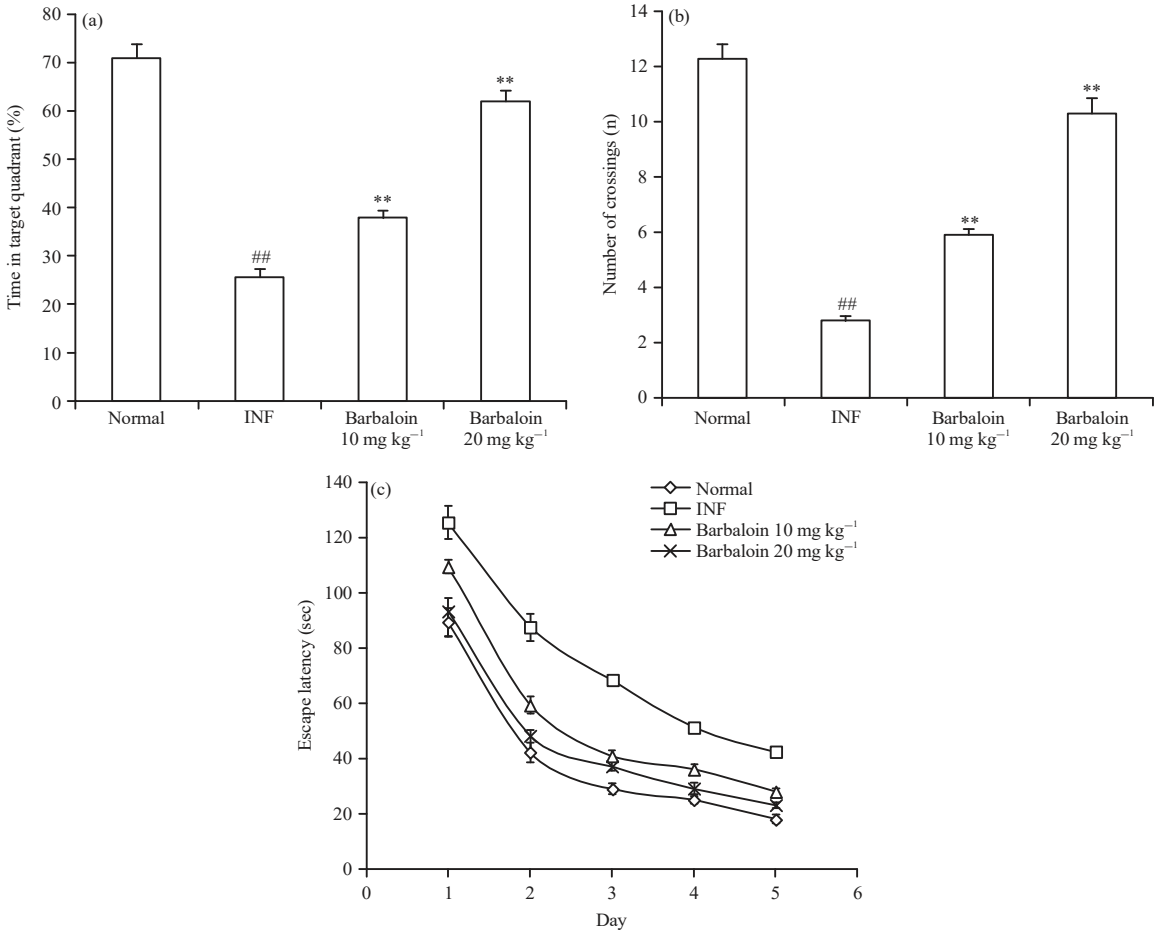

Fig. 1(a-c): Barbaloin improves the cognitive functions evaluated by MWM in anesthetic induced neuronal injured rats, (a) Time spent in the target quadrant, (b) Number of crossing and (c) Escape latency

Mean±SEM (n = 10), ##p<0.01 compared to normal group, **p<0.01 compared to INF group, INF: Isoflurane

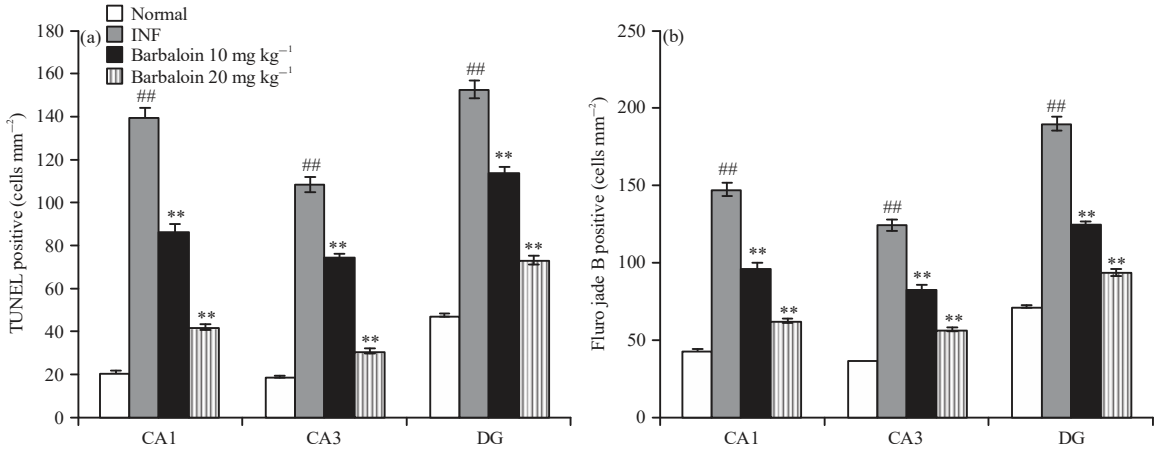

Fig. 2(a-b): Barbaloin ameliorates the neuronal cell apoptosis in anesthetic induced neuronal injured rats, (a) TUNEL staining and (b) Fluro Jade B staining

Mean±SEM (n = 10), ##p<0.01 compared to normal group, **p<0.01 compared to INF group, INF: Isoflurane

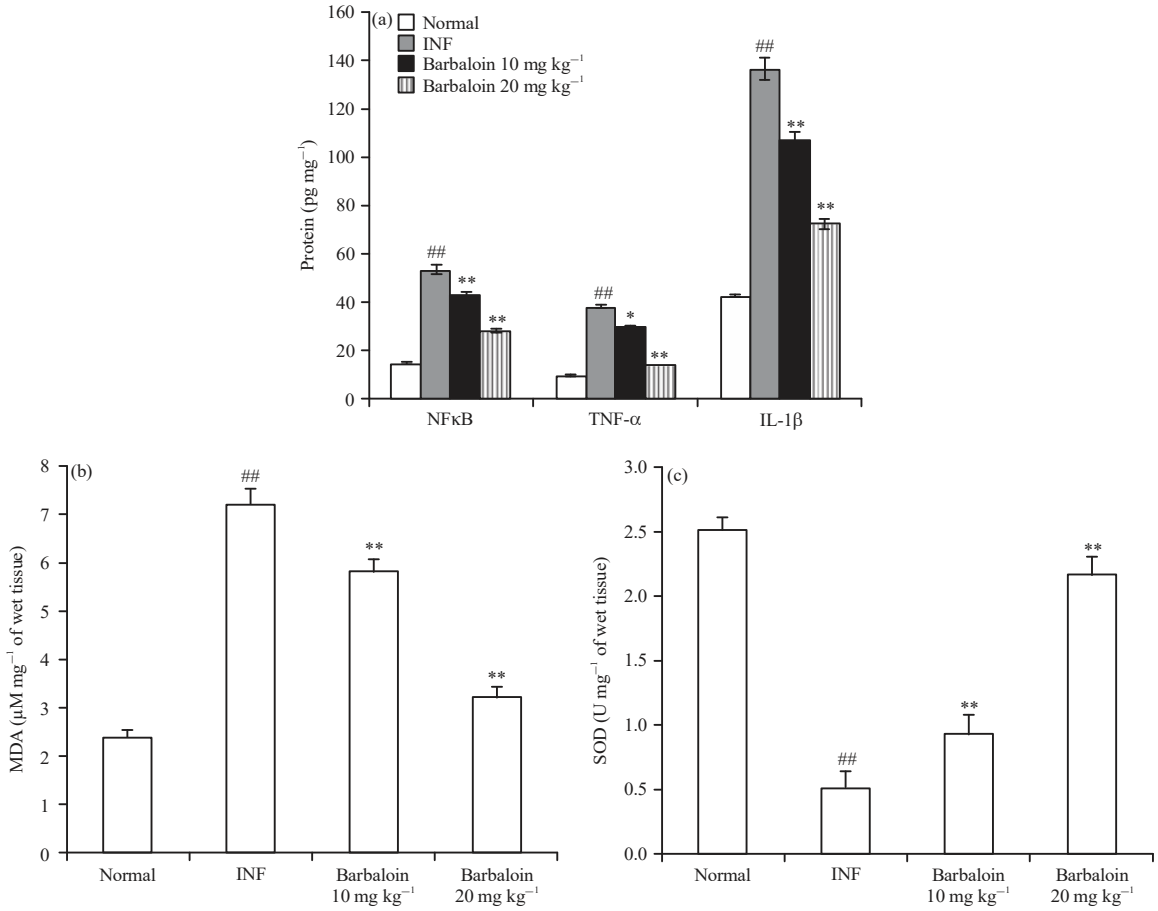

Fig. 3(a-c): Barbaloin effectively reduces mediators of inflammation and parameters of oxidative stress in anesthetic induced neuronal injured rats, (a) Level of inflammatory cytokines in the neuronal tissues, (b) Level of MDA in the neuronal tissues and (c) Activity of SOD in the neuronal tissues

Mean±SEM (n = 10), ##p<0.01 compared to normal group, **p<0.01 compared to INF group, INF: Isoflurane, MDA: Malondialdehyde, SOD: Superoxide dismutase

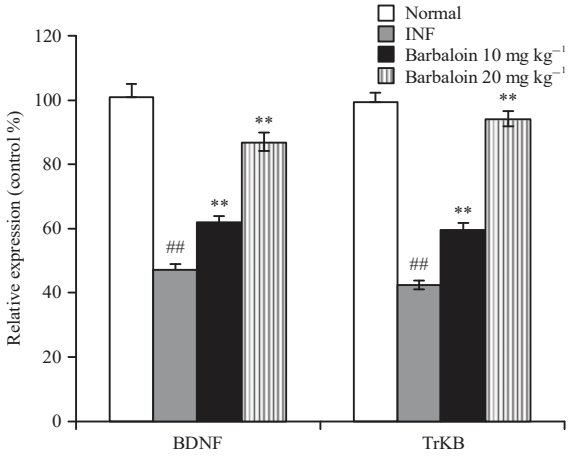

Fig. 4: Barbaloin ameliorates the expression of BDNF and TrKB in anesthetic induced neuronal injured rats

Mean±SEM (n = 10), ##p<0.01 compared to normal group, **p<0.01 compared to INF group, INF: Isoflurane, BDNF: Brain derived neurotrophic, TrKB: Tropomyosin receptor kinase B

significantly (p<0.01) and expression of Bad enhanced in the brain tissues of INF group than normal group. However, Barbaloin ameliorates the altered expression of p-PI3K, p-Akt, Bcl-2, Bad and Bax in anesthetic induced neuronal injured rats (Fig. 6).

Barbaloin ameliorates the level of cAMP: Assessment of level of cAMP in barbaloin and isoflurane treated animals was shown in Fig. 7. There was significant (p<0.01) decrease in the level of cAMP in the brain tissues of INF group than normal group. Treatment with barbaloin effectively increases (p<0.01) the level of cAMP in brain tissue of anesthetic induced neuronal injured rats than INF group.

DISCUSSION

Anesthetic used in the neonates or in children leads to the degeneration of neurons by inducing the apoptosis of

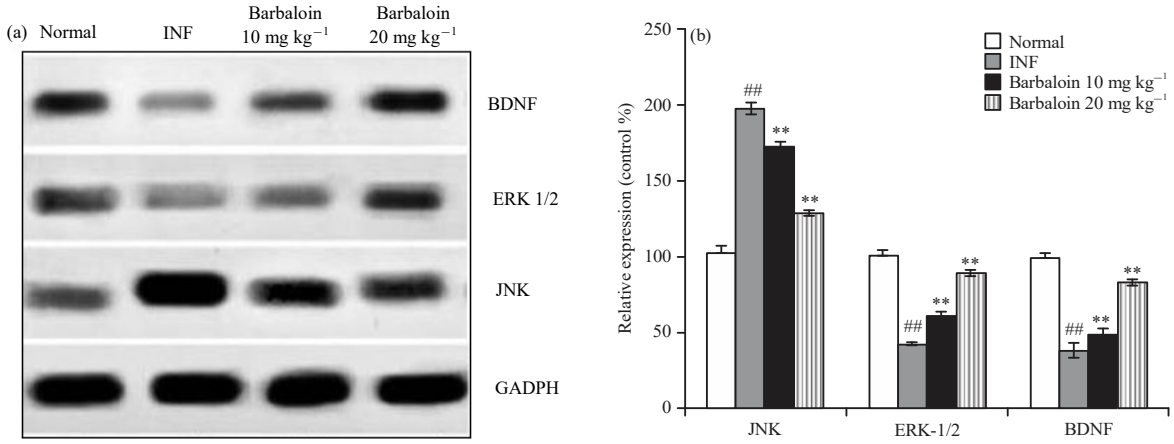

Fig. 5(a-b): (a) Barbaloin ameliorates the expression of JNK, ERK-1/2 and (b) BDNF in anesthetic induced neuronal injured rats

Mean±SEM (n = 10), ##p<0.01 compared to normal group, **p<0.01 compared to INF group, INF: Isoflurane, ERK: Extracellular regulating kinase, JNK: Jun.N-Terminal kinase, GADPH: Glyceraldehyde 3 phosphate dehydrogenase, BNDF: Brain derived neurotrophic factor

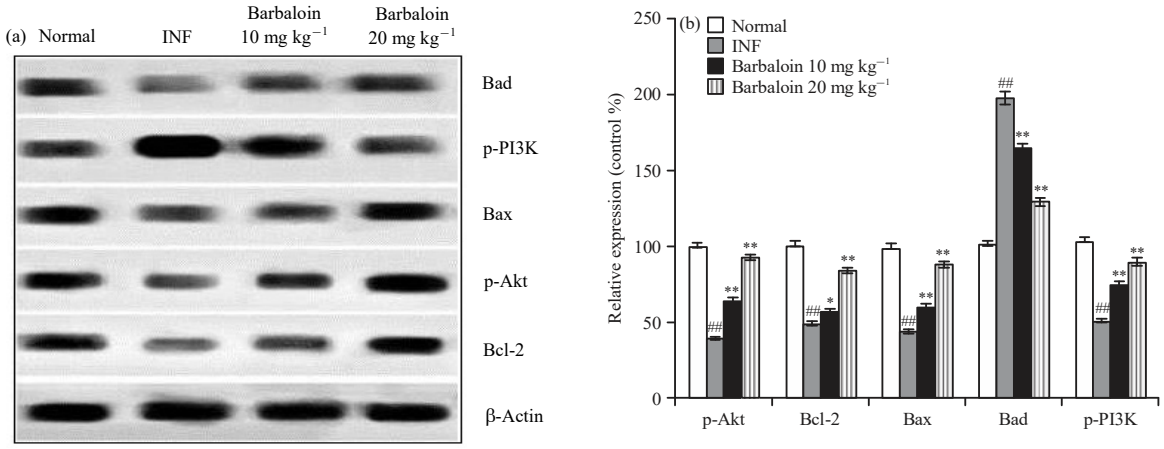

Fig. 6(a-b): (a) Barbaloin ameliorates the expression of p-PI3K, p-Akt, Bcl-2, Bad and (b) Bax in anesthetic induced neuronal injured rats

Mean±SEM (n = 10), ##p<0.01 compared to normal group, **p<0.01 compared to INF group, INF: Isoflurane, P13K: Phosphoinositide-3 kinase, Bcl-2: B-cell lymphoma 2

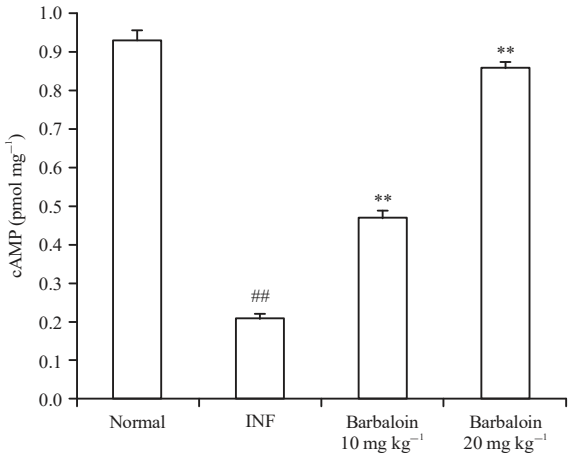

Fig. 7: Barbaloin ameliorates the level of cAMP in anesthetic induced neuronal injured rats

Mean±SEM (n = 10), ##p<0.01 compared to normal group, **p<0.01 compared to INF group, cAMP: Cyclic adenosine monophosphate

neuronal cells. Moreover anesthetic induced neuronal apoptosis causes loss of cognitive function and memory impairment[12]. Current investigation determined the protective effect of barbaloin in the anesthetic induced neuronal injured rats. Cognitive function was evaluated by using MWM and level of mediators of inflammation and oxidative stress was estimated in the brain tissue by using ELISA. Moreover western blot assay was performed for the determination of expression of proteins and apoptosis of neuronal ell was observed by TUNEL assay and Fluoro Jade B staining.

It is well documented that isoflurane alters the balance between anti-inflammatory and pro-inflammatory cytokines in the brain tissues[13]. Pro-inflammatory mediators like NF-κB, TNF-α and IL-1β causes tissue damage by aggregating the granulocytes[14]. Moreover several reports suggested that drugs shows beneficial effect against neuronal damage by reducing the oxidative stress[15]. Data of the present investigation also suggested that treatment with barbaloin ameliorates the altered level of mediators of oxidative stress and inflammation in the brain tissues of anesthetic induced neuronal injured rats.

Reports revealed that isoflurane induces apoptosis by altering the expression protein such as Bcl-2, Bax and Bad involved in controlling the apoptotic pathway[16]. These proteins maintained the cell membranes integrity and releases the factors involved for apoptosis. Moreover ERK and JNK pathway is involved in the regulation of Bcl-2 proteins[17]. Data of present study suggested that barbaloin attenuates the altered expression of Bcl-2, Bax, Bad, JNK and ERK protein in the brain tissues of anesthetic induced neuronal injured rats.

In addition to these, survival of cell is regulated by PI3K/Akt pathway and this pathway activates anti-apoptotic protein which leads to inhibition of apoptosis[18]. Result of given investigation suggested that barbaloin activates PI3K/Akt pathway in the brain tissues of anesthetic induced neuronal injured rats and thereby inhibits the apoptosis of neuronal cells. Data of investigation supported by previously reported studies. Further PI3K/Akt signaling pathway activated by binding of BDNF with TrKB and thus causes decrease in apoptosis of neuronal cells. Literature reveals that BDNF protects the neuronal cells death by decreasing the apoptosis[19]. Data of the investigation supported by previous study reveals that barbaloin reduces apoptosis of neuronal cell by enhances the expression of BDNF protein. Moreover barbaloin decreases apoptosis of neuronal cells by TUNEL assay.

CONCLUSION

Study reveals that barbaloin protects the neuronal degeneration and protects the cognitive function in isoflurane induced neuronal injured neonatal rats. Moreover barbaloin ameliorates the JNK/ERK-1/2/Bcl-2, PI3K/Akt and BDNF/TrKB signaling pathways and thereby it contributes in the reduction of neuronal apoptosis.

SIGNIFICANCE STATEMENT

This investigation discovers the neuroprotective effect of barbaloin against the isoflurane induced neuronal injured rat model. Moreover barbaloin also reported to posse's protective effect on cognitive function in isoflurane induced neuronal injured rat. Present study also postulates the possible mechanism of its action as barbaloin protects neuronal injury by regulating JNK/ERK-1/2/Bcl-2, PI3K/Akt and BDNF/TrKB signaling pathways. These pathway are dysregulated due to increase in the level of inflammatory cytokines and oxidative stress due to isoflurane which leads to neuronal toxicity. Treatment with barbaloin ameliorates the altered level of cytokines and parameters of oxidative stress and thereby regulating BDNF/Bcl-2/PI3K pathway, it protects the neuronal injury.

ACKNOWLEDGMENT

All the author of this manuscript are thankful to Xiangya Hospital Central South University, China for providing the necessary facility for present work.

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