Research Article • DOI: 10.1515/tnsci-2018-0015 • Translational Neuroscience • 9 • 2018 • 88-98

DE GRUYTER

BAICALEIN INHIBITS NEUROAPOPTOSIS VIA PATHWAYS IN SEVOFLURANE INDUCED RATS

Si Wang,
Yu Zhou*

Department of Pediatrics,
Affiliated Hospital of North Sichuan Medical College, Nanchong, Sichuan,
China, 637000

Abstract

Background: Baicalein, a bioactive flavonoid was explored for its capability to attenuate sevoflurane induced neuronal apoptosis and to improve behavioural and cognitive impairments. Sevoflurane is a frequently used inhalation anesthetic in neonates and children. Neonatal sevoflurane exposure causes widespread neurodegeneration and cognitive impairments. Development of compounds that could effectively prevent/ reduce the adverse effects is of tremendous medical value. Methods: Isolated groups of neonatal rats were regulated with baicalein (25, 50 or 100 mg/kg b.wt) from postnatal day 3 (P3) to P21 and were exposed to sevoflurane (3%; 6 h) on P7. Results: Baicalein inhibited sevoflurane induced neuroapoptosis significantly as assessed by TUNEL assay. The raised levels of cleaved caspase-3, Bad and Bax were down-regulated by baicalein with enhanced Bcl-2, Bcl-xL, xIAP, c-IAP-1, c-IAP-2 and survivin expression. Baicalein regulated JNK/ERK signalling and also activated the PI3K/Akt pathway effectively as evident from the increased Akt, phospho-Akt, GSK-3β, phospho- GSK-3β levels. Baicalein, also improved the behaviour of animals in open filed and olfactory tests. The freezing responses and the performance in Morris Water Maze tests were enhanced. Conclusion: Baicalein reduced neurodegeneration and improved learning and memory retention of rats and as well modulated PI3/ Akt/GSK-3β and JNK/ERK signalling pathways.

Keywords

• baicalein • JNK/ERK signalling pathway • neuroapoptosis • PI3K/Akt/GSK-3β signalling • sevoflurane

Received 14 January 2018
accepted 29 May 2018

Introduction

Anesthetics are inevitable part of therapeutic care that demands surgery and related procedures. Inhalational anesthetics such as sevoflurane are most commonly used for general anesthesia, especially in pediatric patients [1]. However, accumulating experimental data on anesthetic-induced neurotoxicity have raised stern concerns on the safety of drugs [2-4]. General anesthetics have been stated to induce neuroapoptosis and potentially disturb neurogenesis [5] and subsequently cause memory deficits and long-term neurocognitive dysfunctions specifically in developing brains [6].

However, the precise mechanism underlying anesthetic-induced neurodegeneration is yet to be completely understood. Previous studies have proposed the involvement of reactive oxygen species, neuroinflammation, mitochondrial dysfunction and neurotransmitter disturbances [7].

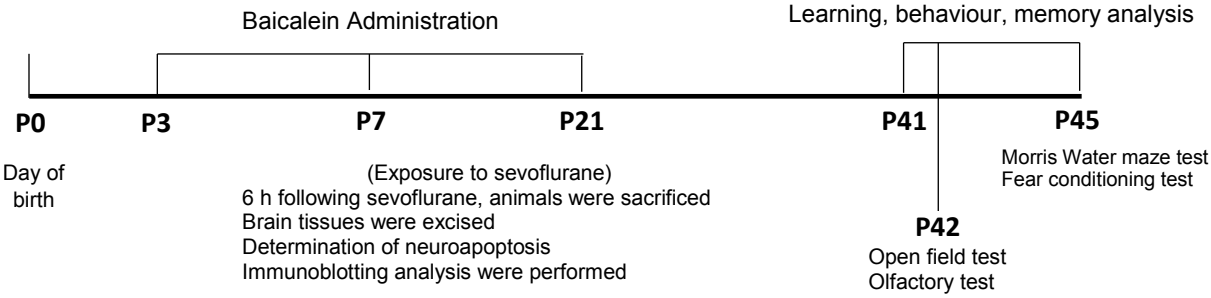
The phosphoinositide 3-kinase (PI3K)/ protein kinase B (Akt) pathway is expressed extensively in the CNS during development. In the neurons, PI3K is effectively induced by many growth factors including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3, as well as cytokines and neurotransmitters. PI3K/Akt pathways crucially regulate cell survival and apoptosis [8]. Further, the mitogen activated protein kinase (MAPK) signalling is been reported to be critically involved in nervous system development [9] and neurodegeneration [10]. Previous studies have shown MAPKs – JNK and ERK1/2 in isoflurane and sevoflurane-induced neurotoxicity [11]. There is also a potential interlink and crosstalk between Akt and JNK signalling pathways [11]. Targeting these pathways could possibly aid in preventing/ treating anesthetic-induced neurotoxicity and neurocognitive dysfunctions.

Many phytochemicals exhibit neuroprotective effects [12]. 5, 6, 7-trihydroxy-2-phenyl-4H-1-benzopyran-4-one, Baicalein is one of the key flavonoid of *Scutellaria baicalensis* that is been widely used for long time in Chinese and Japanese medicine. Anti-viral, anti-hepatotoxicity, anti-inflammatory, neuroprotective have been reported [13] as bioactive effects of baicalein. Herbal preparations of baicalein have been shown to potentiate learning and memory [14]. Wang *et al.* [15] reported that pre-treatment with baicalein attenuated β- amyloid peptide induced- amnesia. In this study baicalein was explored for its capability to protect brain cells against sevoflurane-induced neurotoxicity and improve cognition and memory in neonatal rats.

* *E-mail: yuzhou56@hotmail.com*

Materials and methods

Study design flow chart



Animals

Pregnant Sprague-Dawley rats were procured from North Sichuan Medical College animal care centre for laboratory animals and were maintained under normal conditions (22 ± 1°C; 12-h light/dark cycle) in sterile cages. The rats were provided access to water and were fed on standard pelleted diet and closely monitored for the birth of pups and day of birth was recorded as P0 (post-natal day 0). The pups were carefully monitored and were provided free access to water and pellet food. The study and the work plan were approved by the North Sichuan Medical College animal care committee and were carried out in complete compliance with the National Institutes of Health Guide for the Use of Laboratory Animals.

Reagents and antibodies

Sevoflurane and baicalein were obtained from Sigma-Aldrich, St.Louis, MO, USA. Antibodies against survivin (#2808), cleaved caspase-3 (#9664; 1:1000), Bad (#9239;1:1000), Bax (#5023; 1:1000), Bcl-xL (#2764; 1:1000), Bcl-2 (#3498), JNK (#9252), phospho-JNK (#4668), c-Jun (#9165), p-c-Jun (#3270), ERK1/2, phospho-ERK1/2 (#4094), PI3K (#4249), Akt (#4691), phospho-Akt (#4060), GSK-3β (#9315), phospho-GSK-3β (#9322), phosphatase and tensin homolog (PTEN) (#9549), cyclin D1 (#2978) and inhibitors of apoptosis proteins [xIAP (#14334), cIAP-1 (#4952) and cIAP-2 (#3130)] were from Cell Signaling Technology (Beverly, MA, USA), p-NF-κBp65 (sc-33020), p-IκBα (sc-8404) and β-actin (sc-47778) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used for expression analysis. Other chemicals that were used in this study were of analytical grade and were purchased from Sigma-Aldrich, St.Louis, MO, USA otherwise are mentioned.

Anesthetic exposure and dosing

Baicalein (25, 50 or 100 mg/kg b.wt) was administered to isolated groups of rat pups from day P3 and continued till P21 with standard pelleted diet. On P7, pups measuring about 16-20 g of weight were exposed to sevoflurane (3%) for 6 h in 60% oxygen or air in a temperature-controlled chamber (33 - 35°C) [16]. P7 rats were preferred based on earlier studies which suggested that the 1st week after birth is equivalent to the rapid period of brain development in humans which is from 3rd trimester to approximately 2 to 3 years. During this period of rapid brain development, neurogenesis is utmost susceptible to anesthesia-induced neuronal damages [2,17]. Control group received no anesthesia or baicalein. After 6 h of anesthetic exposure, rat pups of each group (n = 6) were sacrificed and brain tissues were excised and used for assessment of apoptosis and for protein expression analysis by western blotting. Separate groups of rats (n=12/group) were treated with 50 mg or 100 mg baicalein day P3 and continued till P21 along with standard pellet. The baicalein alone treated rats were not exposed to anesthetic. The rat pups exposed to sevoflurane and not treated with baicalein served as anesthetic control pups.

Analysis of neuroapoptosis - TUNEL fluorescent assay

Subsequent 6 h of exposure to sevoflurane, neuroapoptosis in the hippocampal regions was assessed by TUNEL studies. Hippocampal sections of 5 µm thickness were sliced (200 µm apart) and subjected to analysis [18]. As per manufacturer's directions DeadEnd TM fluorometric TUNEL system kit (Promega, Madision, WI, USA) was used. The TUNEL positive cells in hippocampal CA1, CA3 and Dentate gyrus (DG) were imagined and additionally investigated utilizing NIS-Elements BR picture handling and examination programming (Nikon Corporation, Japan).

Immunohistochemistry staining

By immunohistochemical staining, apoptosis was assessed for activated caspase-3. Expression of caspase-3 is measured as an indicator for apoptosis. Briefly, the brain sections of 4 µm thickness are embedded in paraffin-wax blocks and were blocked for endogenous peroxidase by incubating in PBS-Triton (PBST) containing 0.1% H_2O_2. Following washing with PBST, the segments were incubated overnight with anti-cleaved caspase-3 prime antibody at 4°C and further incubated for 40 min with secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and with avidin-biotinylated peroxidase complex (Vectostain ABC-Kit, Vector Lab, Burlingame, CA, USA) for 40 min. The tissue sections were then treated with diaminobenzidine and were analyzed for caspase-3 positive cells with NIS-Elements BR image processing and analysis software.

Immunoblotting

The tissue sections that were harvested were subjected to western blotting to assess protein expression following sevoflurane exposure as described previously [19,20]. In brief, the brain tissue sections were carefully blended on ice using immunoprecipitation buffer (10 mM Tris–HCl of pH 7.4, 2 mM EDTA, 150 mM NaCl, and 0.5% Nonidet P-40) containing protease inhibitors (1 mg/mL leupeptin, 1 mg/mL aprotinin and 1 mg/mL pepstatin A). Protein concentrations in the collected cell lysates were determined using BCA protein assay kit (Bio-Rad, Hercules, CA, USA). Equal amount of proteins (60 µg) were electrophoretically separated on SDS-PAGE and electro-transferred on to polyvinylidene difluoride membranes. The membranes were incubated with primary antibodies, overnight at 4°C. Following incubation, the blots were washed and further incubated with appropriate secondary antibodies. The immunoreactive bands were imagined and the images were scanned using Image Master II scanner (GE Healthcare, Milwaukee, WI, USA). The band densities of the positive bands were analysed further by ImageQuant TL software (GE Healthcare, Milwaukee, WI, USA). The expression of proteins was normalized with that of β-actin.

Behavioural analysis and memory assessment

Several studies have reported sevoflurane exposure induced cognitive dysfunctions and memory impairments [2,3]. Here a series of tests were conducted to assess the effects of baicalein on the behaviour, memory retention and learning capacity of the rats following sevoflurane exposure.

Open field test

P42 rats that were exposed to sevoflurane on post-natal day 7 (P7) were assessed for their emotional responses to a novel field. As previously described by Satoh *et al*. [21] the open-field test was performed. The behavioural responses of each rat were observed and noted as the distance travelled in the field in 10 min. The movement of the animals was automatically documented using XR-XZ301 video tracking system (Shanghai Soft maze Information Technology Co, Ltd).

Fear conditioning test

For determining the hippocampal-dependent and -independent responses, a reliable measure is the fear conditioning test. The rats on post-natal day 45, received stimuli consisting of both conditioned and unconditioned stimulus pair which was separated by 1 min each. Rats were placed in chambers, the stainless-steel bars on the floor were linked to a shockwave delivery system (Coulbourn, Whitehall, PA, USA) and electrical shock was distributed through the steel bars. The unconditioned stimulus involved of 1mA foot shock with 1 sec duration while the conditioned stimulus involved of an 80-db white noise of 20 sec duration. Fear responses were quantified with freezing (absence of any movement), a distinctive defensive fear response in rodents and is a consistent measure of fear to learned stimuli. Electric shock was distributed during the last few seconds of white noise. A contextual fear test was performed 24 h after conditioning, where for a period 5 min the rats remained in the conditioning chamber in the absence of white noise. Cued tests (for the same set of rats) were directed by offering a cue (80 db white noise, 3-min duration) alternatively while giving distinct visual and tactile cues. The freezing responses of the animals were scored using FreezeView software (Coulbourn). Movement of the animals was also recorded as in open field test [16].

Olfactory test

On post natal day 42 (P42), the rats were familiarized to the flavour of a novel food (garlic cheese) for 3 days. On fourth day the rats were subjected to fasting for 24 h and on the fifth day a portion of garlic cheese was hidden (4 cm) under bedding in a fresh cage. The animals were positioned in the cage and time taken by the rats to find the food was observed.

Morris water maze test

The Morris water maze (Shanghai Jiliang Software Technology Co. Ltd., China) was used to assess learning and memory of rats [22]. The rat pups that were exposed to sevoflurane on post-natal day 7 (P7) and/ or administered baicalein were trained in the maze for a period of 4 days between P41 and P45. The pool was filled with warm water (23 ± 2°C) and a transparent round platform of 10 cm diameter was placed at 0.5 cm below the surface of water. The rats were allowed to explore the pool and were subjected to training sessions. The animals undertook 2 training sessions a day. Each session consisted of 2 trials. The rats were allowed a take a time of 60 sec to trace the hidden platform in the pool. If the rats failed to trace the platform in 60 sec, they were gently guided. The rats were permitted to remain on the platform for 30 sec during each trial. The swimming path of the rats were recorded using ANY-maze video tracking system (Stoelting Co., Wood Dale, IL, USA) that records and also calculates the time taken (latency) by each rat to locate the hidden platform, and also any other behavioural information.

Cued trials

On post natal day 45 (P45), the animals were exposed to cued trials for testing any non-cognitive performance impairments such as visual impairments and/or swimming complications. The visual cues around the pool were hidden by covering the pool with a white cloth and the rats were trained with 4 trials a day. Through each trial, each rat was placed in a precise position of the pool and was permitted to swim to locate the platform that had an attached rod which aided as cue. The rod was fixed at about 20 cm above water level. As described before, rats were allowed for 60 sec to find out and trace the inundated platform with the aid of the rod and time taken by each rat to trace the cued platform was documented.

Place trials

Capacity to learn and understand the association between cues and the submerged platform and to recall the position of the platform was assessed by place trials. For place trials, the white cloth was detached and the rats were allowed to locate the platform with no cue rod that was placed in a fixed position as through the place trails in one of the four quadrants. The rats were located at random starting points on the swimming pool and the time taken to locate the platform was documented.

Probe trials

The probe trials were conducted for the same set of rats 24 h later place trials. To evaluate

memory, the underwater platform (without cue rod) was detached from the target quadrant (the quadrant where the platform was located during place trials). Rats were permitted to swim for 60 sec and the period spent by each rat in the target quadrant looking out for the underwater platform were documented. The results are presented as percentage of time spent by the rat in the target quadrant, as a measure of memory retention.

Statistical analysis

The observed results are given as mean ± SD, taken from six independent experiments. The results of the various experimental groups were subjected to multiple group comparison and analysed by ANOVA (one-way Analysis of variance) which followed post-hoc analysis using DMRT (Duncan's Multiple Range Test) with SPSS statistical package (version 22.0). The values at $p < 0.05$ were significant statistically.

Results

Baicalein reduced sevoflurane-induced neuroapoptosis

Several studies have shown widespread apoptotic neurodegeneration in developing rat brain following sevoflurane exposure [3,23,24]. In line with previous studies, apoptosis was observed ($p < 0.05$) subsequent after 6 h of sevoflurane experience in the hippocampal regions CA1, CA3 and DG (Figure 1). Pre-treatment with baicalein significantly ($p < 0.05$) reduced apoptosis and is seen as decreased TUNEL positive cell counts, suggesting the efficacy of baicalein in preventing neurodegeneration induced by sevoflurane. Baicalein at 100 mg dose showed maximum protective effects in comparison with lower doses of 25 mg and 50 mg.

A major executioner protein of apoptosis is caspase-3 and its expression is measured as an important marker for neuroapoptosis [23]. Raised caspase-3 positive cells in the hippocampus of sevoflurane-exposed rat pups were observed (Figure 2). Expression level of caspase-3 was further assessed by western blotting. Significantly visible increase ($p < 0.05$) in the protein level of cleaved caspase-3 was observed (Figure 3) following sevoflurane exposure. This raised expression was in line with the apoptotic counts seen in TUNEL assay. Interestingly, baicalein at 25, 50 and 100 mg doses effectively reduced caspase-3 expression in a dose-dependent manner.

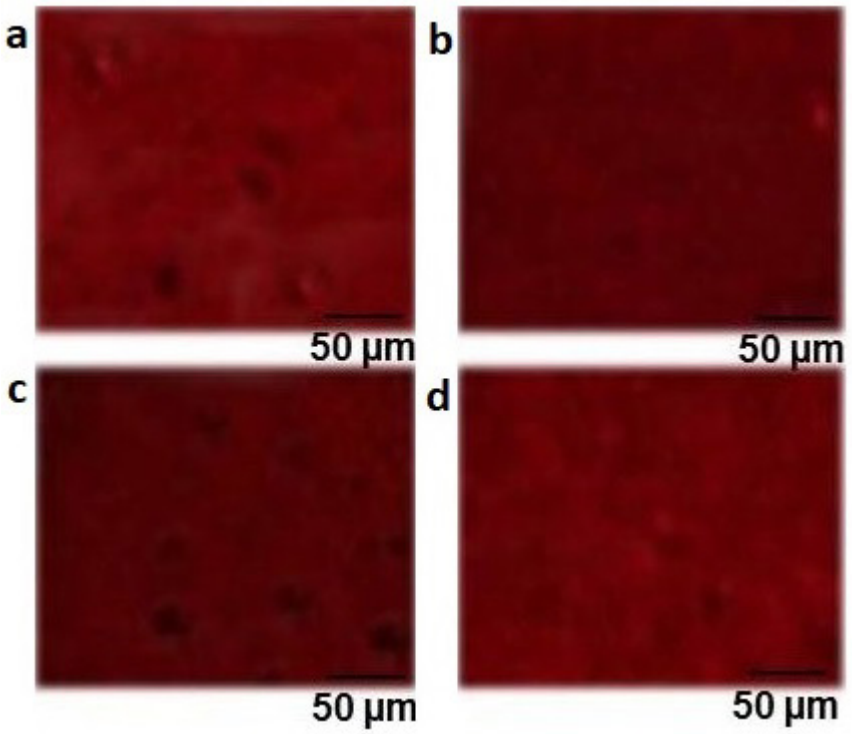


Figure 1A. Influence of baicalein on sevoflurane-induced neuroapoptosis as determined by TUNEL assay. a-Control; b-Sevoflurane; c-Sevoflurane + 50 mg Baicalein; d-Sevoflurane + 100 mg Baicalein

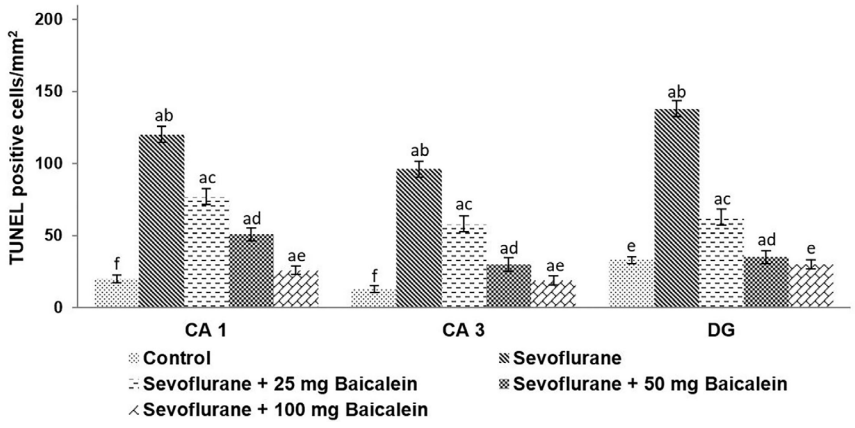


Figure 1B. Influence of baicalein on sevoflurane-induced neuroapoptosis. Results are stated as mean ± SD (n = 6). [a] represents $p < 0.05$ vs control; [b-f] represents mean values from different experimental groups that differ at $p < 0.05$ as derived by one-way ANOVA and DMRT analysis.

Baicalein modulated expression of apoptotic pathway proteins

Sevoflurane-induced neuroapoptosis in the developing brain is well documented [25]. Previous studies have demonstrated mitochondrial apoptotic pathway in

sevoflurane-induced neurotoxicity [26,27]. It was assessed whether baicalein influenced sevoflurane-modulated expression of apoptotic pathway proteins specifically the proapoptotic proteins (Bax and Bad) and Bcl-2 and Bcl-xL (anti-apoptotic proteins). Sevoflurane exposure caused a multi-fold increase in Bax and Bad levels with significantly down-regulated Bcl-2 and Bcl-xL expression (Figure 3). Further expression of xIAP, c-IAP-1, c-IAP-2 and survivin were also severely down-regulated on exposure to 3% sevoflurane (Figure 3), suggesting up-regulated neuroapoptosis. However, baicalein treatment modulated the expressions of pro and anti-apoptotic proteins. Baicalein at all the 3 doses improved the expression of cell survival proteins - Bcl-xL, Bcl-2, xIAP, c-IAP-1, c-IAP-2 and survivin while down-regulating the expression of caspase-3, Bad and Bax.

Baicalein potentially regulated PI3K/Akt pathway

PI3K/Akt is a key pathway that involves in neuronal cell existence and is widely expressed in developing brain. Song *et al.* [28] reported that Akt may be deactivated during the process of apoptosis. In this study, sevoflurane strikingly down-regulated the pathway as the levels of GSK-3β, Akt and as well as phosphorylated Akt and phosphorylated- GSK-3β were reduced (Figure 4). Baicalein treatment significantly caused activation of the pathway as demonstrated by up-regulated expression of Akt and GSK-3β. Baicalein at 100 mg dose showed extreme effect as compared to other lower doses.

Furthermore, the expression of - cyclin D1, p-NF-κβ(p65) and p-IκBα, were down-regulated by sevoflurane (Figure 4). Reduced expression and of Akt and p-GSK-3β could have led to decreased expression of the down-stream proteins of the target pathway. Nevertheless, activation of the pathway by baicalein resulted in raised expression of cyclin D1, p-NF-κβ(p65) and p-IκBα. However, in rats that were treated with baicalein alone and not exposed to sevoflurane, the expression levels of the proteins remained negligibly altered, suggesting that baicalein did not influence the pathway under normal conditions.

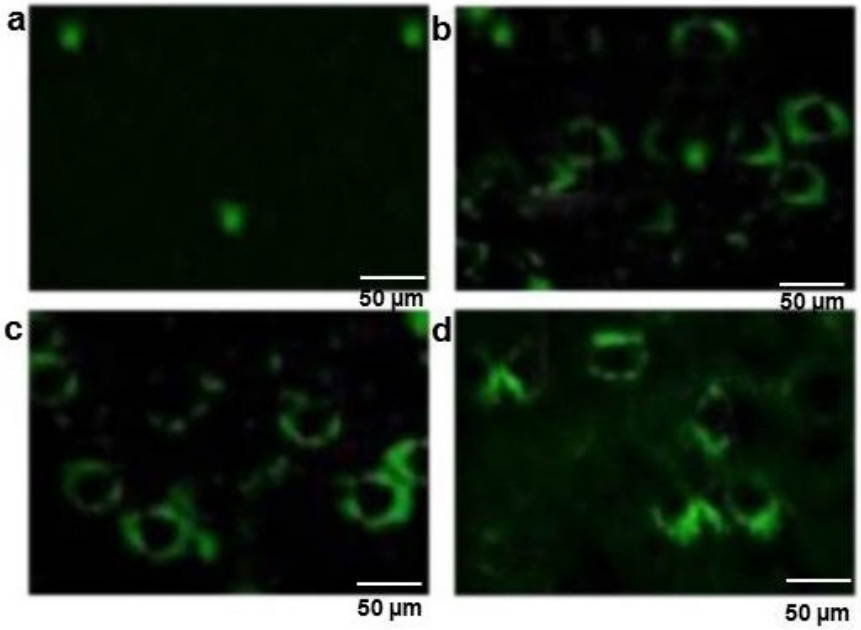


Figure 2A. Baicalein effectively reduced activated caspase-3 expression as determined by immunohistochemical analysis. a-Control; b-Sevoflurane; c-Sevoflurane + 50 mg Baicalein; d-Sevoflurane + 100 mg Baicalein

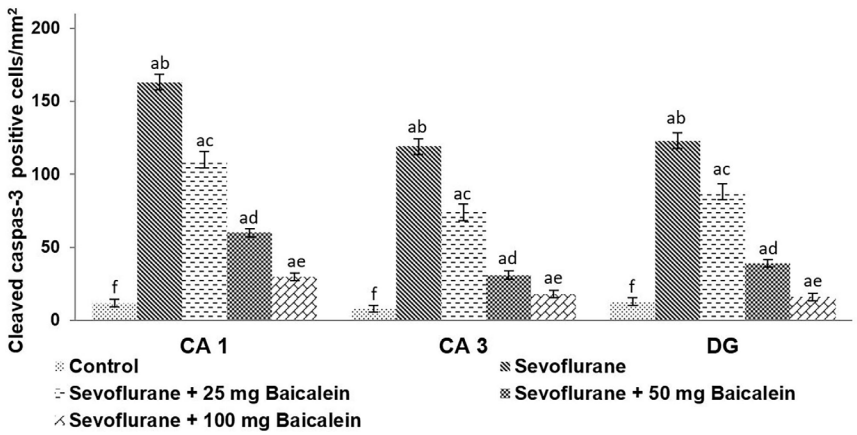


Figure 2B. Baicalein effectively reduced activated caspase-3 expressions. Results are stated as mean ± SD (n = 6). [a] represents $p < 0.05$ vs control; [b-f] represents mean values from different experimental groups that differ at $p < 0.05$ as derived by one-way ANOVA and DMRT analysis.

Baicalein modulated JNK/ERK signalling

The MAPkinaes - ERK and JNK kinases critically regulate neuronal survival, proliferation, apoptosis, development and synaptic plasticity in the brain [9,10]. The influence of sevoflurane and baicalein on the expression of ERK and JNK kinases was studied. Obtained results showed elevated ($p < 0.05$) JNK and c-Jun levels following sevoflurane exposure, while sevoflurane caused notable suppression in the levels of ERK1/2 and p-ERK1/2 (Figure 5). It was also noticed that pre-treatment with baicalein up-regulated levels of ERK1/2 and p-ERK1/2. However, baicalein treatment in sevoflurane exposed rats caused considerable reduction in JNK, p-JNK, c-Jun and p-c-Jun expressions, suggesting inhibition of JNK signalling while activating ERK pathway. Further, interestingly baicalein when administered alone did not cause any changes in the expression levels of JNK, ERK1/2 and c-Jun and also the phosphorylated forms.

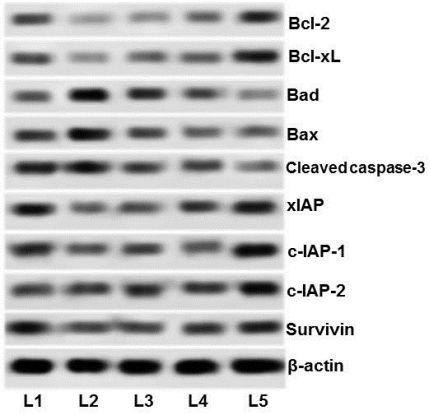


Figure 3A. Baicalein modulates the expression of apoptosis pathway proteins. Baicalein pre-treatment effectively reduced expressions of Bad and Bax and enhanced expressions of Bcl-2 and Bcl-xL. L1-Control; L2-Sevoflurane; L3-Sevoflurane + 25 mg Baicalein; L4-Sevoflurane + 50 mg Baicalein; L5-Sevoflurane + 100 mg Baicalein

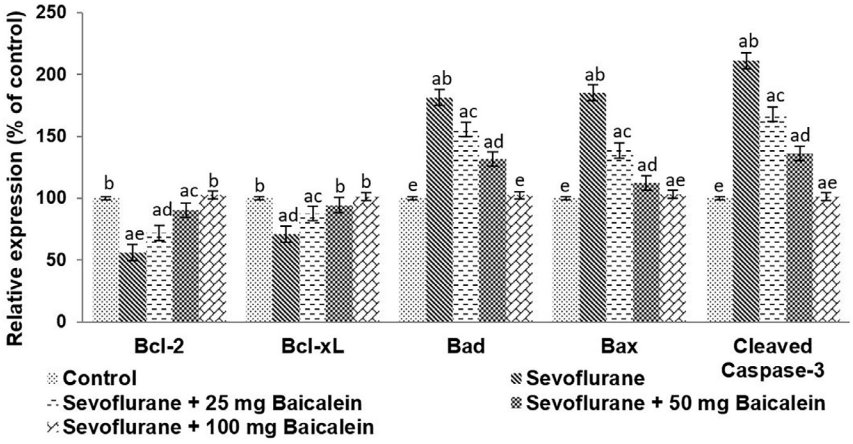


Figure 3B. Baicalein modulates the expression of apoptosis pathway proteins. Baicalein pre-treatment effectively reduced expressions of Bad and Bax and enhanced expressions of Bcl-2 and Bcl-xL. Results are stated as mean ± SD (n = 6). [a] represents $p < 0.05$ vs control; [b-f] represents mean values from different experimental groups that differ at $p < 0.05$ as derived by one-way ANOVA and DMRT analysis.

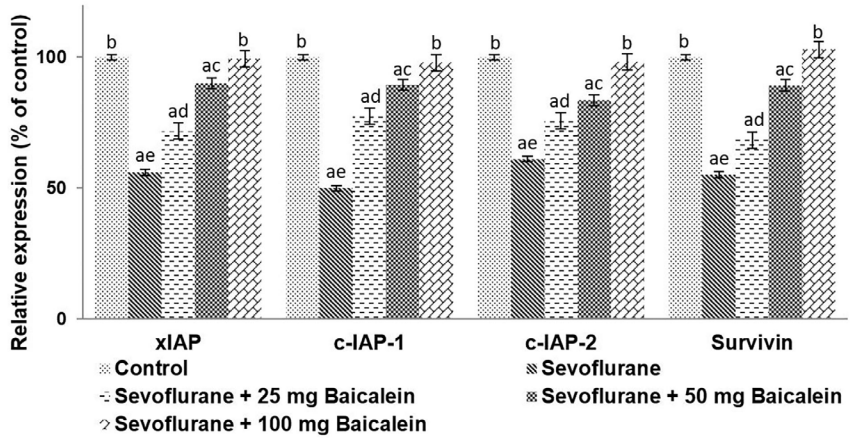


Figure 3C. Baicalein modulates the expression of apoptosis pathway proteins. Baicalein significantly up-regulated the expressions of survivin and IAPs. Results are stated as mean ± SD (n = 6). [a] represents $p < 0.05$ vs control; [b-f] represents mean values from different experimental groups that differ at $p < 0.05$ as derived by one-way ANOVA and DMRT analysis.

Baicalein improved the general behaviour and memory of rats

Behavioural responses of the study rats in a novel environment were observed. Sevoflurane-alone administered pups did not exhibit changes in behaviour; however, the distance covered across the open field were observed to be slightly lesser than the rats that were pre-treated with baicalein. Rats that received baicalein at 50 and 100 mg presented behavioural responses similar to normal control rats (Figure 6A).

In the olfactory test, changes in the time consumed by the animals to find out the cheese buried inside was observed (Figure 6B). Nevertheless, baicalein treated animals were able to trace out cheese very quick as compared to sevoflurane-alone exposed animals.

To evaluate the influence of baicalein on long-term recall following sevoflurane on P7, the P45 rats were exposed to contextual/ cued fear conditioning. In the training sessions, the animals are exposed to white noise (neutral auditory cues) and electric foot shock (context). The memory of the experimental context (hippocampus dependent) and an auditory cue (hippocampus independent) were measured based on the freezing reaction of the animals to the context and conditioned cue. The freezing responses of the rats with neonatal exposure to sevoflurane were found to be reduced significantly compared to controls (Figure 6C). Interestingly the responses were substantially improved in rats that were treated with 100 mg baicalein exhibiting stronger influences on the freezing responses. Further we noticed baicalein alone (50 mg or 100 mg) treated rats exhibited responses similar to normal control rats.

Baicalein potentially improved spatial learning and working memory of rats

To assess the effects of new-born exposure with sevoflurane on learning and recall deficits, the rats were exposed to Morris water maze test (MWM). The rats subjected to sevoflurane on P7 were allowed to discover the swimming pool and were trained to reach the buried submerged platform and the time consumed by each rat to reach the platform was documented as escape latency. While the latency time decreased with

every training session, there were not many differences in the escape latency between various experimental groups (Figure 7A). Nevertheless, rats exposed to sevoflurane alone presented slight dissimilarities from the animals that received baicalein and exposed to sevoflurane on P7.

The cued and place trials were directed to assess spatial navigation skills and visual abilities. The P45 rats that were subjected to sevoflurane anesthesia on P7 were found to take significantly a much-extended time to reach the underwater platform as against the rats that received no anesthesia (Figure 7B). Baicalein supplementation at 50 and 100 mg was found to improve the performance of the rats. The rats were able to identify the cue (rod) and also able to navigate faster to reach the platform. Further, 100 mg baicalein gave the determined enhancement in the performance of the rats.

The ability of retaining memory was evaluated by probe trials wherein the platform was removed from target quadrant and was placed in a random quadrant. Capability to look out for the submerged platform in the target quadrant was detected. Sevoflurane exposure on P7 had substantially affected the memory of rats. The animals exposed to sevoflurane alone spent significantly lesser time in the target quadrant as compared to animals of the control group and baicalein treated groups. These results suggest that baicalein administration remarkably improved memory of the rats. The 100 mg dose of baicalein presented more enhanced performance than the lower doses. Further similar to results of fear conditioning tests and general behavioural analysis, baicalein at 50 mg or 100 mg given alone had no adverse effects on the behaviour and learning of rats. The responses observed were almost comparable to normal control rats.

Discussion

Sevoflurane has been reported to exert neurotoxic effects to the developing brain [1,7] and also cause cognitive deficits [3]. In this study investigation was done to determine whether baicalein was able to attenuate sevoflurane-induced toxicity at doses of 25, 50 or 100 mg.

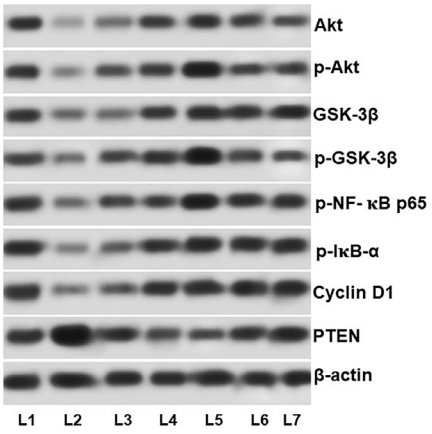


Figure 4A. Baicalein modulates PI3K/Akt signaling pathway. Baicalein significantly activated PI3/Akt pathway as evidenced by increased phosphorylation of Akt, GSK-3β and elevated levels of down-stream target proteins of Akt. L1-Control; L2-Sevoflurane; L3-Sevoflurane + 25 mg Baicalein; L4-Sevoflurane + 50 mg Baicalein; L5-Sevoflurane + 100 mg Baicalein; L6-50 mg Baicalein; L7-100 mg Baicalein

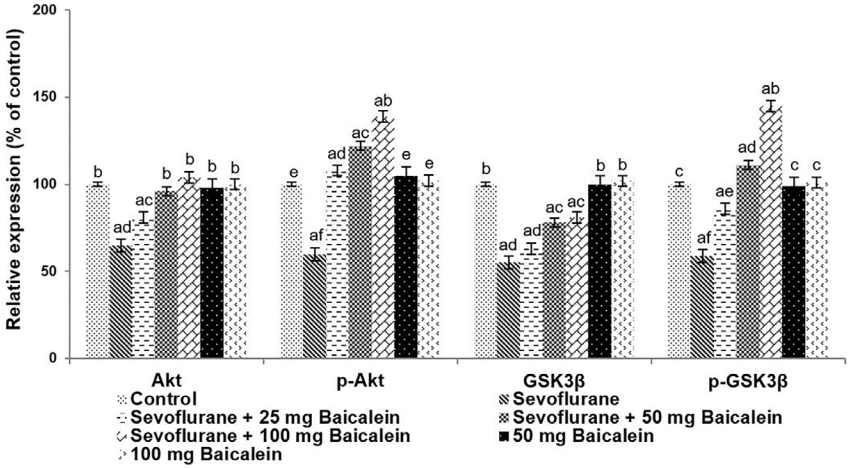


Figure 4B. Baicalein modulates PI3K/Akt signaling pathway. Baicalein significantly activated PI3/Akt pathway as evidenced by increased phosphorylation of Akt, GSK-3β. Results are stated as mean ± SD (n = 6). [a] represents $p < 0.05$ vs control; [b-f] represents mean values from different experimental groups that differ at $p < 0.05$ as derived by one-way ANOVA and DMRT analysis.

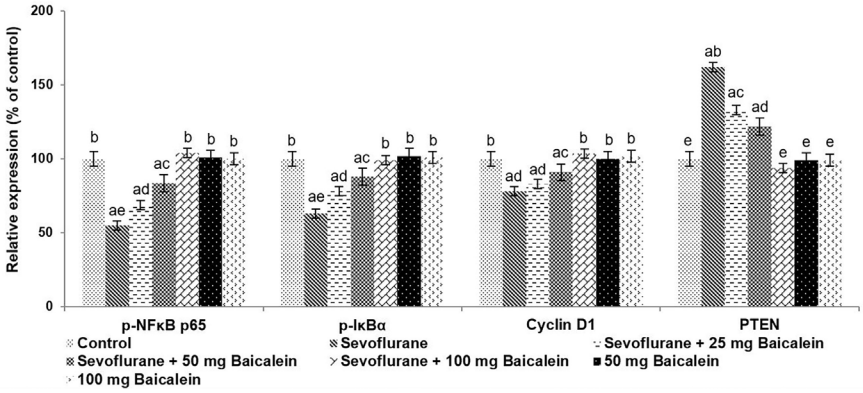


Figure 4C. Baicalein modulates PI3K/Akt signaling pathway. Baicalein significantly activated elevated levels of down-stream target proteins of Akt. Results are stated as mean ± SD (n = 6). [a] represents $p < 0.05$ vs control; [b-f] represents mean values from different experimental groups that differ at $p < 0.05$ as derived by one-way ANOVA and DMRT analysis.

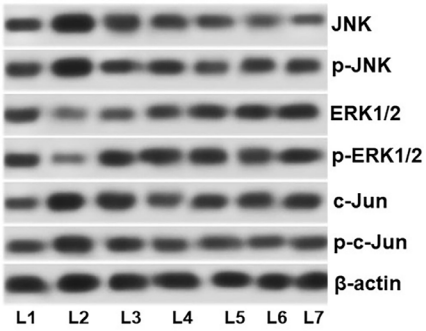


Figure 5A. Baicalein modulates JNK/ERK signalling. JNK signalling was significantly down-regulated by baicalein with mᵃrkedly enhanced ERK1/2 expressions, suggesting the involvement of JNK and ERK signalling in sevoflurane-induced neurotoxicity. L1-Control; L2-Sevoflurane; L3-Sevoflurane + 25 mg Baicalein; L4-Sevoflurane + 50 mg Baicalein; L5-Sevoflurane + 100 mg Baicalein; L6-50 mg Baicalein; L7-100 mg Baicalein

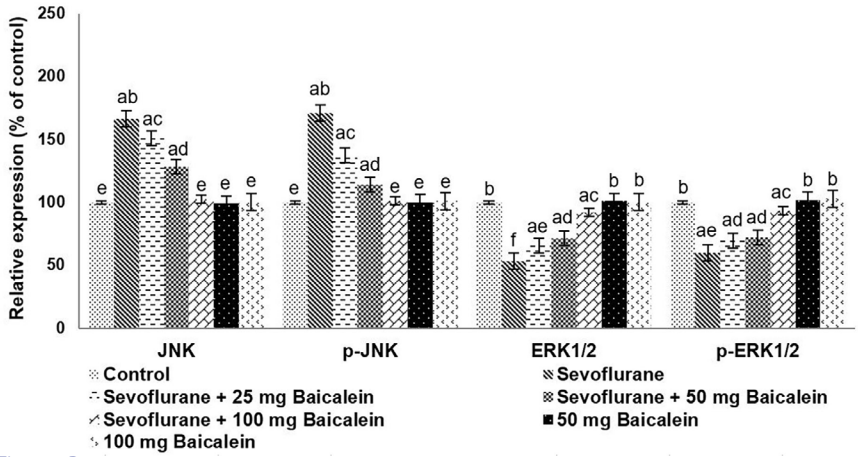


Figure 5B. Baicalein modulates JNK/ERK signalling. JNK signalling was significantly down-regulated by baicalein with markedly enhanced ERK1/2 expressions, suggesting the involvement of JNK and ERK signalling in sevoflurane-induced neurotoxicity. Results are stated as mean ± SD (n = 6). [a] represents $p < 0.05$ vs control; [b-f] represents mean values from different experimental groups that differ at $p < 0.05$ as derived by one-way ANOVA and DMRT analysis.

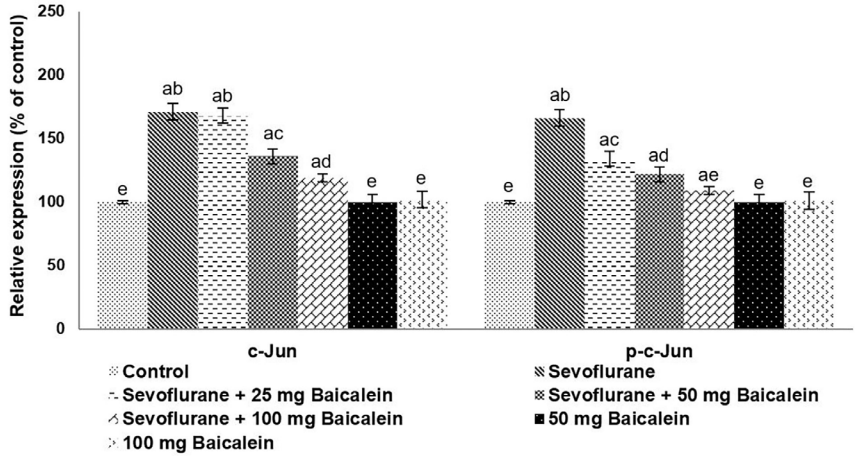


Figure 5C. Baicalein modulates JNK/ERK signalling. Baicalein treatment in sevoflurane exposed rats caused considerable reduction in c-Jun and p-c-Jun expression, suggesting inhibition of JNK signalling while activating ERK pathway. Results are stated as mean ± SD (n = 6). [a] represents $p < 0.05$ vs control; [b-f] represents mean values from different experimental groups that differ at $p < 0.05$ as derived by one-way ANOVA and DMRT analysis.

Severe neuroapoptosis was detected on 6 h of sevoflurane contact in the hippocampal regions of P7 rat pups. Though sevoflurane causes widespread neuroapoptosis in the brain, studies have shown hippocampus to be more sensitive to anesthetic-induced neurotoxicity [29,30]. Further, immunohistochemistry (IHC) and western blot analysis exposed significantly elevated cleaved-caspase-3 expression in the hippocampi following sevoflurane exposure. Caspase-3 is a chief executioner enzyme of the apoptotic pathway and activated caspase-3 expression is measured as a potent marker of neuronal degeneration [31]. Interestingly, the experimental data indicates effective reduction in cleaved caspase-3 expression and TUNEL positive cells in the hippocampi of P7 rats treated with baicalein.

Further investigation on the apoptotic pathway expression revealed effective regulation of Bcl-2 family proteins, the major proteins that control mitochondrial membrane integrity and regulate cytochrome C release and other apoptogenic factors from mitochondria [32]. Baicalein significantly up-regulated anti-apoptotic proteins expression while down-regulating Bax and Bad. Bcl-xL has been reported to widely enhance cell survival and inhibit cytochrome C release, while Bax in response to several apoptotic stimuli enhances release of cytochrome C, leading to activation of caspase cascade [32]. Baicalein also enhanced expression of IAPs-xIAP, c-IAP-1 and c-IAP-2. Thus, the observed modulation of expression could possibly be responsible in baicalein mediated reduction in apoptosis and as well in activated caspase-3 expression.

Several studies have described that the expression of Bcl-2 family proteins is controlled by JNK [33] and ERK signalling [34]. JNK signalling helps apoptosis via transcriptional regulation of Bcl-2 family proteins [35]. Activated JNK phosphorylates c-Jun that leads to rise of AP-1 transcription action and regulated transcription of genes of apoptotic pathway [30]. Further, Bcl-2 is constitutively phosphorylated by ERK1/2 [34]. Additionally, ERK1/2 directly phosphorylates pro-caspase-9 subsequently inhibiting caspase-3 activation [36]. Enhanced JNK signalling and supressed ERK1/2 in the P7 rats following sevoflurane

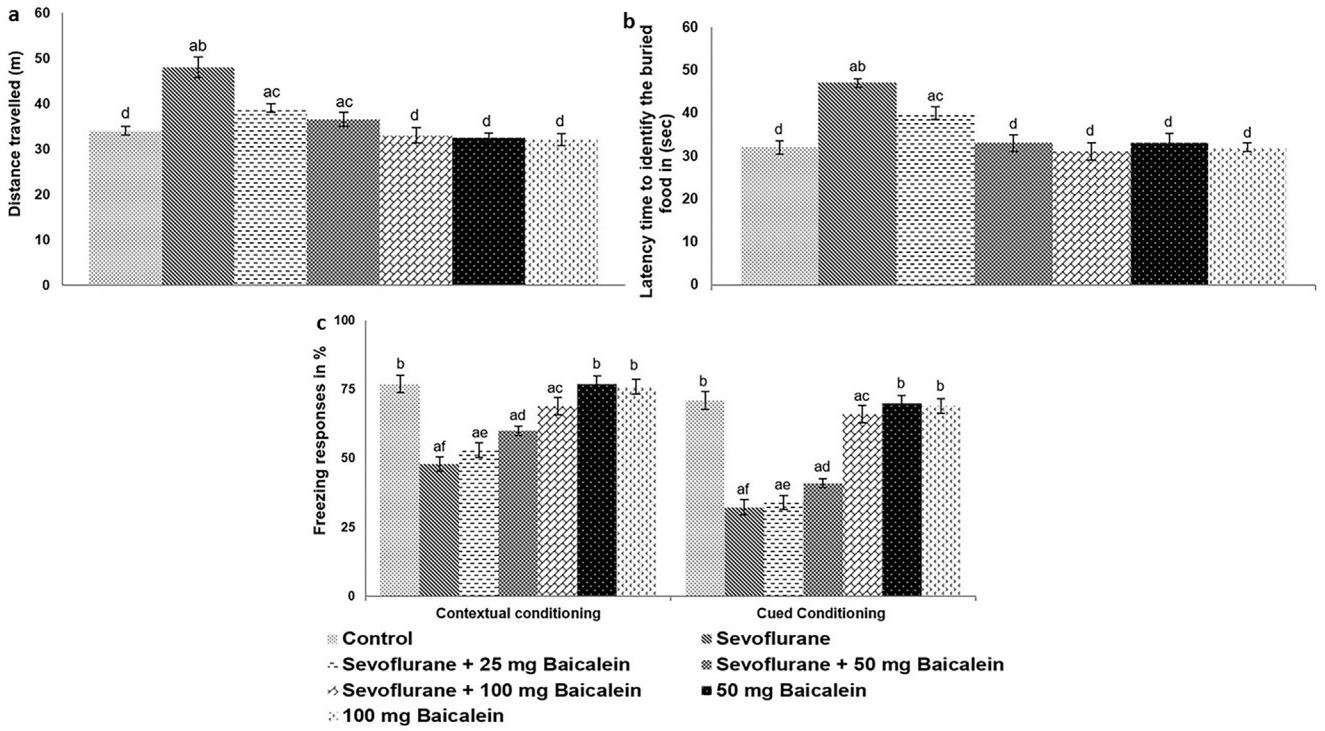


Figure 6. Influence of baicalein on the behaviour of sevoflurane exposed rats - Baicalein treatment was found to (A) improve the behaviour of rats exposed to novel environment, (B) decrease the latency time to find the buried food and (C) improve freezing responses on contextual and cued fear conditioning following anesthesia exposure on P7. Results are stated as mean ± SD (n = 6). [a] represents $p < 0.05$ vs control; [b-f] represents mean values from different experimental groups that differ at $p < 0.05$ as derived by one-way ANOVA and DMRT analysis.

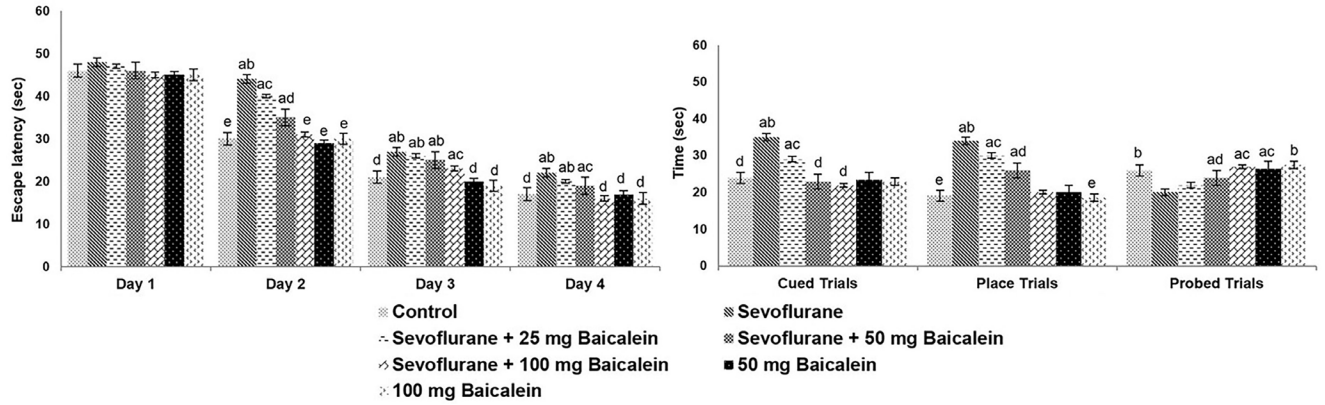


Figure 7. Baicalein effectively improved learning and memory of sevoflurane-exposed rats – (A) Escape latency of P45 rats following exposure to anesthesia on P7 were improved by baicalein and (B) Learning and memory of P45 rats subjected to cued, place and probe trials with Morris water maze were remarkably improved on baicalein treatment. Results are stated as mean ± SD (n = 6). [a] represents $p < 0.05$ vs control; [b-f] represents mean values from different experimental groups that differ at $p < 0.05$ as derived by one-way ANOVA and DMRT analysis.

exposure was observed. Results showed that baicalein pre-treatment prevent sevoflurane-induced increased phosphorylation of JNK and c-Jun while enhance the ERK1/2 levels suggesting improved cell survival. The results indicate the involvement of JNK and ERK1/2 pathways in sevoflurane-induced neurodegeneration.

Several experimental studies have shown that PI3K/Akt pathway plays a pivotal role in neurite initiation, growth and stability [6]. Earlier reports have showed inactivation of Akt during apoptosis [28] and sevoflurane inhibit GSK-3β phosphorylation [37]. Sevoflurane exposure for 6 h reduced phosphorylation of Akt and GSK-3β. It is known that phosphorylation of Akt suppresses caspases [38] and inactivates Bad that causes release of Bcl-xL thus promoting cell survival [39]. In our study, baicalein administration significantly enhanced phosphorylation of Akt and GSK-3β

and supressed PTEN, the negative regulator of the pathway [40]. Also, interestingly, we noticed 50mg dose of baicalein caused more markedly elevated expressions of total Akt, p-AKT, GSK-3β and p-GSK-3β as compared to 100 mg doses. Considering these observations, the effects of doses between 50 and 100 mg could be investigated to understand the effectiveness. Further, baicalein enhanced expression of other down-stream molecules of Akt such as cyclin D1, IκBα nuclear factor-κB (NF-κB). xIAP, cIAP-1 and survivin the important target genes of NF-κB [41]. It has been reported that GSK-3β causes activation of NF-κB [40]. Thus, enhanced levels of p-NF-κBp65, xIAP, cIAP-1 and survivin suggests activation of the pathway by baicalein.

Neurobehavioral deficits and cognitive dysfunctions persisting well into adulthood have been documented following neonatal exposure to sevoflurane [2,3]. The influence of baicalein over the general behaviour and cognition of rats following sevoflurane exposure on P7 was assessed. Minor variations in the general behaviour were noticed following 6 h sevoflurane. Olfactory test did not reveal many changes in behaviour. Fear conditioning tests were carried out to assess the influence of sevoflurane on long term memory. The results of the context conditioning (hippocampal-dependent) and cued conditioning (non-hippocampal-dependent) indicated markedly altered responses of sevoflurane-alone exposed rats. Freezing responses were much lesser suggesting memory impairment and neurocognitive dysfunction.

Further, spatial working memory and reference memory were assessed by MWM test. The escape latencies of the rats that received sevoflurane were to some extent longer than the controls. Working memory involves complex tasks involving planning and procedural behaviours and any deficits in working memory reflects in behavioural flexibility [42]. Differences in the functioning of the sevoflurane exposed rats in identifying the submerged platform in MWM tests was observed, indicating severe impact of sevoflurane on spatial navigation and working memory. Nevertheless, effective improvement in the cognitive behaviour and memory was observed in sevoflurane exposed rats that were administered with baicalein. At all the tested doses, baicalein strikingly improved the general behaviour of rats in novel environment and also in MWM. Thus, the significant reduction in neurodegeneration and regulation of the JNK/ERK and PI3K/Akt signalling on baicalein administration in part could have contributed to the improvements in performance. Also, baicalein when administered alone did not cause any adverse effects. Baicalein did not alter the protein expressions or the behaviour, learning and memory of rats under normal conditions. These observations illustrate the beneficial effects of baicalein.

Conclusion

The study data reveals the efficacy of baicalein in inhibition of neonatal sevoflurane-induced neuroapoptosis and neurobehavioral dysfunctions. Baicalein could be explored further in therapy against inhalation anesthetic-induced neurotoxicities.

Acknowledgements

Funding support: This study was supported by Natural Science Foundation of China (No: 81300528).

References

[1] Boscolo A, Starr JA, Sanchez V, Lunardi N, DiGruccio MR, Ori C, et al. The abolishment of anesthesia-induced cognitive impairment by timely protection of mitochondria in the developing rat brain: the importance of free oxygen radicals and mitochondrial integrity. Neurobiol Dis. 2012;45:1031-41.

[2] Jevtovic-Todorovic V, Hartman RE, Izumi Y, Benshoff ND, Dikranian K, Zorumski CF, et al. Early exposure to common anesthetic agents causes widespread neurodegeneration in the developing rat brain and persistent learning deficits. J Neurosci. 2003;23:e876-82.

[3] Satomoto M, Satoh Y, Terui K, Miyao H, Takishima K, Ito M, et al. Neonatal exposure to sevoflurane induces abnormal social behaviors and deficits in fear conditioning in mice. Anesthesiology 2009;110:628-37.

[4] Shu Y, Patel SM, Pac-Soo C, Fidalgo AR, Wan Y, Maze M, et al. Xenon pretreatment attenuates anesthetic-induced apoptosis in the developing brain in comparison with nitrous oxide and hypoxia. Anesthesiology 2010;113:360-8.

[5] Brambrink AM, Evers AS, Avidan MS, Farber NB, Smith DJ, Zhang X, et al. Isoflurane-induced neuroapoptosis in the neonatal rhesus macaque brain. Anesthesiology 2010;112:834-41.

[6] Flick RP, Katusic SK, Colligan RC, Wilder RT, Voigt RG, Olson MD, et al. Cognitive and behavioral outcomes after early exposure to anesthesia and surgery. Pediatrics 2011;128:e1053-61.

[7] Vlisides P, Xie Z. Neurotoxicity of general anesthetics: an update. Curr Pharm Des. 2012;18:6232-40.

[8] Staal SP. Molecular cloning of the AKT oncogene and its human homologues AKT1 and AKT2: amplication of AKT1 in a primary human gastric adenocarcinoma. PNAS 1987;84:5034-7.

[9] Mousa A, Bakhiet M. Role of cytokine signaling during nervous system development. Int J Mol Sci. 2013;14:13931-57.

[10] Harper SJ, Wilkie N. MAPKs: new targets for neurodegeneration. Expert Opin Ther Targets 2003;7:187-200.

[11] Li Y, Wang F, Liu C, Zeng M, Han X, Luo T, et al. JNK pathway may be involved in isoflurane-induced apoptosis in the hippocampi of neonatal rats. Neurosci Lett. 2013b;545:17-22.

[12] Menard C, Bastianetto S, Quirion R. Neuroprotective effects of resveratrol and epigallocatechin gallate polyphenols are mediated by the activation of protein kinase C gamma. Front Cell Neurosci. 2013;7:281.

[13] Ahn HC, Lee SY, Kim JW, Son WS, Shin CG, Lee BJ. Binding aspects of

baicalein to HIV-1 integrase. Molecules and Cells 2001;12:127-30.

[14] Wang W, Wang F, Yang YJ, Hu ZL, Long LH, Fu H, et al. The flavonoid baicalein promotes NMDA receptor-dependent long-term potentiation and enhances memory. Br J Pharmacol. 2011;162:1364-79.

[15] Wang SY, Wang HH, Chi CW, Chen CF, Liao JF. Effects of baicalein on beta-amyloid peptide-(25-35)-induced amnesia in mice. Eur J Pharmacol. 2004;506:55-61.

[16] Lei X, Zhang W, Liu T, Xiao H, Liang W, Xia W, et al. Perinatal supplementation with omega-3 polyunsaturated fatty acids improves sevoflurane-induced neurodegeneration and memory impairment in neonatal rats. PLoS ONE 2013;8:e70645.

[17] Yuede CM, Wozniak DF, Creeley CE, Taylor GT, Olney JW, Farber NB. Behavioral consequences of NMDA antagonist-induced neuroapoptosis in the infant mouse brain. PLoS ONE 2010;5:e11374.

[18] Li Y, Liang G, Wang S, Meng Q, Wang Q, Wei H. Effects of fetal exposure to isoflurane on postnatal memory and learning in rats. Neuropharmacology 2007;53:942-50.

[19] Wu J, Dong L, Zhang M, Jia M, Zhang G, Qiu L, et al. Class I histone deacetylase inhibitor valproic acid reverses cognitive deficits in a mouse model of septic encephalopathy. Neurochem Res. 2013;38:2440-9.

[20] Ji M, Dong L, Jia M, Liu W, Zhang M, Ju L, et al. Epigenetic enhancement of brain derived neurotrophic factor signaling pathway improves cognitive impairments induced by isoflurane exposure in aged rats. Mol Neurobiol. 2014;50: 937-44.

[21] Satoh Y, Endo S, Ikeda T, Yamada K, Ito M, Kuroki M, et al. Extracellular signal-regulated kinase 2 (ERK2) knockdown mice show deficits in long-term memory; ERK2 has a specific function in learning and memory. J Neurosci. 2007;27:10765-76.

[22] Zhao YL, Xiang Q, Shi QY, Li SY, Tan L, Wang JT, et al. GABAergic excitotoxicity injury of the immature hippocampal pyramidal neurons' exposure to isoflurane. Anesth Analg. 2011;113:1152-60.

[23] Fang F, Xue Z, Cang J. Sevoflurane exposure in 7-dayold rats affects neurogenesis, neurodegeneration and neurocognitive function. Neurosci Bull. 2012;28:499-508.

[24] Piehl E, Foley L, Barron M, D'Ardenne C, Guillod P, Wise-Faberowski L. The effect of sevoflurane on neuronal degeneration and $GABA_A$ subunit composition in a developing rat model of organotypic hippocampal slice cultures. J Neurosurg Anesthesiol. 2010;22:220-9.

[25] Zheng SQ, An LX, Cheng X, Wang YJ. Sevoflurane causes neuronal apoptosis and adaptability changes of neonatal rats. Acta Anaesthesiol Scand. 2013;57:1167-74.

[26] Zhang Y, Dong Y, Wu X, Lu Y, Xu Z, Knapp A, et al. The mitochondrial pathway of anesthetic isoflurane-induced apoptosis. J Biol Chem. 2010;285:4025-37.

[27] Wang WY, Yang R, Hu SF, Wang H, Ma ZW, Lu Y. N-stearoyl-l-tyrosine ameliorates sevoflurane induced neuroapoptosis via MEK/ERK1/2 MAPK signaling pathway in the developing brain. Neurosci Lett. 2013;541:167-72.

[28] Song G, Ouyang G, Bao S. The activation of Akt/PKB signaling pathway and cell survival. J Cell Mol Med. 2005;9:59-71.

[29] Sanders RD, Xu J, Shu Y, Januszewski A, Halder S, Fidalgo A, et al. Dexmedetomidine attenuates isoflurane-induced neurocognitive impairment in neonatal rats. Anesthesiology 2009;110:1077-85.

[30] Liao Z, Cao D, Han X, Liu C, Peng F, Zuo Z, et al. Both JNK and P38 MAPK pathways participate in the protection by dexmedetomidine against isoflurane-induced neuroapoptosis in the hippocampus of neonatal rats. Brain Res Bull. 2014;107:69-78.

[31] Zimmermann KC, Green DR. How cells die: Apoptosis pathways. J Allergy Clin Immunol. 2001;108:S99-103.

[32] Zhao H, Yenari MA, Cheng D, Sapolsky RM, Steinberg GK. Bcl-2 overexpression protects against neuron loss within the ischemic margin following experimental stroke and inhibits cytochrome c translocation and caspase-3 activity. J Neurochem. 2003;85:1026-36.

[33] Guan QH, Pei DS, Zhang QG, Hao ZB, Xu TL, Zhang GY. The neuroprotective action of SP600125, a new inhibitor of JNK, on transient brain ischemia/reperfusion-induced neuronal death in rat hippocampal CA1 via nuclear and non-nuclear pathways. Brain Res. 2005;1035:51-9.

[34] Tamura Y, Simizu S, Osada H. The phosphorylation status and anti-apoptotic activity of Bcl-2 are regulated by ERK and protein phosphatase 2A on the mitochondria. FEBS Lett. 2004;569:249-55.

[35] Jeong HS, Choi HY, Choi TW, Kim BW, Kim JH, Lee ER, et al. Differential regulation of the antiapoptotic action of B-cell lymphoma 2 (Bcl-2) and B-cell lymphoma extra long (Bcl-xL) by c-Jun N-terminal protein kinase (JNK) 1-involved pathway in neuroglioma cells. Biol Pharm Bull. 2008;31:1686-90.

[36] Allan LA, Morrice N, Brady S, Magee G, Pathak S, Clarke PR. Inhibition of caspase-9 through phosphorylation at Thr 125 by ERK MAPK. Nat Cell Biol. 2003;5:647-54.

[37] Liu XS, Xue QS, Zeng QW, Li Q, Liu J, Feng XM, et al. Sevoflurane impairs memory consolidation in rats, possibly through inhibiting phosphorylation of glycogen synthase kinase-3b in the hippocampus. Neurobiol Learn Mem. 2010;94:461-67.

[38] Manning BD, Cantley LC. AKT/PKB signalling: navigating downstream. Cell 2007;129:1261-74.

[39] Koh PO. Nicotinamide attenuates the ischemic brain injury-induced decrease of Akt activation and Bad phosphorylation. Neurosci Lett. 2011;498:105-9.

[40] Zhang J, Yu XH, Yan YG, Wang C, Wang WJ. PI3K/Akt signaling in osteosarcoma. Clin Chim Acta. 2015;444:182-92.

[41] Hunter AM, LaCasse EC, Korneluk RG. The inhibitors of apoptosis (IAPs) as cancer targets. Apoptosis 2007;12:1543-68.

[42] Kodama M, Satoh Y, Otsubo Y, Araki Y, Yonamine R, Masui K, et al. Neonatal desflurane exposure induces more robust neuroapoptosis than do isoflurane and sevoflurane and impairs working memory. Anesthesiology 2011;115:979-91.