Mangiferin Potentiates Neuroprotection by Isoflurane in Neonatal Hypoxic Brain Injury by Reducing Oxidative Stress and Activation of Phosphatidylinositol-3-Kinase/Akt/Mammalian Target of Rapamycin (PI3K/Akt/mTOR) Signaling

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Data Collection B
Statistical Analysis C
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Background: Hypoxic-ischemic brain injury in the perinatal period is a main cause of perinatal mortality and neurologic complications in neonates and children. Recent studies have focused on the neuroprotective effect of anesthetic drugs. The volatile anesthetic isoflurane has been shown to exert neuroprotective effects in cerebral ischemia. Mangiferin is a natural polyphenol with various pharmacological properties, including antioxidant and anttumor effects. This study aimed to determine whether mangiferin potentiates the neuroprotective effects of isoflurane and also if mangiferin when administered alone exerts neuroprotective effects following hypoxic-ischemic brain injury.

Material/Methods: Sprague-Dawley rats were subjected to cerebral hypoxic ischemia on postnatal day 10 (P10). Mangiferin (50, 100, or 200 mg/kg b.w.) was intragastrically administered from P3 to P12 and 1 h prior to insult on the day of ischemic induction. At 3 h after hypoxia-ischemia (HI) insult, separate groups of rat pups were exposed to isoflurane (1.5%) for 6 h. Following 48 h of HI, the rats were sacrificed and brain tissues were used for analysis.

Results: Mangiferin treatment attenuated neuronal apoptosis and reduced cerebral infarct volume. The expression of cleaved caspase-3 and apoptotic cascade proteins were regulated. The levels of reactive oxygen species (ROS) and malondialdehyde were reduced by mangiferin and/or isoflurane exposure. The levels of antioxidant glutathione were considerably raised under HI injury, which was modulated by mangiferin and isoflurane exposure. The PI3K/Akt signaling pathway, which was downregulated following HI insult, was activated by mangiferin and/or isoflurane.

Conclusions: This study reveals the potent neuroprotective efficacy of mangiferin against HI-induced brain injury via effectively modulating apoptotic pathways, ROS levels, and PI3K/Akt cascades while potentiating protective effects of isoflurane.

MeSH Keywords: Brain Injuries • Cell Hypoxia • Isoflurane • Phosphatidylinositol 3-Kinases

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Background

Perinatal hypoxia-ischemia (HI) has high global incidence of 1–8 per 1000 live births and is the main cause of mortality and neurological complications such as epilepsy, cerebral palsy, cognitive deficits, and visual and hearing impairments in neonates and children [1–6]. The pathophysiology of neonatal HI includes excitotoxicity, oxidative stress, neuroinflammation, and apoptosis [5,7].

Identification and development of effective neuroprotective compounds could be of immense clinical value. Recent studies have explored the neuroprotective effects of sevoflurane and isoflurane, which are commonly used volatile anesthetics. Isoflurane, administered prior to or after experimental cerebral ischemia has been found to exert neuroprotective effects in rodent models [8–12]. Neuroprotective effects of the inhalation anesthetic, sevoflurane, following cerebral ischemic injury has been reported to be mediated via activation of the PI3K/Akt (phosphoinositide 3-kinase/Protein kinase B) pathway [13,14].

The PI3K/Akt (phosphoinositide 3-kinase/Protein kinase B) signaling pathway regulates various processes, including cell growth, survival, and metabolism [15]. Wang et al. [16] demonstrated the neuroprotective role of the PI3K/Akt signaling pathway in an ischemic stroke model. This pathway is negatively regulated by phosphatase and tensin homolog (PTEN) [15]. Mammalian target of rapamycin (mTOR), one of the main downstream effectors of the pathway, exists as a multiprotein complex of mTORC1 and mTORC2 [15]. mTORC1, upon activation by Akt, subsequently leads to cell cycle progression. mTORC2 activates Akt in turn, providing a positive feedback loop aiding cell proliferation and survival [17]. Additionally, Akt can phosphorylate and deactivate glycogen synthase kinase 3β (GSK3β), a serine/threonine kinase, which leads to increased expressions of the cell cycle protein cyclin D1 [11]. Nuclear factor-κB (NF-κB), a central transcriptional factor, is also regulated and activated by Akt; it also activates the inhibitor of κB (IκB) kinase (IKK) and is activated by Akt. Upon activation, it causes phosphorylation and degradation of IκB, leading to subsequent release of NF-κB, which translocates to the nucleus and triggers transcription target genes. Akt is also documented to suppress apoptosis. The activation of PI3K/Akt signaling pathway leads to cell survival and to reduced cellular apoptosis [15].

It is well documented that oxidative stress, an imbalance between oxidant and antioxidant factors, is a chief contributor to ischemic brain injury [18,19]. Excessive production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) is detrimental and causes damage to the biomolecules, ultimately leading to neuronal degeneration and death [20,21]. The developing neonatal brain is susceptible to oxidative stress and damage, due to immature radical scavenging systems,

following injury [22]. Antioxidants counteract with free radicals and terminate the chain reaction [21]. Thus, it is rational to assume that exogenous antioxidant therapy could help reduce the associated cellular damage [23].

Recent studies have demonstrated the neuroprotective efficacy of plant-derived compounds in stroke and ischemic injury [7,24,25]. Mangiferin (C-glucopyranoside 1,3,6,7-tetrahydroxyxanthone) is a phytopolyphenol naturally present in numerous plant species such as *Mangifera indica* and *Iris unguicularis* [26]. Mangiferin exhibits various pharmacological properties such as antioxidant [27,28], anti-inflammatory [29], anti-tumor [30,31], and anti-diabetic effects [32]. Considering its wide array of bioactive properties, the present study explored the effects of mangiferin against experimental neonatal hypoxic brain injury.

Material and Methods

Experimental animals

The experimental design and protocols were approved by the Institutional Animal Ethics Committee of Provincial Hospital Affiliated to Shandong University. Pregnant Sprague-Dawley rats were housed in separate sterile plastic cages with 12 h light/12 h dark cycle at 25±1°C temperature and 50–60% humidity and were provided with water and standard pellet diet. The animals were carefully monitored for the birth of pups. The day of birth of pups was noted as postnatal day 0 (P0). On postnatal day 10 (P10) (weight, 22 to 30 g), the pups were subjected to hypoxic insult. All the experiments were carried out in compliance with the guidelines of the law of China for use of laboratory animals and also the National Institute of Health Guide for the Use of Laboratory Animals [33].

Antibodies and chemicals

Primary antibodies against Bcl-2, Bax, Bad, and Bcl-xL were procured from Abcam (USA). Akt, p-Akt, mTORC1, p-mTORC1, mTORC2, p-mTORC2, phosphatase and tensin homolog (PTEN), glycogen synthase kinase 3β (GSK-3β), p- GSK-3β (Ser9), NF-κBp65, and cleaved caspase-3 were procured from Cell Signaling Technology (Danvers, MA, USA). CyclinD1, β-actin, and horseradish- and peroxidase-labeled IgG secondary antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA) and used for expression analysis. Cell lysis buffer for Western blotting analysis was purchased from Beyotime Institute of Biotechnology (Beijing, China). Mangiferin and isoflurane were procured from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals used for analysis were obtained from Sigma-Aldrich.

Experimental design and dosing

The experimental animals were randomly separated to groups (n=12/group) as follows: Group 1 – Control – were not subjected to HI and received no isoflurane or mangiferin, Group 2 – HI control – animals were subjected to HI and did not receive isoflurane or mangiferin, Groups 3–5 – animals were subjected to HI and received mangiferin alone at (50, 100, or 200 mg/kg bodyweight); Group 6 – animals subjected to HI were exposed to isoflurane alone (1.5%); Groups 7–9 – animals received mangiferin (50, 100, or 200 mg/kg bodyweight) and exposed to isoflurane.

A separate group of rat pups (n=12 per group) were administered mangiferin (50, 100 or 200 mg/kg body weight) via oral gavage every day from P3 to P12, and on the day of HI, mangiferin was administered 1 h prior to insult. The doses of mangiferin were chosen based on the previous experiments conducted in our laboratory with varying doses of mangiferin (data not included). Cerebral HI was induced as previously described by Vannucci et al. [1] on P10 with slight variations. In brief, rats were anesthetized (3% halothane) and a 3-mm midline cervical incision was made and the left common carotid artery was isolated and ligated using 6-0 surgical silk at 2 locations. The common carotid artery was then transected between the ligatures to ensure that there was no blood flow through the ipsilateral carotid. The incision was sutured and the pups were placed in their respective cages and were allowed to recover for 2 h. The pups were then placed in a temperature-controlled chamber and subjected to 8% O_2 and 92% N_2 for 2 h at 37°C. At 3 h after insult, a separate group of pups were exposed to isoflurane (1.5% with air as carrier; gas flow 2 L/min) continuously for 6 h [1,34]. After 6 h of isoflurane exposure, the rats were removed from the chamber and exposed to air. The animals were observed for free movements and then placed back in their respective maternal cages.

On P12, the rats were killed by with transcardial perfusion of 10% formalin in PBS. The brains were excised immediately and stored in buffered formalin (10%) for 48 h and then frozen and used for analysis.

Histological studies

Selected sections from the hippocampal regions embedded in paraffin wax were sectioned to 2-mm sections after dehydration using a Leica SM2000R microtome (Leica, Germany). The sections were then stained with hematoxylin and eosin (H&E) as per standard protocol. The sections were then examined for morphological changes using a light microscope (Olympus BX 50 light microscope, ×400).

TTC staining

The brains were excised immediately after sacrifice and the wet weight was measured. 2,3,5-triphenyltetrazoliumchloride (TTC) staining was performed to evaluate brain viability and to measure the infarct size. The brain tissues were sectioned (2-mm sections) and were incubated with TTC for 30 min (37°C). The tissue sections were then immersed in 4% paraformaldehyde in the dark overnight. The infarct area was determined using NIH Image J software (Version 1.42; National Institutes of Health, Bethesda, MD). Normal areas of the brain stain with TTC, while the infarct areas remain unstained. Infarct volume was calculated by adding the infarct area of every section x thickness of the sections. The results are presented as percentage infarction/ipsilateral hemisphere.

Determination of apoptosis by TUNEL analysis

Terminal transferase-mediated dUTP nick-end labeling (TUNEL) staining was performed to assess apoptosis following hypoxic insult using the DeadEnd TM fluorometric TUNEL system kit (Promega, Madison, WI, USA) according to manufacturer's protocol. In brief, 5-µm-thick paraffin-embedded brain tissue sections were subjected to analysis. The TUNEL-positive cells were visualized and analyzed by NIS-Elements BR imaging processing and analysis software (version 4.0, Nikon Corporation, Japan).

Immunohistochemistry

Neuroapoptosis was further assessed by detecting cleaved caspase-3 expression levels by immunohistochemistry (IHC) as described previously [35]. The brain sections (10-µm slices) were incubated with cleaved caspase-3 primary antibody (1: 1000, Cell Signaling Technology) overnight at 4°C. The tissue sections were then washed with PBS and blocked with 1.6% H_2O_2 in PBS for 10 min at room temperature. After washing with PBS, the sections were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1: 500, Santa Cruz Biotechnology, Dallas, TX, USA) for 60 min at room temperature and then treated with diaminobenzidine. Cleaved caspase-3 positive cells were quantified and analyzed as described in TUNEL assay.

Assay of malondialdehyde and glutathione levels

Brain tissue (n=6) was homogenized with 1: 10 (w/v) PBS on ice and centrifuged (3000 rpm; 15 min, 4°C) and the supernatant was collected and used for assay of ROS and antioxidant levels. Concentration of the total protein in the supernatant was determined using the Bradford protein assay kit (Bio-Rad, Hercules, CA, USA). The concentrations of glutathione (GSH) and malondialdehyde (MDA) in the samples were determined
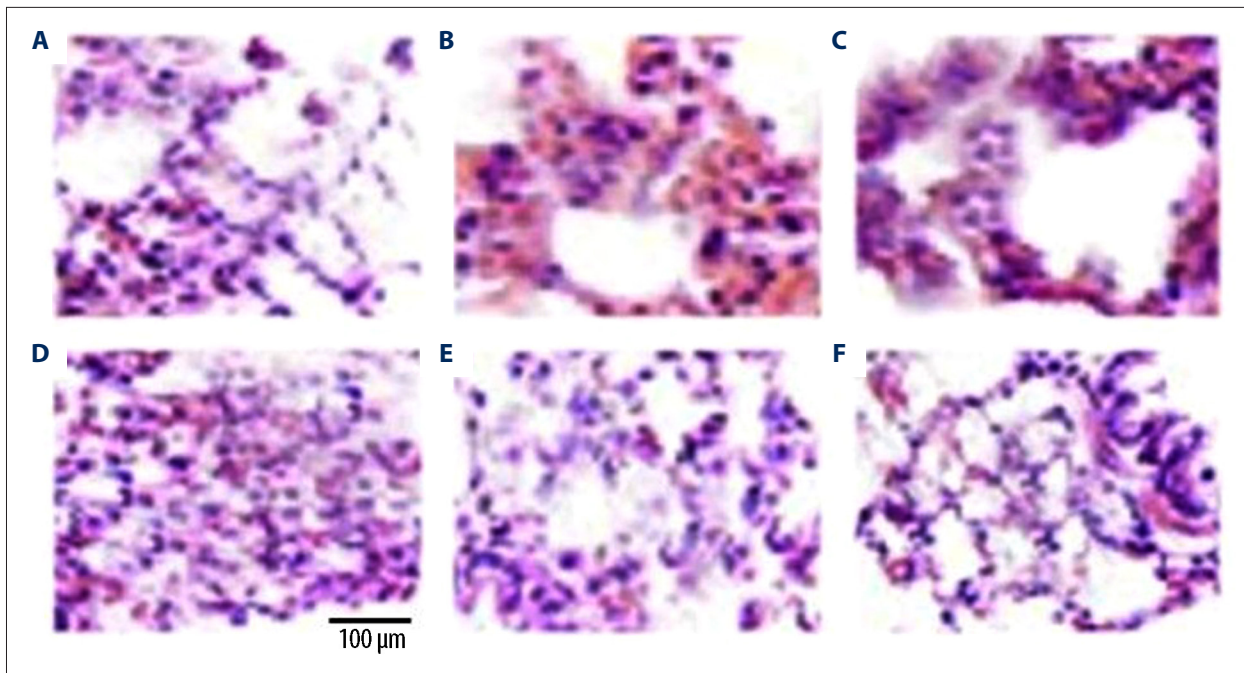
Figure 1. Effect of mangiferin and isoflurane on histological changes in the brain tissue following HI using hematoxylin and eosin (H&E) staining. (**A** – Control; **B** – HI control; **C** – Mangiferin (200 mg/kg) + HI; **D** – HI + Isoflurane (1.5%); **E** – Mangiferin (100 mg/kg) + HI + Isoflurane; **F** – Mangiferin (200 mg/kg) + HI + Isoflurane). Representative plates were obtained at 400× magnification.

using assay kits from Sigma-Aldrich, according to the manufacturer's protocols.

Determination of ROS

The *in vitro* ROS/RNS assay kit (OxiSelect™) (Cat No – STA-342-5; Cell Bio Labs, Inc.) was used to determine ROS levels in the brain tissues. A fluorogenic probe dichlorodihydrofluorescin DiOxyQ (DCFH-DiOxyQ) that is specific to ROS/RNS and which is similar to 2', 7'-dichlorodihydrofluorescein diacetate was used in the assay. DCFH-DiOxyQ is stabilized first to highly reactive DCFH that reacts with ROS and RNS and becomes oxidized to highly fluorescent DCF. The fluorescence intensity was measured (480 nm excitation and 530 nm emission) using the Synergy™ 2 Multi-function Microplate Reader.

Western blot analysis

The brain hemispheres (n=6) following HI were homogenized on ice using cell lysis buffer and were centrifuged at 15 000 rpm and 4°C for 10 min. Concentration of the total protein in the supernatant was determined using a bicinchoninic acid (BCA) protein assay kit (Bio-Rad, Hercules, CA, USA). Further, to assess NF-κB (p65) expression both in the cytosolic and nuclear fractions, an aliquot of the homogenate was separated into fractions using an NE-PER nuclear and cytoplasmic extraction reagents kit (Pierce Biotechnology, Rockford, IL, USA) according

to the manufacturer's instructions. Equal amounts (50 μg) of protein samples (for NF-κB, p65) from both the fractions/group from different experimental groups were electrophoretically separated on SDS-PAGE (2%) and electrotransferred on to polyvinylidene difluoride (PVDF) membranes (Invitrogen). Using 5% BSA (fetal bovine serum albumin) in Tris-buffered saline containing Tween-20, 0.1% (TBST), the membrane was blocked for 2 h at room temperature and then incubated overnight at 4°C with respective primary antibodies, after which, the blots were washed using TBST and further incubated for 2 h at room temperature with HRP-conjugated secondary antibodies. The immunoreactive bands were visualized and scanned using an Image Master II scanner (GE Healthcare, Milwaukee, WI, USA). The band densities were further analyzed by ImageQuant TL software (GE Healthcare, Milwaukee, WI, USA). The expression of the test proteins was standardized with that of β-actin.

Statistical analysis

The results are expressed as mean ± standard deviation calculated from 6 individual experiments. The statistical analysis was carried out using SPSS software (version 22.0, SPSS Inc., Chicago, IL, USA). Multiple group comparisons were analyzed using analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT). A *p*-value <0.05 was considered significant.
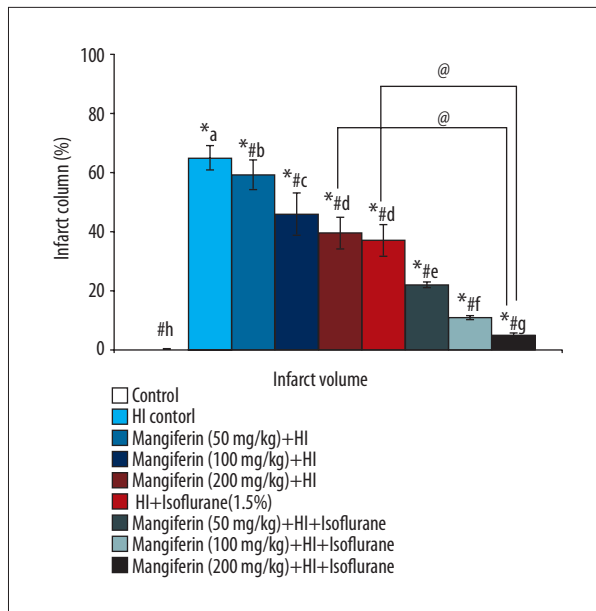
Figure 2. Mangiferin and isoflurane reduced cerebral infarct area. Values are mean ±SD, n=6. p<0.05 as determined by one-way ANOVA followed by DMRT analysis. * Represents p<0.05 *vs.* control; # represents p<0.05 *vs.* HI control; @ represents HI + Mangiferin (200 mg) + Isoflurane *vs.* HI + Isoflurane and Mangiferin (200 mg) + HI at p<0.05 *vs.* isoflurane. Different letters in different experimental groups indicate significant differences at p<0.05.

Results

Mangiferin and isoflurane improve histological changes in the brain tissue following HI

The histological analysis using HE staining (Figure 1) revealed severe neuronal damage in the hippocampal tissues. The pyramid neurons in the hippocampus region were observed to be swollen with no distinct boundaries between neurons. The nuclei were shrunken and the neuronal density was reduced. HE data suggest nerve cell damage following HI. Isoflurane exposure was found to restore the neuronal architecture. We also noticed that mangiferin treatment substantially improved the histology of the damaged tissues in a dose-dependent manner. Neuronal density was observed to be increased with substantially distinct boundaries between the neuronal cells. However, in animals that were exposed to isoflurane and mangiferin, the tissue morphology was more comparable to control animals than in animals that were exposed to isoflurane or mangiferin alone.

Mangiferin and isoflurane reduced infarct area

TTC staining was performed to assess the extent of infarction following HI. The observations revealed the severe infarction

(Figure 2). Isoflurane post-treatment resulted in reduction of infarct volume as compared to the HI control group and the pups that were treated with mangiferin exhibited considerably (p<0.05) smaller infarct volume as compared to the HI control group. However, the infarct volume was markedly smaller in rats treated with mangiferin and exposed to isoflurane in comparison to rats that were treated with either mangiferin or exposed to isoflurane.

Isoflurane and mangiferin reduced HI-induced apoptosis

TUNEL assay was performed to explore the neuroprotective effects of mangiferin and isoflurane. The results indicated that treatment with mangiferin (50, 100, or 200 mg) significantly (p<0.05) inhibited ischemia-induced neuronal cell death (Figure 3A). Further, IHC analysis revealed significantly (p<0.05) reduced cleaved caspase-3 expression in rats that were treated with mangiferin (Figure 3B). Isoflurane post-treatment also resulted in substantial decrease in cleaved caspase-3-positive cells as compared to the HI control group. However, the cleaved caspase-3-positive cell counts were higher in animals treated with mangiferin or isoflurane alone as compared to rats that were treated with mangiferin and exposed to isoflurane.

Further, Western blot analysis revealed significantly (p<0.05) enhanced cleaved caspase-3 expression along with elevated (p<0.05) levels of pro-apoptotic proteins Bax and Bad at 48 h after HI injury (Figure 4). Bcl-2 and Bcl-xL expression was observed to be substantially inhibited, indicating activation of the apoptotic cascade. Interestingly, post-treatment with isoflurane and/or treatment with mangiferin downregulated cleaved caspase-3, Bax and Bad expression, while the levels of Bcl-xL and Bcl-2 were elevated. In line with TUNEL-positive counts, the results of protein expression also illustrated that mangiferin treatment and exposure to isoflurane offered significant neuroprotective effects compared to treatment with mangiferin or isoflurane. We observed 200-mg mangiferin to be more effective in comparison to lower doses, irrespective of whether administered alone or followed by isoflurane post-treatment on P10.

Isoflurane and mangiferin decreased ROS levels following HI

Oxidative stress as a major contributor to hypoxic-ischemic brain injury is well documented [7]. Our data show a significant (p<0.05) increase in ROS levels following HI (Figure 5A). Interestingly, isoflurane reduced ROS levels considerably as compared to the HI control group. However, the levels of ROS in the mangiferin-treated group rats were markedly (p<0.05) lower as compared to the isoflurane alone group. Further, isoflurane and mangiferin treatment resulted in lower ROS levels compared to mangiferin alone groups, illustrating the
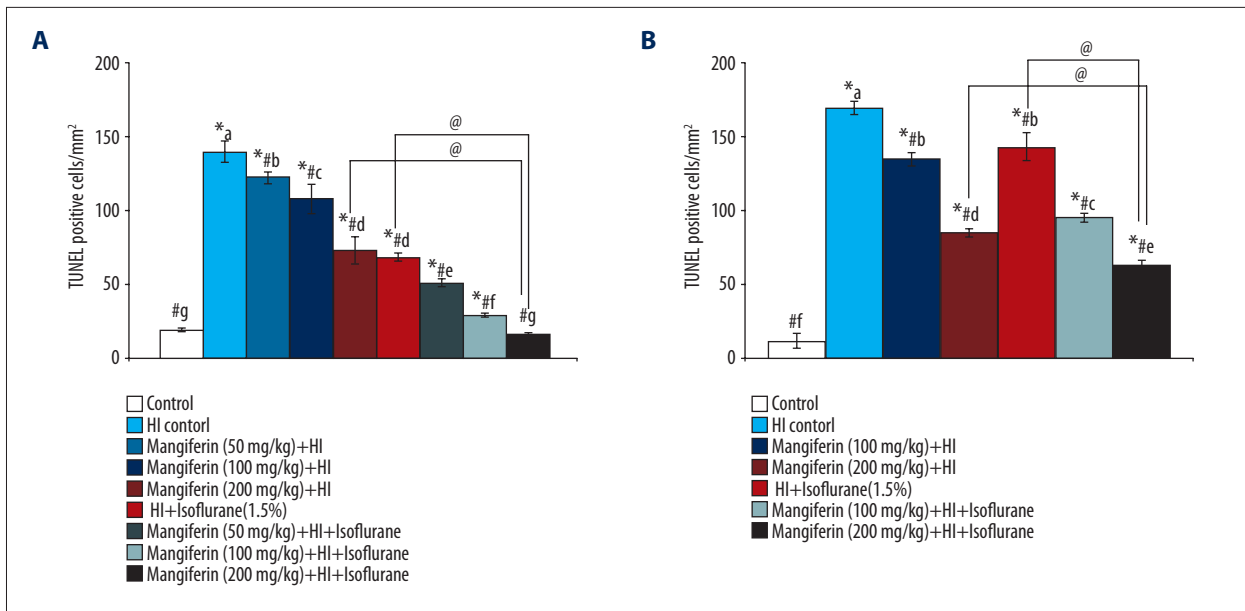
Figure 3. (**A**) Mangiferin and isoflurane decreased neuronal apoptosis as determined by TUNEL assay. Values are mean ±SD, n=6. p<0.05 as determined by one-way ANOVA followed by DMRT analysis. * Represents p<0.05 *vs.* control; # represents p<0.05 *vs.* HI control; @ represents HI + Mangiferin (200 mg) + Isoflurane *vs.* HI + Isoflurane and Mangiferin (200 mg) + HI at p<0.05 *vs.* isoflurane. Different letters in different experimental groups indicate significant differences at p<0.05. (**B**) Mangiferin and isoflurane decreased neuronal apoptosis expressions of cleaved caspase-3. Values are mean ±SD, n=6. p<0.05 as determined by one-way ANOVA followed by DMRT analysis. * Represents p<0.05 *vs.* control; # represents p<0.05 v HI control; @ represents HI + Mangiferin (200 mg) + Isoflurane *vs.* HI + Isoflurane and Mangiferin (200 mg) + HI at p<0.05 *vs.* isoflurane. Different letters in different experimental groups indicate significant differences at p<0.05.
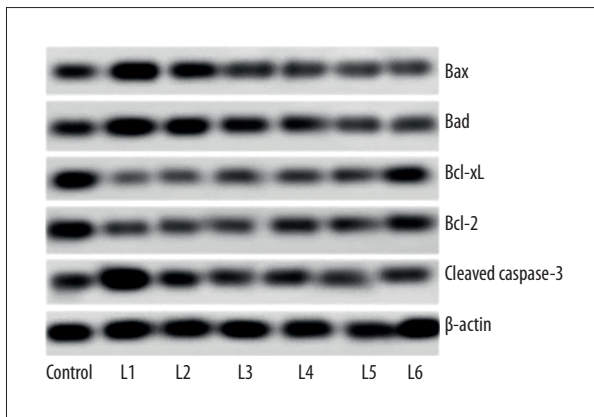

Figure 4. Mangiferin and isoflurane regulated apoptotic pathway proteins. Representative Western blot images. L1 – HI control; L2 – Mangiferin (100 mg/kg) + HI; L3 – Mangiferin (200 mg/kg) + HI; L4 – HI + Isoflurane (1.5%); L5 – Mangiferin (100 mg/kg) + HI + Isoflurane; L6 – Mangiferin (200 mg/kg) + HI + Isoflurane.

antioxidant potential of mangiferin that could have potentiated the effects of isoflurane on neutralizing ROS levels. In line with the ROS levels, MDA content was markedly higher (p<0.05) in the HI control group compared to normal controls. Elevated GSH levels (Figure 5B, 5C) neutralized increased ROS.

Interestingly, mangiferin treatment significantly (p<0.05) reduced MDA content, but no such effects were seen in the GSH levels. Isoflurane caused a marked decrease in GSH levels as opposed to the HI alone group. In the mangiferin and isoflurane groups, considerable increases in GSH levels were noticed. Further, the reduction in MDA levels was more pronounced in mangiferin treatment compared to isoflurane post-treatment. However, in rats exposed to mangiferin and isoflurane, MDA levels were significantly (p<0.05) reduced as compared to the isoflurane alone group.

Mangiferin and isoflurane activate the PI3K/Akt pathway

To further assess if the PI3K/Akt signaling pathway underlies mangiferin and isoflurane-induced neuroprotection in the neonatal HI model, we measured the expression of major effector proteins of the pathway. HI caused marked down-regulation in the levels of p-Akt, p-GSK-3β, p-mTORC1, and NF-κB p65, and significantly upregulated PTEN levels (Figure 6). Expression levels of cyclinD1 and p-IκBα were also reduced. However, the levels of phosphorylation of Akt, GSK-3β, mTORC1, and IκBα were significantly (p<0.05) increased, with suppressed PTEN expression after isoflurane post-treatment, suggesting activation of the pathway. Mangiferin treatment also significantly (p<0.05) increased phosphorylation of Akt, GSK-3β, mTORC1,

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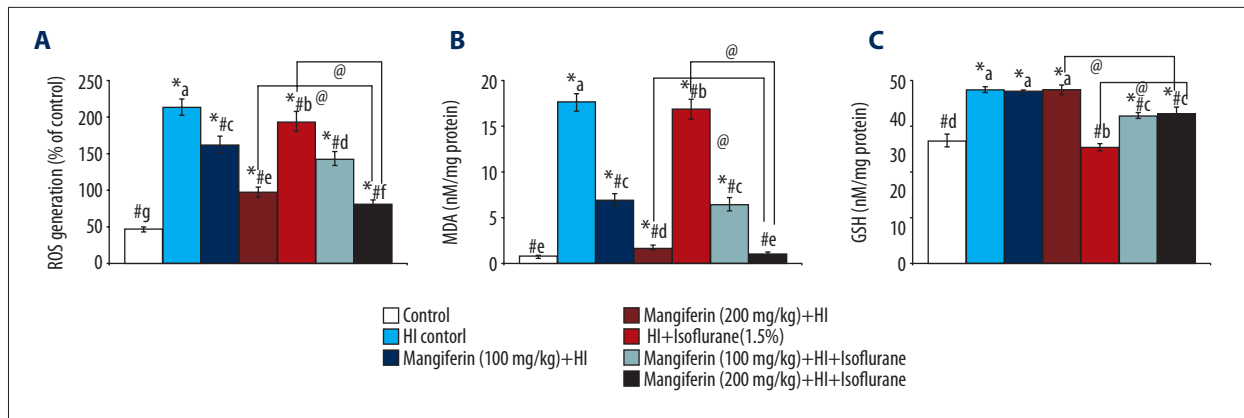
Figure 5. Mangiferin and isoflurane reduced HI-induced ROS. (**A**) Values are mean ±SD, n=6. p<0.05 as determined by one-way ANOVA followed by DMRT analysis. * Represents p<0.05 *vs*. control; # represents p<0.05 *vs*. HI control; @ represents HI + Mangiferin (200 mg) + Isoflurane *vs*. HI + Isoflurane and Mangiferin (200 mg) + HI at p<0.05 *vs*. isoflurane. Different letters in different experimental groups indicate significant differences at p<0.05. (**B**) Mangiferin and isoflurane reduced HI-induced MDA levels. Values are mean ±SD, n=6. p<0.05 as determined by one-way ANOVA followed by DMRT analysis. * Represents p<0.05 *vs*. control; # represents p<0.05 *vs*. HI control; @ represents HI + Mangiferin (200 mg) + Isoflurane *vs*. HI + Isoflurane and Mangiferin (200 mg) + HI at p<0.05 *vs*. isoflurane. Different letters in different experimental groups indicate significant differences at p<0.05. (**C**) Mangiferin and isoflurane reduced HI-induced ROS, MDA levels and enhances GSH levels. Values are mean ±SD, n=6. p<0.05 as determined by one-way ANOVA followed by DMRT analysis. * Represents p<0.05 *vs*. control; # represents p<0.05 *vs*. HI control; @ represents HI + Mangiferin (200 mg) + Isoflurane *vs*. HI + Isoflurane and Mangiferin (200 mg) + HI at p<0.05 *vs*. isoflurane. Different letters in different experimental groups indicate significant differences at p<0.05.
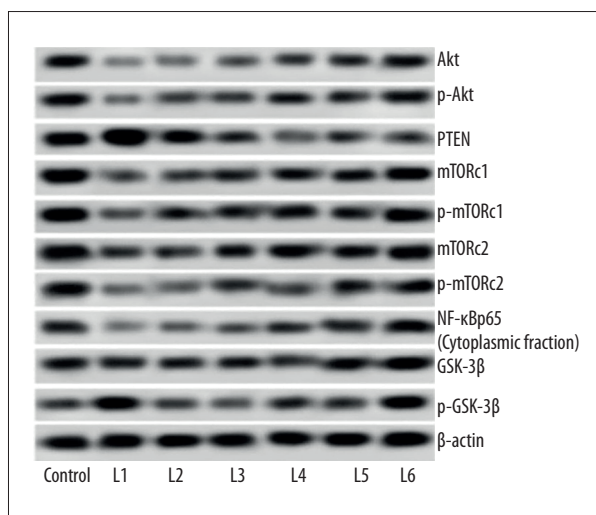

Figure 6. Effect of mangiferin and propofol on expression of major effector proteins of the PI3K/Akt pathway – representative Western blot images. L1 – HI control; L2 – Mangiferin (100 mg/kg) + HI; L3 – Mangiferin (200 mg/kg) + HI; L4 – HI + Isoflurane (1.5%); L5 – Mangiferin (100 mg/kg) + HI + Isoflurane; L6 – Mangiferin (200 mg/kg) + HI + Isoflurane.

and IκBα in a dose-dependent manner. However, in animals treated with mangiferin and exposed to isoflurane, activation of the pathway was more marked as compared to isoflurane or mangiferin treatment.

Discussion

Hypoxic-ischemic brain injury is a major cause of neonatal death and neurologic sequelae in neonates and infants. Neonatal HI presents high morbidity rates and survivors live with long-term neurological impairments, such as epilepsy and cerebral palsy [36,37]. Several pathophysiological factors such as excitotoxicity, inflammatory mediators, and oxidative stress have been associated in hypoxia-ischemia [5]. The volatile anesthetic isoflurane has been demonstrated to exert protective effects against ischemic injury in various organs [38–40]. The present study explored whether mangiferin improved the effects of isoflurane in a neonatal HI model.

We found that isoflurane (1.5%) exposure immediately after HI significantly restored histology of hippocampal tissue and reduced infarct volume and number of TUNEL-positive cells. Further, mangiferin administration was found to reduce neuronal loss and infarct volume. These observations suggest that mangiferin augmented the neuroprotective effects of isoflurane. Further, immunohistochemical analysis revealed downregulated expression of cleaved caspase-3, a marker of apoptotic cell death. It is known that the balance between the pro-apoptotic proteins (Bad and Bax) and anti-apoptotic proteins (Bcl-2 and Bcl-xL) critically regulates cell survival [41]. The effect of upregulated expression of Bcl-2 and Bcl-xL, along with downregulated Bad and Bax, on mangiferin and/or isoflurane treatment illustrates promotion of cell survival. Interestingly, exposure

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to both mangiferin and isoflurane was found to reduce apoptosis and improve cell survival, indicating that mangiferin potentiated neuroprotective effects of isoflurane.

Oxidative stress is well documented as a major contributor in the pathology of HI [42]. The neonatal brain is highly vulnerable to oxidative stress due to lack of adequate antioxidants to combat the excessive ROS, leading to neurotoxicity [42–45]. Elevated ROS levels and MDA levels in our study reflect oxidative stress following HI. Raised levels of the antioxidant GSH indicates cellular defence responses to stress. Mangiferin administration was found to attenuate the oxidative stress. Mangiferin reduced ROS production and MDA levels but mangiferin did not cause such effects on the GSH levels. This is probably due to the effective contribution of mangiferin in neutralization of ROS, which contributed to decreased oxidative stress levels. Reduced stress levels as observed in mangiferin treatment may have not induced GSH levels. Excessive ROS causes response from native antioxidant defence and the reduction in ROS levels by mangiferin reflects reduced oxidative stress condition. These results indicate the antioxidant potential of mangiferin.

Isoflurane exposure following HI insult slightly increased the antioxidant levels and reduced the ROS levels; nevertheless, the changes were significantly less than the effects of mangiferin. Further, isoflurane was observed to decrease GSH levels. Studies have reported that prolonged exposure to isoflurane reduces GSH levels [46,47]. The observed decrease could be attributed to the raised oxidative stress conditions on induction of HI injury and, in part, isoflurane could have contributed to decreased GSH levels, as reported in previous studies. This reduction in GSH reflects overutilization of the enzyme against ROS. Nevertheless, mangiferin and isoflurane administration enhanced GSH and reduced MDA and ROS levels, reflecting the capacity of mangiferin in aiding the potential of isoflurane as a neuroprotective agent. Increased GSH levels on administration of mangiferin and isoflurane *vs.* the isoflurane alone group suggests the efficacy of mangiferin in improving the antioxidant status, which in part could have aided in reduced ROS and MDA levels apart from direct neutralization of ROS by mangiferin, illustrating the effects of mangiferin in potentiating the effects of isoflurane. This reduction in ROS could have also aided in reduction in neuronal apoptosis. These effects could be attributed to the antioxidant capacity of mangiferin.

The phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway is expressed widely in the CNS during development; it mediates cell survival and differentiation and cellular metabolisms, which are involved in neurocyte nutrition [48–50]. GSK-3β, an important downstream protein of Akt, is reported to be involved in HI brain damage. GSK-3β exerts pro-apoptotic effects in HI brain injury via activation of p53, thus making proteins of the PI3K/Akt/GSK-3β signaling

pathway targets in treatment of HI [51–53]. Studies have reported that activation of the PI3K/Akt pathway increases neuroprotection and neuronal cell survival [54,55].

In the present study, HI-induced significant suppression of the pathway was observed to be markedly activated by isoflurane and/or mangiferin. mTORC2 causes phosphorylation and activation of Akt. Mangiferin and/or isoflurane mediated enhances expression of mTORC2, and p-mTORC2 could have caused enhanced phosphorylated Akt levels. Phosphorylated Akt inactivates GSK-3β and inhibits apoptosis through phosphorylation of Bad and GSK-3β [56,57], indicating activation of the pathway. Isoflurane and mangiferin also raised the expression of p-mTORC1 and NF-κB, the downstream targets of Akt. NF-κB, is an important transcription factor that is associated with cell proliferation and cell cycle progression [58–60]. In our study we observed suppressed phosphorylation of Akt and GSK-3β with enhanced PTEN, the chief negative regulator of the pathway following ischemic insult [15], thus down-regulating PI3KAkt signaling. Isoflurane and/or mangiferin-mediated enhanced expression of p-Akt and downstream molecules of Akt as – GSK-3β, nuclear factor-κB (NF-κB) and mTORC1. Increased expressions of mTORC2 indicates effective activation of Akt, leading to activation of the pathway. It is known that activated mTORC1 and mTORC2 eventually increase cell cycle progression and cell survival [15]. Further, the expression levels of phosphorylated forms of Akt, GSK-3β, mTORC1, and mTORC2 were more pronounced in animals that were exposed to isoflurane and treated with mangiferin compared to rats treated with isoflurane or mangiferin alone. Interestingly, anesthetic sevoflurane post-conditioning improved memory of neonatal rats following HI brain damage via activation of the PI3K/Akt signaling pathway [61]. The experimental data suggest that PI3K/Akt pathway activation is a potential mechanism involved in the protective effects of mangiferin and/or isoflurane. Furthermore, mangiferin significantly potentiated the neuroprotective effects of isoflurane via enhancing the PI3K/Akt pathway. These findings demonstrate mangiferin is a promising therapeutic agent in the treatment of neonatal ischemic brain injury, administered alone or with isoflurane.

Conclusions

The experimental data illustrate the neuroprotective effects of mangiferin and isoflurane in experimental neonatal HI and also show that administration of mangiferin clearly potentiated the protective effects of isoflurane. The molecular mechanisms underlying these protective effects warrant further study.

Conflicts of interest

None.

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