Naringenin pre-treatment inhibits neuroapoptosis and ameliorates cognitive impairment in rats exposed to isoflurane anesthesia by regulating the PI3/Akt/PTEN signalling pathway and suppressing NF-κB-mediated inflammation

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Abstract. The volatile anaesthetic isoflurane is one of the most frequently employed general anaesthetics in neonates, children and adults. Accumulating evidence demonstrated that exposure to anaesthetics is associated with widespread neurodegeneration and cognitive impairment. Thus, the identification and development of compounds capable of preventing or reducing these adverse effects is of great clinical importance. For this purpose, the present study aimed to assess the effects of a flavonoid, naringenin, on isoflurane-induced neuroapoptosis and cognitive impairment. Separate groups of neonatal rat pups were administered naringenin at 25, 50 or 100 mg/kg body weight from postnatal day 1 (P1) to P21. On P7, the pups were exposed to 6 h of isoflurane (0.75%) anaesthesia. Neuroapoptosis was examined using the TUNEL assay. The expression of cleaved caspase-3, the apoptotic pathway proteins (Bad, Bax, Bcl-2 and Bcl-xL), the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) pathway proteins [Akt, phosphorylated (-)Akt, glycogen synthase kinase 3β (GSK-3β), p-GSK-3β, phosphatase and tensin homolog (PTEN)] and nuclear factor-κB (NF-κB)-mediated signalling proteins were determined by western blot analysis. General behaviour, as well as the learning ability and memory of the pups were assessed. Naringenin significantly inhibited isoflurane-induced neuroapoptosis and markedly decreased the protein expression of caspase-3, Bad, Bax, NF-κB, tumor necrosis factor-α, interleukin (IL)-6 and IL-1β. Furthermore, naringenin increased the expression of Bcl-xL and Bcl-2 and activated the PI3K/Akt pathway. Significant improvements in learning capacity and memory retention were observed following naringenin treatment. Naringenin effectively ameliorated cognitive dysfunction and reduced isoflurane-induced apoptosis as well as modulating the PI3/Akt/PTEN and NF-κB signalling pathways.

Introduction

General anaesthetics are commonly employed in operative settings for surgery and/or for pain relief. Volatile anaesthetics such as isoflurane and sevoflurane are widely used in adults as well in infants and young children for surgical procedures and other medical treatments that require anaesthesia (1). Previous findings have demonstrated that prolonged exposure to the anaesthetics isoflurane and sevoflurane leads to apoptotic neurodegeneration in developing animal brains and also affects learning and memory (2-7). Liang *et al* (8) reported that isoflurane anaesthesia induced more robust apoptosis of cells in the neonatal rat brain than sevoflurane when administered at an eqivalent dose. Further clinical retrospective studies have demonstrated that children <4 years of age exposed to anaesthesia are possibly at a higher risk of developing learning disabilities (9,10).

The increased expression of activated caspase-3, the principal enzyme in the cellular apoptotic pathway, and β-amyloid peptide (Aβ) levels were observed in murine brains following isoflurane exposure (11,12). Studies have also suggested that inflammation may possibly be involved in anaesthetic-mediated neurodegeneration, particularly cognitive dysfunction (13,14). Wu *et al* (15) observed that isoflurane increased pro-inflammatory cytokine levels in both *in vitro* and *in vivo* experiments. Inflammation involving microglial activation and increased levels of pro-inflammatory cytokines, particularly tumor necrosis factor-α (TNF-α) and interleukin (IL)-6 in the brain, may lead to cognitive impairment (16-21).

It is well documented that the nuclear factor-κB (NF-κB)-dependent signalling pathway is required for cytokine gene transcription (22). The NF-κB family of

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transcription factors play essential roles in inflammation and innate immunity, and regulate many genes, particularly those involved in inflammation, injury and stress (23). Isoflurane has been reported to induce neuronal apoptotic degeneration via $[Ca^{2+}]_i$ overload through gamma-aminobutyric acid (GABA)$_A$ receptor-mediated synaptic voltage-dependent calcium channels (VDCCs). The excessive release of Ca^{2+} from the endoplasmic reticulum through the activation of inositol-1,4,5-trisphosphate (IP3) receptors (24,25), activates the NF-κB signalling pathway (26-28), eventually leading to increased levels of pro-inflammatory cytokines (27,29).

In addition, the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) pathway is widely expressed in the central nervous system and affected by growth factors, such as nerve growth factor, brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor, as well as cytokines and neurotransmitters. The pathway plays a crucial role in neuronal survival (30). Akt, a serine/threonine kinase, regulates neuronal survival and is activated by growth factors (31). On activation, Akt inhibits apoptosis by phosphorylating and inactivating Bad and glycogen synthase kinase 3β (GSK-3β) (32-34). Another target modulated by Akt is NF-κB (35). Thus, targeting the PI3/Akt pathway may significantly modulate neuronal cell survival and inflammation.

The flavonoid naringenin is found in oranges, grapefruits and tomatoes (36). It has been proven to possess health benefits such as anti-inflammatory, antioxidant and free radical scavenging properties (37,38) as well as to reduce lipid levels (39) and to prevent dyslipidemia (40). Taking into consideration the biological effects of naringenin, in the present study, we examined the ability of naringenin to inhibit isoflurane-induced neuroapoptosis and to ameliorate inflammation-induced cognitive dysfunction.

Materials and methods

Experimental animals. The study was approved by the Institutional Animal Care Committee of Nanchang University (Nanchang, China) and was performed in accordance with the National Institutes of Health Guidelines for the Use of Laboratory Animals. Pregnant Sprague-Dawley rats (Guangdong Medical Laboratory Animal Center, Guangzhou, China) were housed separately in sterile cages under animal room conditions maintained at 22±1˚C on a 12-h light/dark cycle. The rats were provided with standard pelleted diet and water *ad libitum* and closely monitored for the date of birth of the pups, that was noted as postnatal day 0 (P0). The pups were maintained carefully with free access to water with their littermates.

Chemicals and reagents. Isoflurane (0.75%) and naringenin were obtained from Sigma-Aldrich (St. Louis, MO, USA). The following antibodies were used for expression analysis: antibodies against cleaved caspase-3 (#9661), Bcl-2 (mouse mAb #15071), Bad (rabbit mAb #9268), Bcl-xL (rabbit mAb #2764), Bax (rabbit mAb #5023), Akt (rabbit mAb #4691), phosphorylated (p-)Akt (rabbit mAb #4060), GSK-3β (rabbit mAb #9315), p-GSK-3β (rabbit mAb #9322), phosphatase and tensin homolog (PTEN; rabbit mAb #9188), the inhibitors of apoptosis proteins (IAP) xIAP (rabbit mAb #2045), cIAP-1 (rabbit mAb #3130), survivin (rabbit mAb #2808),

TNF-α [rabbit mAb (mouse specific) #11948] and β-actin (mouse mAb #3700) (all from Cell Signalling Technology, Beverly, MA, USA); NF-κB p65 (#sc-8008) and p-IκBα (#sc-8404) (both from Santa Cruz Biotechnology, Santa Cruz, CA, USA); and IL-6 (ab100712) and IL-1β (ab197742) (both from Abcam, Cambridge, MA, USA). All other chemicals used were of analytical grade and purchased from Sigma-Aldrich, unless specified otherwise.

Anaesthetic exposure and dosing. Sixty pups were used and 12 pups were randomly allocated to each group. Separate groups of pups were administered naringenin [25, 50 or 100 mg/kg body weight (b.wt], orally from P3 until P21 along with a standard diet. On P7, pups weighing about 16-20 g were exposed to 0.75% isoflurane [approximately 0.3 minimum alveolar concentration (MAC)] (41) in 30% oxygen or air in a temperature controlled chamber for 6 h (42,43). P7 rats were selected on the basis of previous studies suggesting that this period was most vulnerable to anaesthesia-induced neuronal damage (2). The control rats received no anaesthesia or naringenin. At the end of 6 h of isoflurane exposure, the rat pups were sacrificed and brain tissues were excised in order to perform protein expression analysis and the TUNEL assay. Briefly, P7 rat pups were anaesthetized with isoflurane and transcardially perfused with ice-cold saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The brain tissues were excised and embedded in paraffin. The rat pups not exposed to either anaesthesia or treated with naringenin were segregated as control rats. The isoflurane-exposed pups not administered naringenin were grouped as anaesthetic control pups. The animals were anesthetised with pentobarbital (40 mg/kg body weight) and were intracardially perfused with 0.01M PBS. The brains were immediately excised and the hippocampi were isolated. The brain tissues were kept at -80ºC until required for use in subsequent experiments.

Assessment of neuroapoptosis by TUNEL assay. Following 6 h of exposure to isoflurane, neuroapoptosis was assessed by the TUNEL assay as previously described by Li *et al* (44). Three sections 5 μm thick were sliced (200 μm apart) on the same plane of the hippocampus from each rat pup and were further analysed. Apoptosis was assessed using a DeadEnd™ fluorometric TUNEL System kit (Promega, Madison, WI, USA). The slides were carefully protected from exposure to direct light during the experiment. The Hoechst stain was used to stain nuclei. The TUNEL-positive cells in the regions of hippocampal CA1, CA3 and dentate gyrus (DG) were further analysed using NIS-Elements Basic Research (BR) imaging processing and analysis software (Nikon Corporation, Tokyo, Japan).

Immunohistochemistry (IHC) staining. Cleaved caspase-3 expression was used as a marker of apoptosis and cell death. Immunohistochemical analysis was performed to assess caspase-3 expression in the hippocampi of isoflurane-exposed rats as previously described by Li *et al* (42,44). Briefly, the brain sections were incubated with anti-cleaved caspase-3 primary antibody overnight at 4˚C and further incubated with a secondary antibody (Santa Cruz Biotechnology) for 40 min. Following treatment with avidin-biotinylated peroxidase complex (Vectastain ABC-kit; Vector Laboratories,

Burlingame, CA, USA) for 40 min, the tissue sections were treated with diaminobenzidine and were analysed with NIS-Elements BR imaging processing and analysis software. The density of cleaved caspase-3-positive cells in CA1, CA3 and DG regions was calculated by dividing the number of caspase-3-positive cells by the area of that brain region.

Western blot analysis. Protein expression in the hippocampi of rat pups was examined in order to determine the effect of naringenin on the outcome of isoflurane exposure according to previously described procedures (42,43). Briefly, the tissues were homogenised and proteins were isolated. Protein concentrations in the tissue samples were determined using a BCA protein assay (Bio-Rad, Hercules, CA, USA). Sixty micrograms of each protein sample were subjected to SDS-PAGE separation and blotted on to polyvinylidene difluoride membranes. The blots were incubated with primary antibodies overnight at 4˚C and washed and further incubated with appropriate secondary antibodies. The immunoreactive bands were visualized and the images were scanned using an ImageMaster II scanner (GE Healthcare, Milwaukee, WI, USA). The densities of the bands were further analysed by ImageQuant TL software v2003.03 (GE Healthcare). Protein expression was normalized to β-actin.

Memory and learning studies. Isoflurane induction has been documented to cause cognitive dysfunction affecting learning and memory (2). In the present study, the effects of naringenin on the behaviour and learning of rat pups were assessed.

Open-field test. An open-field test was performed to assess the emotional response of the rats to a novel environment. P42 rats exposed to isoflurane on P7 were subjected to an open-field test as previously described (45). The response was noted as the movement of the rats in the open field. The total distance travelled in meters over 10 min was recorded. The movement of the animals was monitored and analysed using a computerized video tracking system (SMART; Panlab S.L., Barcelona, Spain).

Elevated plus-maze test. The elevated plus-maze test was performed to evaluate anxiety-related behaviour in rodents. The test was conducted according to the procedure described by Satoh *et al* (45). The apparatus consisted of two open (25x5 cm) and two closed arms. The arms were placed at 50 cm above the floor. The responses of the animals were recorded using a computerized video tracking system (SMART). The behaviour of P42 rats was observed for a period of 10 min. The percentage of time spent in the open arm was noted and this was considered as an anxiety index.

Y-maze test. This study was performed to assess spatial working memory and was performed as previously described by Satoh *et al* (45). The symmetrical, acrylic Y-shaped maze consisted of three arms (25x5 cm) that were separated by 15-cm-high transparent walls. The rats were placed at the centre of the maze and each rat was allocated 8 min to freely explore the maze. The total number of arms entered and sequence of entry were noted too. The calculations were performed as described by Kodama *et al* (46).

Fear conditioning test. The P49 rats were subjected to a fear response test. The fear conditioning test is a reliable, sensitive test used to assess the effect of a hippocampal-dependent and hippocampal-independent conditioned stimulus-unconditioned stimulus pair that were separated by 1 min each. The unconditioned stimulus consisted of 1 mA foot shock of a 1 sec duration whereas the conditioned stimulus consisted of 80 dB of white noise for 20 sec. The unconditioned stimulus was delivered during the last few seconds of the conditioned stimulus. A contextual fear test was performed in the conditioning chamber for a period of 5 min in the absence of white noise 24 h after conditioning. A cued test (for the same set of rats) was conducted by presenting a cue (80 dB white noise, 3-min duration) while presenting distinct visual and tactile cues. The movement of the animals was monitored using the computerized video tracking system (SMART). The freezing response rate of the rats, which was identified as the absence of movement in any part of the body for 1 sec, was scored automatically and the data were analysed and used as a measure of fear memory (4).

Morris water maze (MWM) test. The effect of naringenin on isoflurane-induced alterations in memory and learning, spatial reference memory and learning were evaluated using the MWM and experiments were performed as described previously by Li *et al* (44).

The P7 rat pups that were exposed to isoflurane and/or naringenin were trained for a period of 4 days between P31 and P35 in the MWM. A platform 10 cm in diameter was submerged in a circular pool (200 cm diameter, 60 cm depth) that was filled with warm water (23±2˚C). The rats were subjected to 2 training sessions a day. The rats were allowed 60 sec to locate the hidden platform in the pool. If the animals were unable to locate the platform within 60 sec, they were gently guided. The performance of the rats and swim paths were recorded using the ANY-maze video tracking system (Stoelting Co., Wood Dale, IL, USA). The tracking system records and measures the time taken (latency) by each rat to find the platform(s), and also information on other behaviours.

P35 rats were subjected to cued trials to test for any non-cognitive performance impairments such as visual impairments and/or swimming difficulties. The circular pool was covered by a white cloth in order to conceal the visual cues. The rats were subjected to 4 trials per day. During each trial, the animals were placed in a specific position in the swimming pool and allowed to swim to the platform which had a rod attached, that served as the cue. The rod was placed approximately at 20 cm above water level in any one of the four quadrants of the swimming pool. The rats were given a time of 60 sec to locate the submerged platform with the help of the cue and a time of 30 sec to sit on the platform. The rats that was unable to locate the platform in the given period of time, were gently guided and allowed to remain there for 30 sec. The time taken by each animal to find the cued platform was recorded.

Place trials were also performed. The curtains that surrounded the pool for the cued trials were removed and the same set of rats were assessed for their ability to learn the spatial relationship between cues and the submerged platform (with no cue rod), that was kept in one of the four quadrants. The platform was placed in the same position throughout the place trials. The rats were placed at random starting points and the time taken to reach the platform was noted.

Furthermore, the rats were subjected to probe trials in order to assess memory. The probe trials were conducted 24 h after place trials. The submerged platform with no cue rod was removed from the target quadrant (the quadrant in which the

platform was placed throughout the place trials). Rats were allowed to swim for 60 sec and the time that each rat spent in the target quadrant looking for the submerged platform was recorded. The data are presented as the percentage of time spent in the quadrants. The time spent in the target quadrant by the rats in comparison with the time spent in other quadrants is used as an indication of memory retention.

Statistical analysis. The results are presented as the means ± SD, taken from three or six independent experiments. The data were subjected to statistical analysis using the SPSS statistical package (22.0). The values at $p < 0.05$ were considered to indicate a statistically significant difference as determined by one-way analysis of variance (ANOVA) at $p < 0.05$ followed by Duncan's multiple range test (DMRT) for post hoc analysis.

Results

Naringenin reduces isoflurane-induced neuroapoptosis. Previous studies have demonstrated that isoflurane exposure induces neuroapoptosis (5,47). In agreement with the findings of earlier studies, in our study, an increase ($p < 0.05$) in apoptotic cell counts was observed following 6 h of isoflurane exposure in the CA1, CA3 and the DG regions or the rat hippocampi (Fig. 1). However, the administration of naringenin caused a marked decline in TUNEL-positive cell counts indicating that it effectively provided protection to the neuronal cells exposed to anaesthesia. Naringenin at 100 mg exhibited maximum protective effects as compared with lower doses of 25 and 50 mg.

Cleaved caspase-3 expression is considered as a significant marker of apoptosis (2,6). To determine whether naringenin modulated the expression of caspase-3 in the hippocampus, we assessed the expression of cleaved caspase-3 by IHC staining. An increase in the number of caspase-positive cells was observed in the hippocampus of rats exposed to isoflurane (Fig. 2). Analysis of cleaved caspase-3 protein expression by western blot analysis also revealed significantly enhanced expression ($p < 0.05$) following 6 h of isoflurane exposure (Fig. 3). Elevated caspase-3 expression may have contributed to the enhanced apoptotic counts evaluated by the TUNEL assay. Notably, naringenin at doses of 25, 50 and 100 mg downregulated caspase-3 protein expression and caspase-3-positive cell counts which was in agreement with the TUNEL-positive cell counts. Furthermore, caspase-3 expression was found to be dose-dependent with a 100 mg dose of naringenin producing the greatest decline in caspase-3 expression.

Naringenin effectively modulates the expression of apoptotic pathway proteins. Isoflurane has been reported to cause robust neurodegeneration and apoptosis in the developing brain (5). The balance between Bcl-2 pro-apoptotic proteins (Bax and Bad) and anti-apoptotic proteins (Bcl-2 and Bcl-xL) regulates mitochondrial membrane integrity and the release of apoptogenic factors that critically affect cell survival and death (48). Isoflurane exposure for 6 h in P7 rat pups caused a significant increase in the expression of Bax and Bad with a decrease in the expression of Bcl-2 and Bcl-xL (Fig. 3). The administration of naringenin at doses of 50 and 100 mg induced a marked downregulation of the expression of Bax and Bad and significantly ($p < 0.05$) elevated the expression of the anti-apoptotic proteins Bcl-xL
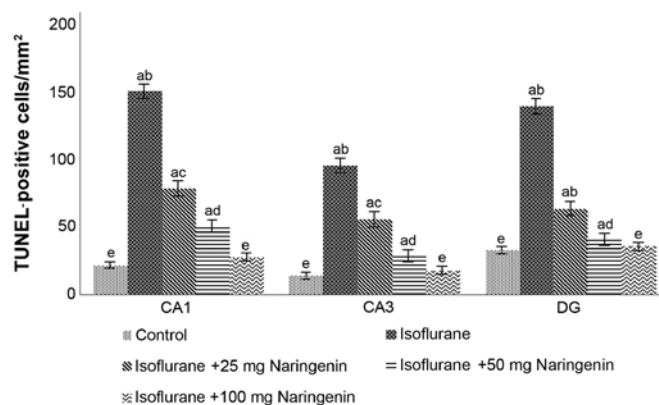

Figure 1. Effect of naringenin on isoflurane-induced neuroapoptosis. Naringenin effectively reduced neuroapoptosis in the hippocampus of isoflurane-exposed rat pups. Values are presented as the means ± SD, n=6. a, $p < 0.05$ compared with the control; b-f, represents mean values within the same group that differ from each other at $p < 0.05$ as determined by one-way ANOVA followed by Duncan's multiple range test analysis.
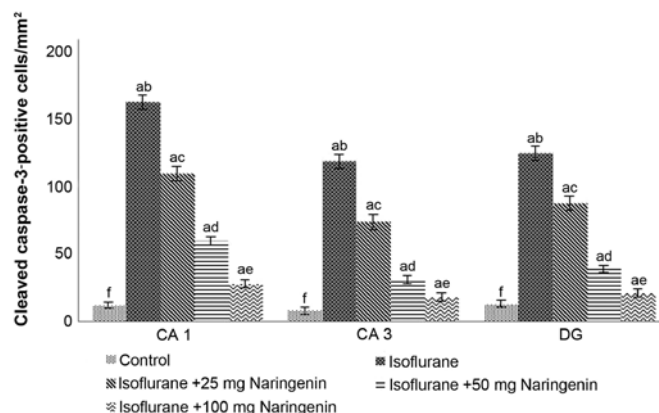

Figure 2. Naringenin reduces the expression of cleaved caspase-3. Cleaved caspase-3 expression was markedly reduced by naringenin in a dose-dependent manner, thus reducing apoptosis. Values are presented as the means ± SD, n=6. a, $p < 0.05$ compared with the control; b-f, represents mean values within the same group that differ from each other at $p < 0.05$ as determined by one-way ANOVA followed by Duncan's multiple range test analysis.

and Bcl-2. Although a 25 mg dose of naringenin modulated protein expression, the higher doses produced more pronounced effects. Taken together, these findings suggest that naringenin effectively regulates the expression of the apoptotic pathway proteins in a dose-dependent manner.

Naringenin potentially activates the PI3K/Akt pathway. Pro-survival pathways such as the PI3K/Akt pathway may be inactivated during the apoptotic process (33). The pathway is widely expressed in developing brains. In our study, isoflurane anaesthesia suppressed the pathway as the levels of Akt, p-Akt, GSK-3β and p-GSK-3β were reduced in the rat pups exposed to anaesthesia and not treated with naringenin. Treatment with naringenin significantly upregulated ($p < 0.05$) the protein levels of Akt, p-Akt, and p-GSK-3β . However, GSK-3β expression was not markedly altered by the administration of naringenin (Fig. 4). PTEN, the negative regulator of the pathway, was increased by isoflurane. Naringenin caused a significant decline in the expression of PTEN in a
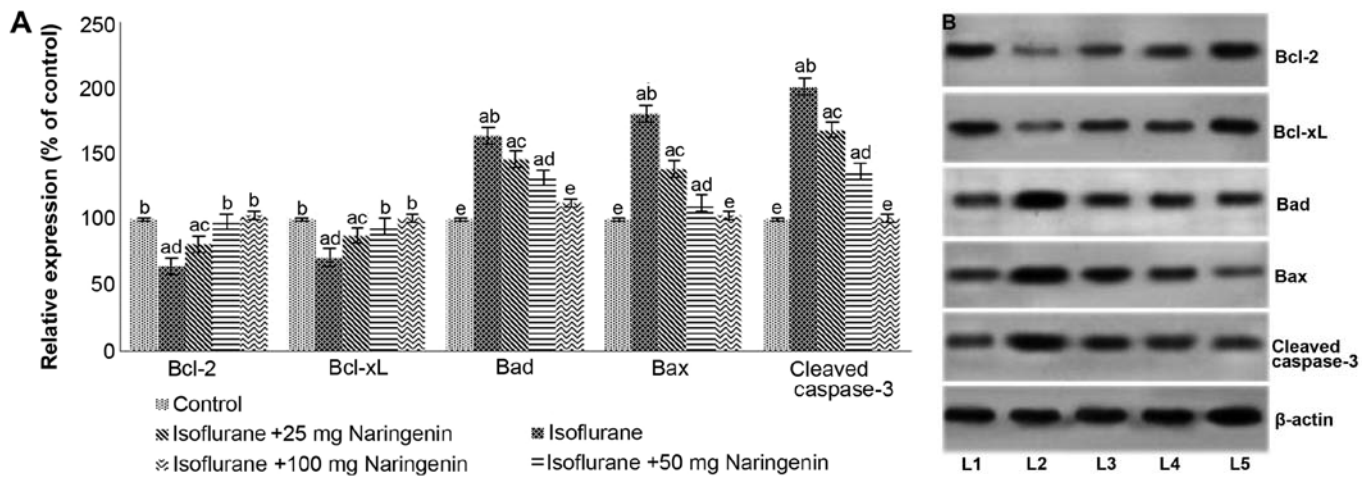
Figure 3. Naringenin modulates the expression of apoptosis pathway proteins. (A) Quantitative analysis of relative protein expression. Naringenin effectively regulated the expression of anti-apoptotic and pro-apoptotic proteins; naringenin enhanced the expression of Bcl-2 and Bcl-xL,and markedly downregulated the expression of Bax, Bad and caspase-3. (B) Representative western blots of the proteins. Values are presented as the means ± SD, n=6. a, p<0.05 compared with the control; b-f, represent mean values within the same group that differ from each other at p<0.05 as determined by one-way ANOVA followed by Duncan's multiple range test analysis. (L1, control; L2, isoflurane; L3, isoflurane +25 mg naringenin; L4, isoflurane +50 mg naringenin; L5, isoflurane +100 mg naringenin).
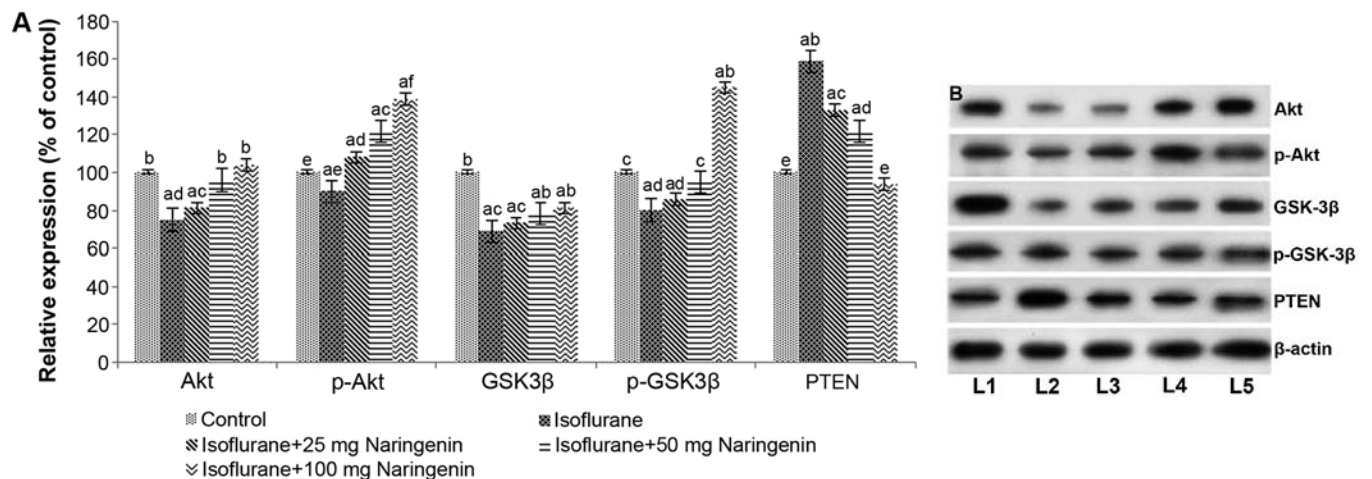

Figure 4. Naringenin modulates the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) signalling pathway. (A) Quantitative analysis of relative protein expression. Naringenin (25, 50 and 100 mg) potentially activated the PI3K/Akt pathway. (B) Representative western blots of the proteins. Values are presented as the means ± SD, n=6. a, p<0.05 compared with the control; b-f, represents mean values within the same group that differ from each other at p<0.05 as determined by one-way ANOVA followed by Duncan's multiple range test analysis. (L1, control; L2, isoflurane; L3, isoflurane +25 mg naringenin; L4, isoflurane +50 mg naringenin; L5, isoflurane +100 mg naringenin).

dose-dependent manner with maximal effects observed at a dose of 100 mg naringenin.

Naringenin significantly regulates the NF-κB signalling pathway. The commonly used anaesthetic isoflurane is known to induce neuro-inflammation (15). It has been reported that the isoflurane-induced elevated cytosolic Ca2+ levels activate the NF-κB signalling pathway (28,29). Activated NF-κB is capable of further activating and regulating various genes involved in inflammatory responses (11). We observed significantly high expression (p<0.05) of NF-κB p65, p-IκBα, TNF-α, IL-1β and IL-6 following isoflurane exposure. However, the levels of xIAP and cIAP were reduced (Fig. 5) which contributes to increased apoptosis. Naringenin at all the tested doses caused marked increases in the expression of xIAP and cIAP.

However, naringenin suppressed the activation of NF-κB and subsequently inhibited the expression of TNF-α, IL-1β and IL-6.

Effects of naringenin on the behaviour and memory of rats exposed to inhalation anaesthesia. The behaviour of the animals exposed to isoflurane was assessed. In the present study, the rat pups exposed to isoflurane on P7 were subjected to an open-field test which involved observing the behaviour of the pups in a novel environment. Isoflurane only-exposed pups did not exhibit noticeable behavioural disturbances; the distance they moved across the new field was comparatively less (p<0.05) than that in the rats exposed to anaesthesia and treated with naringenin. The rats that received 50 and 100 mg doses of naringenin exhibited behaviour similar
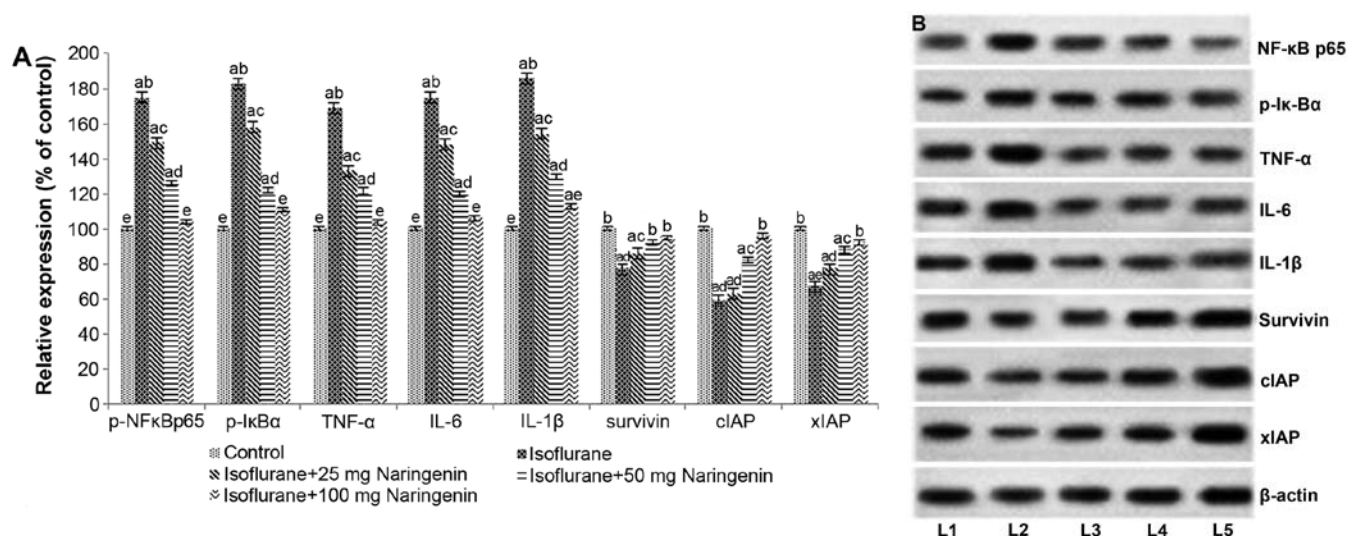
Figure 5. Naringenin modulates the nuclear factor-κB (NF-κB) signalling pathway. (A) Quantitative analysis of relative protein expression. Naringenin caused marked inhibition of NF-κB signalling leading to reduced transcription of cytokines and inflammatory mediators and the increased expression of inhibitors of apoptosis proteins. (B) Representative western blots of the proteins. Values are presented as the means ± SD, n=6. a, p<0.05 compared with the control; b-f, represents mean values within the same group that differ from each other at p<0.05 as determined by one-way ANOVA followed by Duncan's multiple range test analysis. (L1, control; L2, isoflurane; L3, isoflurane +25 mg naringenin; L4, isoflurane +50 mg naringenin; L5, isoflurane +100 mg naringenin).
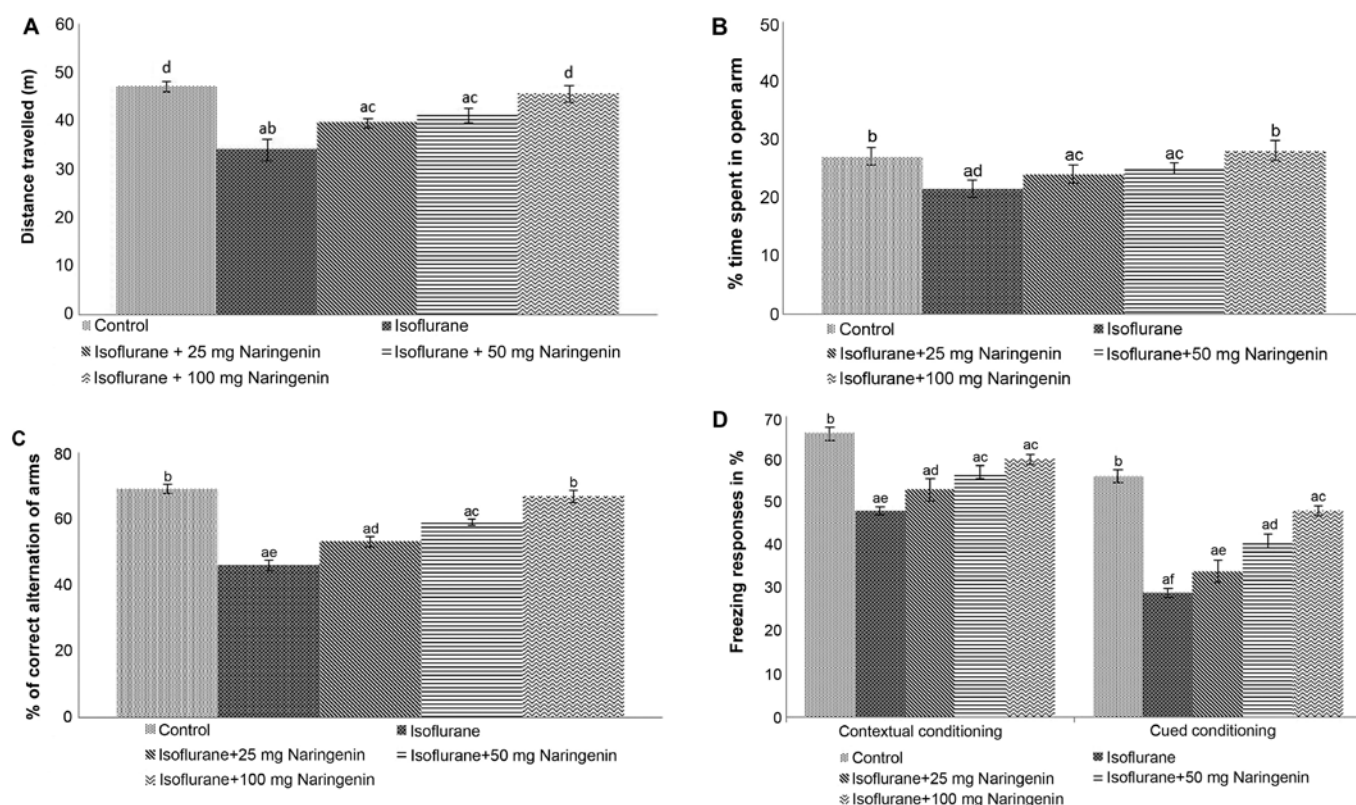

Figure 6. Naringenin improves the cognition and memory of rats following exposure to isoflurane on postnatal day 7 (P7). Behaviour of P42 rats (A) in a novel environment, (B) an elevated maze, (C) in a Y-maze and (D) the freezing response of the rats subjected to contextual and cued fear conditioning. Values are presented as the means ± SD, n=6. a, p<0.05 compared with the control; b-f, represents mean values within the same group that differ from each other at p<0.05 as determined by one-way ANOVA followed by Duncan's multiple range test analysis.

to the control rats that received no anaesthesia or naringenin (Fig. 6A). In order to assess anxiety-related behaviour, an elevated plus-maze test was performed. The percentage of time spent in the open arms was noted. Considerable differences were observed between the behaviour of the animals treated with naringenin compared with that of the animals in the isoflurane control groups (Fig. 6B).

Working memory is involved in holding information temporarily to perform cognitive tasks that are complex and it involves both the hippocampus and prefrontal cortex (49,50).
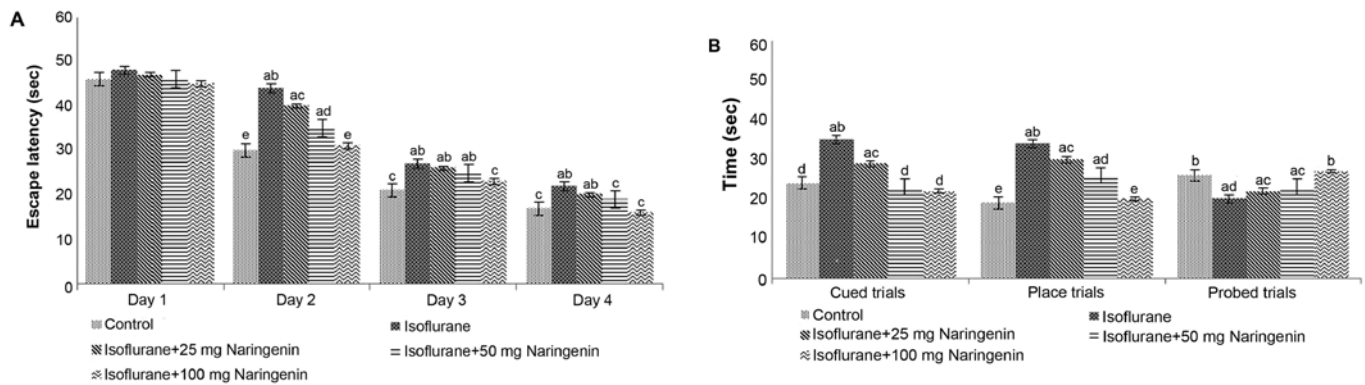
Figure 7. Assessment of learning and memory of postnatal day 35 (P35) rats following exposure to anesthesia on P7 by the Morris water maze (MWM) test. Escape latency of the rats during (A) the training period and (B) cued, place and probe trials with the MWM. Values are presented as the means ± SD, n=6. a, p<0.05 compared with the control; b-f, represents mean values within the same group that differ from each other at p<0.05 as determined by one-way ANOVA followed by Duncan's multiple range test analysis.

To determine whether exposure to isoflurane affected spatial working memory, a Y-maze test was conducted. The tasks examined whether the rats remembered the arm selected in the preceding choice. Rat pups exposed to isoflurane exhibited significantly indifferent behaviour as compared with the control rats not exposed to isoflurane. Naringenin at all the three tested doses considerably improved the performance of the animals (Fig. 6C).

The freezing responses of the animals exposed to isoflurane were significantly (p<0.05) reduced in both the contextual and cued conditioning tests (Fig. 6D). The freezing responses exhibited by the animals treated with naringenin were substantially higher than those of the animals administered with isoflurane alone.

Assessment of learning and memory of animals using MWM tests. To further evaluate the effect of isoflurane exposure on potential learning and memory deficits, the animals were subjected to the MWM test. The MWM is the most frequently used and reliable measure of hippocampus-dependent spatial navigation and reference memory assessment (51). The rats exposed to isoflurane anaesthesia on P7 were trained to explore the swimming pool and reach the hidden submerged platform. The time taken by each animal to find and reach the platform was recorded as the escape latency. The latency time recorded was found to decrease with each training session in all the animals irrespective of whether they were supplemented with naringenin or not (Fig. 7A). Nevertheless, the isoflurane only-treated group exhibited slight variations from the animals that received naringenin and isoflurane exposure on P7.

The cued trials were conducted by hiding the visual cues in order to evaluate swimming and visual abilities. The P35 rats exposed to isoflurane anaesthesia on P7 took considerably longer to reach the submerged platform with the rod compared with the control group of rats that had received no anaesthesia. Naringenin treatment at doses of 50 and 100 mg was observed to improve the performance of the rats; however, the results were not significant at a dose of 25 mg naringenin. The animals that received naringenin (50 and 100 mg) took less time to reach the platform compared with the rats that had received isoflurane alone. Furthermore, 100 mg naringenin produced the maximum improvement in the performance of

the rats (Fig. 7B). Place and probe trials were conducted to assess the ability of the rats to discover and remember the location of the submerged platform without the cue rod. In the place trials, the naringenin-treated rats exhibited a significant improvement (p<0.05) in performance. The rats took significantly less time to reach the platform.

Memory retention was assessed by removing the platform from the target quadrant and placing it in a random quadrant other than the target quadrant. The ability of the rats to look for the platform in the target quadrant was observed. Isoflurane exposure was found to have a significant impact on the memory of the rats, as animals exposed to isoflurane spent significantly less time (p<0.05) in the target quadrant in comparison with the control group not exposed to anaesthesia (Fig. 7B). The observed results suggest that isoflurane induced memory and learning impairments. Naringenin administration markedly improved the memory of the rats as evidenced by the longer duration of the time spent by the rats in the target quadrant looking for the platform, indicating the effectiveness of naringenin in improving the memory of rats. A 100 mg dose of naringenin improved performance more effectively than lower doses of 25 and 50 mg.

Discussion

Isoflurane is a widely used volatile anaesthetic that has been reported to induce neurodegeneration and cognitive impairments in developing brains (2,5,6,43). The hippocampus is the most sensitive region to isoflurane-induced neurotoxicity (52) and earlier studies have demonstrated that neuroapoptosis occurs in the hippocampi following exposure to isoflurane (8,53). In agreement with the findings of previous studies, we observed robust apoptosis in the hippocampi of P7 rats following exposure to isoflurane anaesthesia. It has been demonstrated that the developing brain is particularly vulnerable to anaesthetic-induced neurotoxicity during the period of neurogenesis and synaptogenesis and the P7 rats have been found to be extremely sensitive to neurotoxic challenge (2). In our study, IHC and western blot analysis revealed enhanced cleaved caspase-3 expression in the hippocampal tissues of anaesthetic only-exposed rats. Caspase-3 expression is often used as a potent indicator of apoptosis (2,6) and is also one of

the final steps in apoptosis (54). Protein expression analysis and IHC data revealed that naringenin effectively inhibited caspase-3 expression in a dose-dependent manner, suggesting that this downregulation contributed to the decrease in apoptotic cell counts in the hippocampi.

Furthermore, naringenin was also capable of modulating the expression of the Bcl-2 family proteins that regulate mitochondrial membrane integrity and control the release of apoptogenic factors from mitochondria (48). Isoflurane exposure for 6 h was found to upregulate Bad and Bax proteins and downregulate anti-apoptotic proteins, namely Bcl-xL and Bcl-2. Studies have reported that Bcl-xL enhances cell survival and is widely expressed in the brain. In addition, Bcl-xL maintains mitochondrial membrane integrity and inhibits cytochrome c release together with Bax (48,55), thus inhibiting apoptosis. Earlier studies of isoflurane have also shown similar results (56). Naringenin upregulated the expression of Bcl-xL and Bcl-2 and also caused significant downregulation of Bad and Bax protein expression. These observations indicate that naringenin is effective at inhibiting neurodegeneration.

The results of *in vitro* experiments have demonstrated the critical role of the PI3K/Akt pathway in neurite initiation, growth and stability (57-59), as well as in regulating the branching of dendrites (60). Propofol exerted neuroprotective effects by activating the PI3/Akt pathway (61). In our study, isoflurane was found to inhibit the phosphorylation of Akt, and to eventually reduce the level of p-GSK-3β. Akt plays critical roles in regulating and controlling cell survival and apoptosis. Activation of the PI3K/Akt pathway potentially deactivates the pro-apoptotic factors and activates the anti-apoptotic proteins (62). Isoflurane-induced downregulation of Akt and p-Akt and p-GSK-3β suggests the activation of pro-apoptotic proteins. Activated Akt phosphorylates and inactivates Bad causing the release of the anti-apoptotic protein Bcl-xL that blocks apoptosis by binding to Bax (55,63). In our study, the administration of naringenin significantly induced the phosphorylation of Akt and GSK-3β. Moreover, the isoflurane-induced enhanced expression of PTEN was suppressed by naringenin leading to the activation of the PI3K/Akt signalling cascade. PTEN possesses lipid phosphatase activity and is the negative regulator of the PI3K/Akt pathway (64).

Accumulating evidence has shown that neuro-inflammation is critically involved in anaesthetic-induced neuroapoptosis and cognitive deficits (65-67). Isoflurane induced the release of calcium from the endoplasmic reticulum (68,69), elevated cytosolic calcium levels and this has been suggested to activate the NF-κB signalling pathway which is implicated in inflammatory processes (28,70). This activation increased the expression of TNF-α, IL-1 β and IL-6 (15,66,71). Our results also showed the enhanced expression of NF-κB p65, p-IκBα, TNF-α, IL-1β and IL-6 suggesting activation of the NF-κB pathway. In addition, the expression of NF-κB target genes - IAPs - xIAP, cIAP-1 and survivin (72,73) were suppressed by isoflurane, thus enhancing apoptosis. Naringenin, however, modulated the pathway by effectively suppressing TNF-α, IL-1β and IL-6 suggesting the involvement of the NF-κB signalling pathway in naringenin-mediated neuroprotection.

Cognitive impairments in neonatal rats exposed to isoflurane have been well documented. Moreover, it has been reported that the period of synaptogenesis in the developing brain is highly vulnerable to anaesthetic-induced neurotoxicity (2). Head *et al* (74) demonstrated that isoflurane causes synaptic injury. In this study, tests for working memory as well as long-term memory were performed to assess whether naringenin was capable of ameliorating isoflurane-induced cognitive deficits. We observed minor alterations in the general behaviour of the animals as indicated in the open field and elevated maze test. However, the spatial working memory was greatly affected following anaesthetic exposure. Isoflurane induced profound alterations in the working memory of the animals as evidenced in the Y-maze test. Working memory is concerned with the cognitive functions that are responsible for synchronized temporary storage and also with the analysis and manipulation of information that is required in order to execute intricate cognitive tasks (75).

Working memory is also involved in higher order cognitive functions such as planning and sequential behaviour. Impairment of the working memory is directly associated with behavioural deficits and involves the hippocampus (76). Thus, isoflurane-induced neurodegeneration in the hippocampi may have directly contributed to the deficits in learning and memory. Notably, we observed a marked improvement in the behaviour and memory of the animals treated with naringenin. The rats exhibited a much improved performance in the Y-maze tests indicating a significant improvement in working memory. A naringenin-induced reduction in hippocampal apoptosis may be in part responsible for the significant improvement. Sanders *et al* (52) have demonstrated the neuroprotective effects of dexmedetomidine. Dexmedetomidine reduced isoflurane-induced hippocampal neuroapoptosis and also prevented hippocampal-dependent neurocognitive impairment (52).

The MWM test was conducted in order to evaluate the cognition and behaviour of the P35 rats. It is a reliable test for assessing hippocampal-dependent spatial working and reference memory (51). Jevtovic-Todorovic *et al* (2) reported that the period of neurogenesis is extremely sensitive to anaesthetic challenge. Active neurogenesis is critical for hippocampal-dependent learning and memory and any negative impact affects the process (77,78). The escape latency time of the rats that received isoflurane alone was significantly longer than the controls. More importantly, neuro-inflammation also mediates isoflurane-induced cognitive impairment (66,71). Nevertheless, we observed a marked improvement in the working memory of the rats treated with naringenin. At all the given doses, naringenin ameliorated memory retention and the cognitive behaviour of the rats. These observations suggest that naringenin may improve long-term memory.

Thus, the marked reduction in neurodegeneration and inhibition of inflammation observed following naringenin administration may have contributed to the improvement in the performance of the rats in the MWM tests. In conclusion, naringenin effectively reduced neuronal apoptosis and neuro-inflammation by modulating the NF-κB signalling pathway and also by activateing the PI3K/Akt pathway. Thus, further studies of naringenin are warranted in order to examine the molecular events and targets involved which may aid in developing efficient therapeutic strategies against anaesthetic-induced neuronal toxicity.

Acknowledgements

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