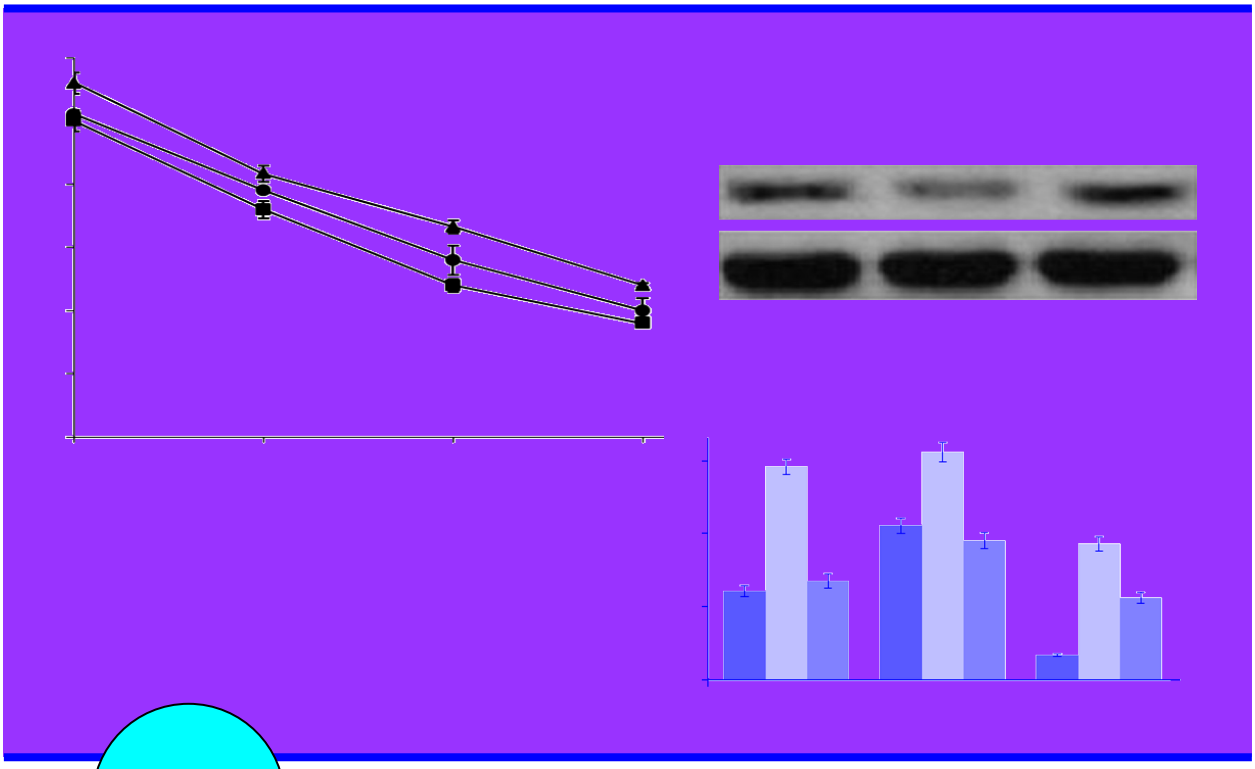

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Effects of vitamin C on inhalation anesthetic isoflurane-induced developmental, neuronal apoptosis in neonatal rats

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Abstract

Developmental abnormalities, neuronal apoptosis and associated cognitive impairment following isoflurane exposure in neonatal rodents have been reported. The study was undertaken to investigate the effect of vitamin C supplementation against isoflurane-induced neurotoxicity. Seven day old rats were exposed to 1.1% isoflurane, or air for 6 hours. Treatment groups were administered with vitamin C (30 mg/kg, orally) from postnatal day 1 (P1) to P10 and were exposed to isoflurane on P7. Isoflurane exposure induced apoptosis was determined by Fluoro-Jade C and terminal deoxynucleotidyl-transferase-mediated 2′-deoxyuridine 5′-triphosphate nick-end labeling assay. Vitamin C considerably improved memory and learning impairments, modulated neuroapoptosis and improved expressions of brain-derived neurotrophic factor, nerve growth factor, Bcl-xL and decreased activated caspase-3 expressions. Thus, vitamin C effectively offered protection against isoflurane-induced neuronal apoptosis, learning and memory disturbances.

Introduction

About 200 million patients worldwide are exposed to anesthesia and undergo surgery each year (Weiser et al., 2008; Moonesinghe et al., 2011). Volatile anesthetics such as isoflurane and sevoflurane are used in millions of young children each year during surgical procedures and imaging studies (Istaphanous and Loepke, 2009). Exposure to anesthetic cocktails (Yon et al., 2005; Lu et al., 2006; Sanders et al., 2009) or volatile agents such as isoflurane even as a single anesthetic (Yon et al., 2005; Ma et al., 2007) has been reported to be associated with widespread apoptotic neurodegeneration in the developing brains and contributes to the post-operative cognitive dysfunction (POCD) (Jevtovic-Todorovic et al., 2003; Satomoto et al., 2009; Brambrink et al., 2010; Kong et al., 2011; Paule et al., 2011; Li et al., 2013a,b).

In rodent models, the effects of anesthesia have been evaluated following exposure during a time of peak brain development and synaptogenesis, typically at postnatal day 7 (P7) (Jevtovic-Todorovic et al., 2003; Stratmann et al., 2009b; Shih et al., 2012). Apoptosis has been observed to occur acutely in the period immediately following anesthesia (Jevtovic-Todorovic et al., 2003; Istaphanous et al., 2011; Shih et al., 2012), and the thalamus and hippocampus are known areas susceptible to extensive neurodegeneration (Jevtovic-Todorovic et al., 2003; Satomoto et al., 2009; Shih et al., 2012).

Retrospective studies in children younger than 4 years old exposed to surgery under general anesthesia for more than once were found to have a higher risk of developing disabilities in reading and learning (DiMaggio et al., 2011; Ing et al., 2012). These observations have led to the concern about the possible detrimental effects of the use of anesthesia and sedation in pediatric population. Thus it becomes crucial to explore the mechanisms of anesthesia-induced neurodegeneration and to identify/develop potential protective strategies.

Previous studies in animals have suggested that exposure to volatile anesthetics can induce oxidative stress (Nazirolu and Gunay, 1999; Nazirolu and Cay, 2001). Our body is equipped with ample cache of defenses against reactive oxygen species (ROS). Concentrations of ROS are kept under strict control by the activity of innate defense systems including enzymes and non-enzymatic anti-oxidants such as vitamins (Kovacic and Somanathan, 2008). Vitamin C protects cellular membrane lipoproteins against oxidative damage caused by toxic free radicals (Netke et al., 1997).

These anti-oxidant functions of vitamin C may prevent certain types of oxidative stress due to anesthetics. Hence, we reconnoitred the protective effects of vitamin C on inhalation anesthetic, isoflurane-induced developmental, neuronal apoptosis in neonatal rats.

Materials and Methods

Animals

This study was approved by the Institutional animal care committee at and performed in accordance with the National Institutes of Health Guide for the Use of Laboratory Animals. Seven-day-old (P7) Sprague-Dawley rat pups (Guangdong Medical Laboratory Animal Co., China) weighing 15-17 g were used. Rats were exposed to 1.1% isoflurane for 6 hours (approximately 0.5 MAC) in P7 rats as determined by Orliaguet et al. (2001) in 30% oxygen or air in a temperature-controlled chamber as described before (Li et al., 2013b).

Treatment groups were administered with vitamin C (30 mg/kg, orally) from postnatal day 1 (P1) and exposed to isoflurane on P7. Vitamin C supplementation was continued till P10. Control group pups received neither vitamin C nor isoflurane but received equal volumes of saline.

All animals were sacrificed 6 hours after termination of isoflurane exposure and their hippocampi were used for western blot analysis of caspase 3 and Bcl-xL (n = 6) and TdT-mediated dUTP nick end labeling (TUNEL) with fluorescent dye and Fluro-Jade C staining (n = 6). For western blot analysis of BDNF and NGF, brain tissues from the animals 48 hours following isoflurane exposure were used.

All the chemicals used in the study were purchased from Sigma-Aldrich, St. Loius, MO, USA, unless otherwise mentioned.

TUNEL fluorescent assay

For TUNEL studies, rat pups were anaesthetized with isoflurane and perfused trans-cardially with 4% paraformaldehyde. Their brains were paraffin embedded and sectioned at 6 µm thickness. As described before (Li et al., 2007), four to five sections (200 µm apart) for each animal at the same plane of the hippocampus were chosen for detecting apoptosis using TUNEL fluorescent method (Promega, Madision, WI, USA). The slides were protected from direct light during experiment. Hoechst was used to stain nuclei. The TUNEL positive cells in CA1, CA3 and DG regions of hippocampus were analyzed immediately with NIS-Elements BR imaging processing and analysis software (Nikon Corporation, Japan). The densities of the TUNEL positive cells in CA1, CA3 and DG were determined by dividing the number of TUNEL positive cells by the area of that brain region.

Fluro-Jade C staining

Brains from treatment and control group rats (n = 6 per group) were assessed for acute neuronal death. Six hours following anesthesia, animals were anesthetized and transcardially perfused with cold 4% paraformaldehyde in phosphate-buffered saline and brains were excised, postfixed, and immersed in sucrose solution. The tissues were sliced into 60 micron-thickness and every other slice was mounted and stained with Fluoro-Jade C, a marker very specific for neurodegeneration (FJC, 0.001%, Millipore, Billerica, MA, USA). FJ-positive cells were counted using Nikon Eclipse 80i microscope under 20x magnification.

Western blot analysis

For western blot analysis, the pups were anaesthetized with isoflurane and sacrificed by decapitation. Hippocampi of rats were isolated immediately on ice and then stored at -80°C until used. Western blotting was performed as described by Li et al. (2013b). In brief, the protein concentrations of samples were determined using the BCA protein assay (Bio-Rad, Herts, UK). Sixty µg of sample were used in western blot analysis using primary antibodies against cleaved caspase-3, Bcl-xL at 1:2000 dilution (Cell Signaling Technology Company, USA.), brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) at 1:200 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Images were scanned by Image Master II scanner (GE Healthcare) and analyzed using Image Quant TL software (v2003.03, GE Healthcare). The band signals of other proteins were normalized to those of β-actin using anti-

β-actin at 1:2000 dilution (Cell Signaling Technology Company, USA.),

MWM test

Rats were trained for 4 consecutive days (postnatal days 31-34) in the Morris water maze. A plat-form (10.3 cm diameter) was submerged in a circular pool (180 cm diameter, 50 cm depth) filled with warm (23-25°C) water. Rats were made to perform two train-ing sessions each day. In each session, rats performed four trials in which they were released from one of four randomly assigned release points while facing the tank wall. This provided two short and two medium swims per session. Animals were allowed 60 sec to locate the hidden platform, and if failed to locate in allotted time, they were guided to the platform. In either case, the rats were removed from the platform after 15 sec. Training sessions were conducted until the rats could locate the hidden platform in less than 15 s (average time per session). All trials were videotaped, and rat swim paths were recorded with ANY-maze video tracking system (Stoelting Co., Wood Dale, IL, USA), that measures the time taken (latency) to find the platform(s), as well as other behavioural information obtained during the spatial reference memory test. The animals were dried and placed beneath a heating lamp after completing each test.

Cued trials

The cued trials were performed only for postnatal rats at P35, to determine whether any non-cognitive performance impairments (e.g. visual impairments and/or swimming difficulties) were present, which might affect performance on the place or probe trials. A white curtain was placed surrounding the pool to hide the visual cues. All rats received 4 trials per day. In each trial, rats were placed in a fixed position of the swimming pool towards the wall and were allowed to swim to a platform with a rod (cue) 20 cm above water level randomly placed in any of the four quadrants of the swimming pool. They were allotted 60 sec to find the platform upon which they sat for 30 sec before being removed from the pool. If a rat did not find the platform within 60 sec, the rat was gently guided to the platform and allowed to remain there for 30 sec. The time for each rat to reach the cued platform and the swim speed was recorded and the data were analyzed.

Place trials

After completion of cued trials, the curtains were removed. The same rats were chosen to perform the place trials to determine the rat's ability to learn the spatial relationship between distant cues and the escape platform (submerged, no cue rod), that was kept in the same place for all place trials. The starting points were random for each rat. The time taken to reach the platform was recorded for each trial.

Probe trials

Probe trials were conducted 24 hours after place trials to assess the memory retention. The platform was removed from the pool and the pups were placed in the opposite quadrant. The pups were allowed to swim 60 sec and the time spent in each quadrant and the swim speed were recorded and analyzed. The data are expressed as the percent time spent in each of the four quadrants.

Statistical analysis

All the values are represented as mean ± SD. Values at

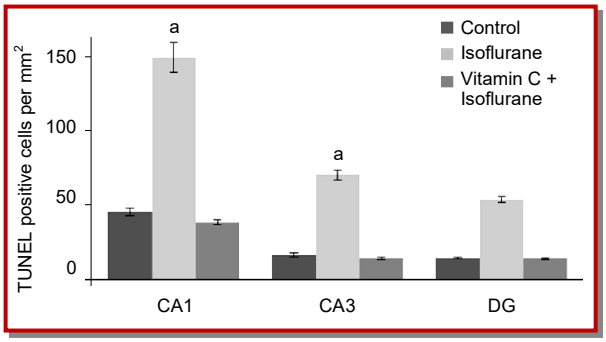

Figure 1: TUNEL positive cells in the hippocampi of P7 rats

Values are represented as mean ± SD, n = 6. [a]represents statistical significance at p<0.05 compared against control as determined by ANOVA

p<0.05 are considered significant as determined by One -way Analysis of variance (ANOVA). The values were analysed using SPSS software, version 17.0.

Results

Neuroapoptosis due to exposure to isoflurane anaesthesia in the hippocampal CA1, CA3 and DG regions of P7 rat pups were assessed by TUNEL assay. Six hours exposure to isoflurane markedly (p<0.05) increased the number of apoptotic cells in CA1, CA3 and in DG as compared against controls that received neither isoflurane nor vitamin C (Figure 1).

The increase in the apoptotic cells was more pronounced in the CA1 region. Vitamin C supplementation to the rat pups was observed to significantly (p<0.05) reduce the number of TUNEL positive cells as against isoflurane alone exposed rat pups. In addition, Fluro-Jade C staining was also performed. The results obtained were in line with the results of the TUNEL assay. Isoflurane markedly (p<0.05) raised the number of the Fluro-Jade C positive cells in the CA1,CA3 and DG regions in the hippocampi of the isoflurane exposed P7 rat pups. Vitamin C supplematation effectively (p<0.05) decreased the number of apoptotic cells (Figure 2).

The levels of caspase-3 protein expression in the hippocampus of the rat pups were assessed. Isoflurane

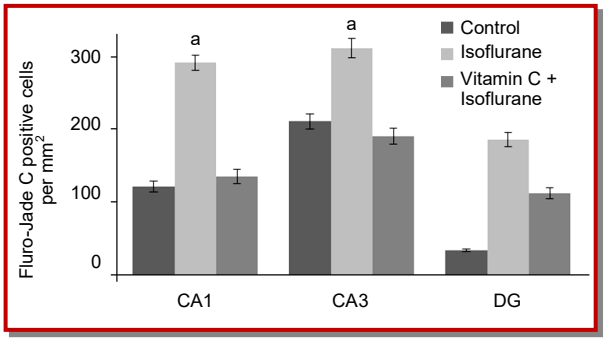

Figure 2: Fluro-Jade C positive cells in the hippocampi of P7 rats

Values are represented as mean ± SD; n = 6; [a]represents statistical significance at p<0.05 compared against control as determined by ANOVA

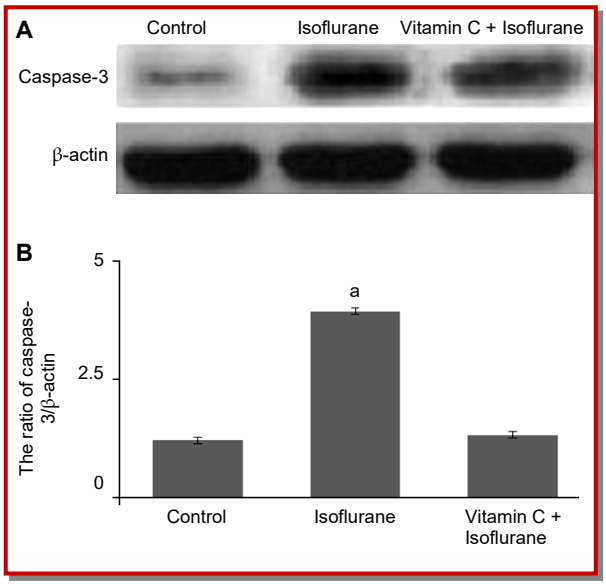

Figure 3: Expression levels of caspase-3 in the hippocampi of P7 rats

Values are represented as mean ± SD, n = 6. [a]represents statistical significance at p<0.05 compared against control as determined by ANOVA

elevated the expression of cleaved caspase-3 markedly (p<0.05). Vitamin C supplementation decreased the expression of cleaved caspase-3 as compared against isoflurane exposure without vitamin C administration (Figure 3). In addition, the expression levels of the anti-apoptotic protein Bcl-xL were detected. A 2-fold decrease in Bcl-xL expression following isoflurane exposure was observed. However supplementation of vitamin C was observed to efficiently prevent the reduction in the expression levels of Bcl-xL (Figure 4).

Isoflurane exposure for 6 hours resulted in a marked decrease in the levels of growth factors (BDNF and NGF) as compared to control group. Vitamin C administration to rat pups from P1 was found to significantly (p<0.05) increase the whole brain expression of BDNF and NGF vs isoflurane control (Figure 5). The expre-

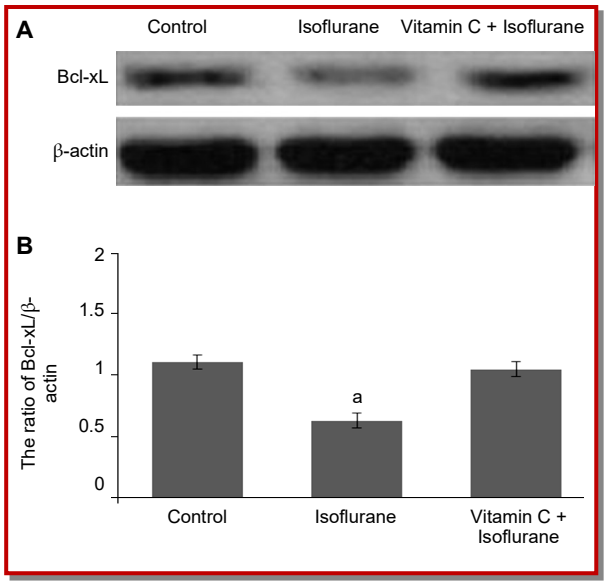

Figure 4: Expression levels of Bcl-xL in the hippocampi of P7 rats

Values are represented as mean ± SD, n = 6. [a]represents statistical significance at p<0.05 compared against control as determined by ANOVA

ssion levels of BDNF and NGF in the brain tissues of rat pups supplemented with vitamin C was observed to be closer to the levels observed in control pups that were not exposed to isoflurane.

To evaluate the effect of neonatal exposure to isoflurane on potential learning and memory deficits, the rat pups were subjected to Morris water maze testing. The rat pups were trained to explore the swimming pool and to reach on the platform. The time taken to reach the platform was noted and the duration to reach up the platform was found to decrease with each training session (Figure 6A).

Cued trials were conducted at postnatal day 35 to evaluate swimming and visual abilities. The rat pups that were exposed to only isoflurane were observed to take a considerably (p<0.05) longer time to reach the platform when compared to control pups that received no isoflurane. The pups that received vitamin C were able to reach the platform much quicker as against isoflurane control pups. (Figure 6B)

Further to establish differences in visual judgments and memory after isoflurane exposure, place and probe trials were performed. Place trials were conducted on P36 to study the ability of the rat pups to learn and remember the location of a new platform (Figure 6B). The rat pups that were supplemented with vitamin C were able to reach the platform in a lesser time than the isoflurane treated rat pups. The isoflurane control pups took a longer timer to find the platform as against control pups that received neither isoflurane nor vitamin C.

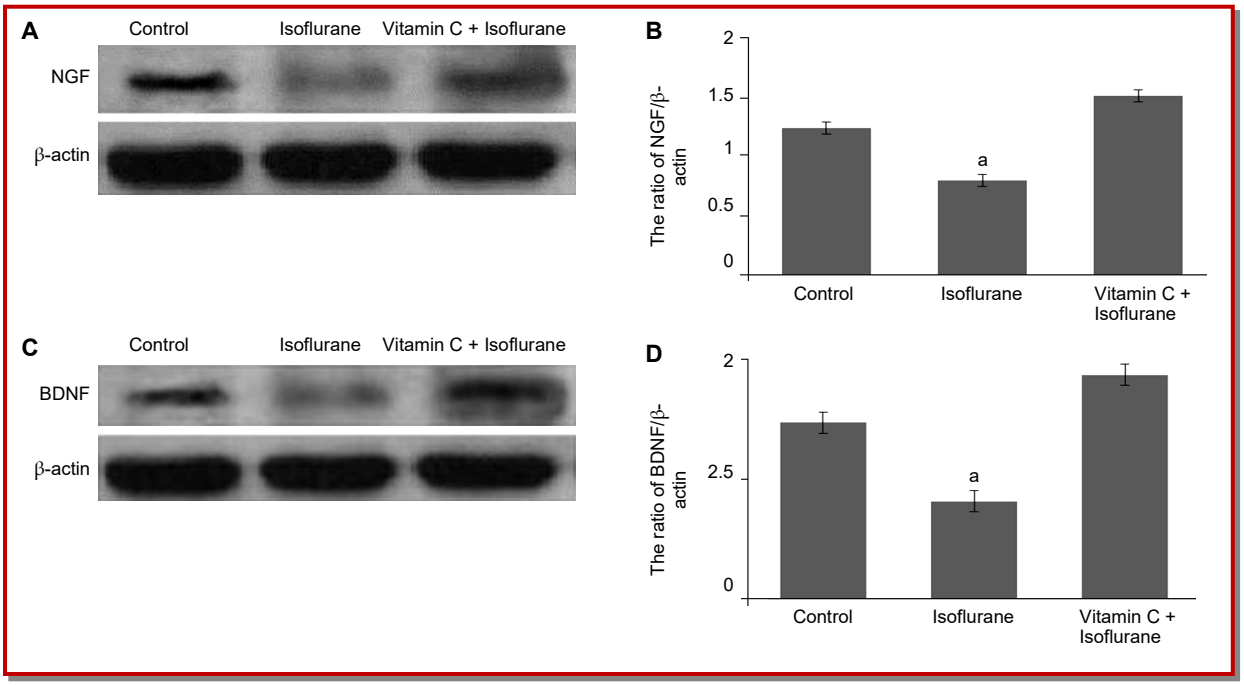

Figure 5: Expression levels of NGF (A, B) and BDNF (C, D)
Data are mean ± SD; n = 6; [a]represents statistical significance at p<0.05 compared against control as determined by ANOVA

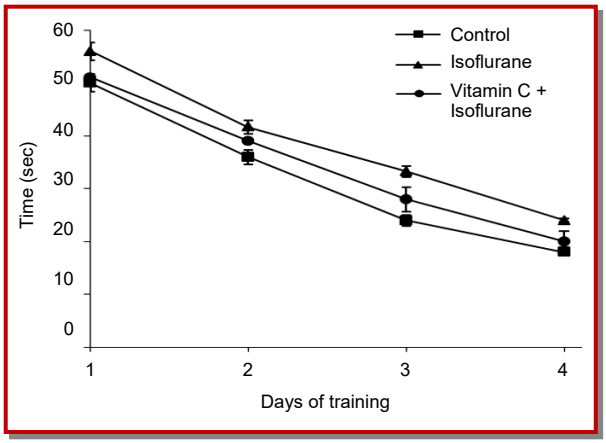

Figure 6A: Morris water maze test -Effects of anesthesia treatments on memory and learning ability

Time taken by the rat pups to reach the platform. Values are represented as mean ± SD (n = 6)

As demonstrated in Figure 6B, pups that were exposed to isoflurane tended to spend less percentage of time in the target quadrant than pups in the control group, with a statistically significant difference between the groups (p>0.05). The rat pups treated with vitamin C spent more time in the target quadrant. Thus the treatment with vitamin C was found to have an influence on memory and learning ability of the pups.

Discussion

Isoflurane is a commonly used volatile anesthetic during surgery. Previous studies have demonstrated that it increases neuroapoptosis and induces long-term cognitive dysfunction in developing animals (Wei et al., 2005; Brambrink et al., 2010; Kong et al., 2011). The present study was undertaken to evaluate the effect of vitamin C supplementation on isoflurane induced neuronal apoptosis and alterations in memory and learning. The rat pups were exposed to isoflurane on P7. The time of exposure to isoflurane in the rats at P7 occurs during a period of peak neural development and synaptogenesis that overlaps with a corresponding stage of development in humans in the late 3rd trimester through the first several months of life (Rice and Barone, 2000).

Previous studies have demonstrated anesthesia induced cell death by apoptosis both in cell cultures (Wei et al., 2005; Xie et al., 2007) and in the developing brains (Jevtovic-Todorovic et al., 2003; Dong et al., 2009). Hippocampus has been reported to be the most sensitive region to isoflurane-induced neurotoxicity (Sanders et al., 2009). Isoflurane exposure resulted in a marked increase in neuronal apoptosis as evidenced by the results of TUNEL assay and Fluro-Jade C staining. Administration of vitamin C in rat pups effectively offered neuroprotection as presented by the decrease in the number of TUNEL positive and Fluro-Jade C positive cells.

Studies have demonstrated that inhalational anesthetics induced neuroapoptosis and activated both the intrinsic and the extrinsic apoptotic pathways (Yon et al., 2005, 2006). As a step forward to understand the molecular level, we investigated the expression of caspase-3 in the

hippocampal regions of brains of experimental rat pups. Activation of caspase-3 has been commonly used in these studies as a biomarker for anesthesia mediated cell death by apoptosis (Jevtovic-Todorovic et al., 2003; Dong et al., 2009). In our study, the raised levels of capase-3 following isofulrane exposure indicates neuronal apoptosis induced by isoflurane. The marked decrease in caspase-3 expressions in the brain tissue of the rat pups supplemented with vitamin C suggests the neuroprotective effects of vitamin against isoflurane induced apoptosis.

The anti-apoptotic protein Bcl-xL is widely expressed in the central nervous system (CNS) and it enhances cell survival by maintaining mitochondrial membrane integrity and inhibits the release of cytochrome C (Zhao et al., 2003). Anesthesia cocktail containing isoflurane, nitrous oxide (N_2O) and midazolam was reported to downregulate Bcl-xL expression and cause neurotoxicity in developing rat brains (Yon et al., 2005). In this study, we observed that isoflurane as a single anaesthetic also caused a decreased expression of Bcl-xL in the hippocampi of P7 rats. Vitamin C supplementation brought a marked raise in the expression of Bcl-xL, thus preventing the mitochondrial membrane alteration and neuronal apoptosis. This result is in agreement with previous studies that isoflurane induced apoptosis through JNK signalling pathway and promotes apoptosis possibly via transcriptional regulation of Bcl-2 family gene, including Bcl-xL (Jeong et al., 2008; Chu et al., 2009; Li et al., 2010). Our results suggest that inhibition of Bcl-xL expression is a crucial step in the isoflurane-induced apoptosis pathway. Thus by effectively increasing the Bcl-xL expression, vitamin C offers protection against isoflurane induced neuroapoptosis.

Neonatal neurogenesis begins as cells proliferate, cells migrate ends as the cells integrate into a neuronal circuit as a functional neuron. It is widely believed that neurogenesis enables hippocampal plasticity and new memories (Denis-Donini et al., 2008). In addition to neuronal apoptosis, several recent studies correlate alterations in neurogenesis with cognitive performance (Zhao et al., 2008; Bianchi et al., 2010).

BDNF is a secreted protein involved in the survival, growth, and development of neurons and proper synapse formation (Bibel and Barde, 2000; Bath et al., 2013). BDNF and NGF are neurotrophins that can prevent neuronal apoptosis (Bibel and Barde, 2000). BDNF has been reported to prevent glutamate-induced apoptosis *in vitro* (Almeida et al., 2005) and *in vivo* (Husson et al., 2005). In a similar way, Nguyen et al. (2010) showed that NGF prevented staurosporine induced apoptosis on neuronal cell lines. The decrease in the levels of BDNF and NGF following 6 hours of isoflurane exposure indicates the neurotoxic effects of isoflurane. The raised expression of BDNF and NGF is indicative of the protective effects of vitamin C against isoflurane induced toxic effects. The results suggest that vitamin C effectively promotes neurogenesis by improving the expressions of BDNF and NGF.

Among the commonly used inhalational general anesthetics, isoflurane is widely reported to induce neurodegeneration in the developing brain and subsequent cognitive dysfunction in several animal models (Jevtovic-Todorovic et al., 2003; Sanders et al., 2010; Kong et al., 2011; Brambrink et al., 2012). In our study, we investigated the effect of supplementation of vitamin C on isoflurane induced cognitive dysfunction in rat pups. Learning and memory was assessed by Morris water maze test by conducting cued, place and probe trials to judge the visual judgements, learning and memory. The results obtained suggest that vitamin C supplementation to the rats prior and post isoflurane exposure offered neuroprotection. Rat pups administrated with vitamin C were observed to take a much lesser time in navigating through the pool and identifying the target platform. In addition, the pups were found to have better memory retention and better visual and spatial judgements.

Conclusion

Vitamin C supplementation effectively offers protection against neuronal apoptosis by reducing the expression of caspase-3 and increasing Bcl-xL expression. Vitamin is also found to improve neurogenesis as evidenced by elevating the expressions of neurotrophic factors-BDNF and NGF and improving the learning and memory of the rat pups as well.

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Conflict of Interest

Authors declare no conflict of interest

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