

Original Article

Neuroprotective properties of vitamin C on equipotent anesthetic concentrations of desflurane, isoflurane, or sevoflurane in high fat diet fed neonatal mice

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Abstract: Obesity has been reported to be one of the significant contributors to various chronic disease conditions. Childhood obesity has been on an alarming increase over recent years leading to various health complications. Millions of children undergo surgery each year as a part of medical care on various health grounds. In the present study, influence of vitamin C on the effect of obesity and over-weight under anaesthetic exposure was analysed. Separate groups of neonatal mice (C57BL/6) were fed on high-fat diet to induce obesity. The mice were administered with vitamin C at 30 and 60 mg/kg b.wt post natal day 1 (P1) to P21. P7 mice were exposed to equipotent doses of isoflurane or sevoflurane or desflurane. Neuroapoptosis was assessed by measuring activated caspase-3 and TUNEL assay. Plasma S100 β levels were detected by ELISA. The mice were assessed for their general behaviour. Morris water maze test was performed to assess the spatial working memory. Anesthesia exposure caused severe neuroapoptosis and also raised the levels of plasma S100 β . Neuroapoptosis, working memory and learning impairments observed following anesthetics were comparatively more profound on high fat diet fed mice. Desflurane exposure resulted in higher apoptotic counts, learning and memory deficits than equipotent dose of isoflurane and sevoflurane. Vitamin C supplementation offered significant protection against anesthetic induced neurotoxicity and behavioural alterations. Vitamin C administration resulted in marked reduction in neurotoxicity induced by anesthesia and as well improved learning and memory of both normal and high fat diet fed mice.

Keywords: Desflurane, isoflurane, neurotoxicity, sevoflurane, vitamin C, obesity

Introduction

The rapid raise in prevalence of obesity has made a significant impact on health and economic burden of our society, with childhood obesity as one of the most crucial public health challenges of 21st century. The incidence of childhood obesity has risen at an alarming rate over the past few years and currently, out of 1.5 billion obese people globally, 200 million are children [1-3]. The chances of developing obesity-related health complications as dyslipidaemia and type 2 diabetes are markedly higher in obese and over-weight children. These children may also possibly become obese adults [4-6] and could also develop the diseases of adults at a much earlier age. Thus obesity could be defined as a state of premature ageing that

which impacts the quality of life on a generation that will live with obesity and its co-morbidities.

A direct result of obesity is that more overweight and obese children are presenting for anaesthesia and surgery. The physiological changes in obesity markedly affect distribution, binding and elimination of anesthetic drugs [7-9]. Severe adverse reactions could possibly arise if drug dosing is based only on the actual body weight of the patient. Additionally obesity can also affect the pharmacokinetic profile of anesthetic drugs, such as the alterations in plasma protein binding [8, 9].

Previous studies have reported safe and effective anesthesia and analgesia [10-12] in high

risk infants [13, 14]. Neuraxial anesthesia may have advantages in preterm-born neonates, susceptible to postoperative apnoea or having co-existing respiratory disease [15-17]. However, there are strong evidences on the apoptotic effect of anesthesia on developing brain cells in rodent and primates and also long-term neurocognitive dysfunction [18-21]. These reports have led to serious concern over the safety of anaesthesia in young children, where reflective studies have demonstrated association between learning disabilities and anesthesia exposure [22, 23].

Isoflurane, sevoflurane and desflurane are halogenated ethers that are widely used to maintain general anesthesia in neonates, children and adults. They either are used alone or in combination with other drugs. While the exact mechanism of these drugs is not completely understood, they are known to exert effects at the γ -aminobutyric acid type A and N-methyl-D-aspartate glutamate receptors [24-26]. These receptors have been reported to be indispensable for mammalian brain development. Therefore, exposure to volatile anesthetics could possibly interfere with normal brain maturation, learning, and later neurocognitive function [27, 28]. Recent studies have indicated the neurotoxic effects of desflurane, isoflurane and sevoflurane on the developing brain [29-32].

Studies have demonstrated the effects of vitamins E and C in controlling certain types of seizures and/or even in preventing adverse effects of antiepileptic drugs [33-35]. With unknown wide array of properties of vitamin C, the present study focused on the neuroprotective properties of vitamin C on equipotent anesthetic concentrations of desflurane, isoflurane, or sevoflurane in high fat diet fed neonatal mice models.

Materials and methods

Animals

The studies were performed in accordance with the National Institutes of Health Guide standards for the use of laboratory animals with the approval from the Institutional animal care committee. The C57BL/6 pregnant mice (Guangdong Medical Laboratory Animal Co., China) used in this study were maintained on a

12 h light-dark cycle (lights on from 07:00 to 19:00) with room temperature at $21^{\circ}\text{C} \pm 1^{\circ}\text{C}$. Mice had *ad libitum* access to water and food. Separate group of C57BL/6 pregnant mice were fed on high fat diet through the course of gestation. The animals were housed individually in separate cages and monitored closely for the day of birth, which was considered as postnatal day 0 (P0). The pups (male and female) were kept in cages in a room on a 12 h light/dark cycle with free access to water with their littermates till P4.

Neonatal overfeeding was done in pups of high fat diet fed mice by reducing litter size to 3 pups per litter (small litter, SL) on P4, while normal litters (NL) were from pregnant mice that were on normal diet and were culled to 10 pups per litter. The pups of SL were provided access to high fat diet from P4 till P21. High fat diet was prepared with butter, milk powder, wheat flour and sugar in equal proportions each. The mice were fed with high fat diet at 2 g/day along with standard chow. The animals of both SL ($n = 80$) and NL ($n = 80$) were monitored carefully.

On P7, mice were grouped separately for experiments. The NL control pups (NLC) received no anaesthesia and were fed on normal standard diet and SL control pups (SLC) received no anaesthesia but were fed on high fat diet. The treatment groups NL and SL pups were induced with isoflurane (NLI and SLI), sevoflurane (NLS and SLS) and desflurane (NLD and SLD). Treatment groups were administered with vitamin C at 30 mg/kg, or 60 mg/kg b.wt orally from postnatal day 1 (P1) and exposed to anesthesia on P7. Vitamin C supplementation was continued till P21. On P35 the body weights of normal litter pups were 18-22 g and that of small litter pups were between 24-31 g.

Anaesthesia exposure

On postnatal day 7 (P7), mice were placed in a humid chamber with manipulating gloves and exposed to anesthetics. The total gas flow was 2 l/min, using 25% O_2 as a carrier. We measured the oxygen and anesthetic agent fractions using a gas analysis system (Capnomac Ultima, GE Healthcare, Tokyo, Japan). During exposure to the anesthetic, the mice were kept warm on a mat heated to $38^{\circ}\text{C} \pm 1^{\circ}\text{C}$. Neonatal littermate mice were assigned to receive the

following 7.4% desflurane or, 2.9% sevoflurane, or 1.5% isoflurane for 6 h in 30% oxygen [30].

Chemicals

All the chemicals used in the study were of analytical grade and were procured from Sigma-Aldrich, St. Louis, MO, USA unless otherwise mentioned.

Evaluation of neuroapoptosis

Apoptosis was evaluated by immunohistochemical staining for activated caspase-3 and by TUNEL assay. Mice were transcardially perfused with 0.1 M phosphate buffer containing 4% paraformaldehyde immediately or 6 h after anesthesia, for activated caspase-3 staining and for TUNEL, respectively. The brain tissue sections were paraffin-embedded (5 μ m thick). Immunohistochemistry was performed as described previously [36]. Briefly, the brain sections were incubated overnight with anti-cleaved caspase-3 primary antibody (1:200; monoclonal antibody, Cell Signaling Technology, Beverly, MA, USA) at 4°C, followed by incubation with a secondary antibody (1:200, Santa Cruz Biotechnology Inc., CA, USA) for about 40 min and then with avidin-biotinylated peroxidase complex (Vectostain ABC-Kit, Vector Lab, Burlingame, USA) for 40 min. Following which the tissue sections were stained with diaminobenzidine (DAB, Vector Laboratories, Burlingame, USA). Cleaved caspase-3 positive cells in the hippocampal CA1, CA3 and dentate gyrus (DG) were analyzed using NIS-Elements BR imaging processing and analysis software (Nikon Corporation, Japan). The density of cleaved caspase-3 positive cells in the three hippocampal region was calculated by dividing the number of caspase-3 positive cells by the area of that brain region.

TUNEL assay was performed using the Dead End TM fluorometric TUNEL system kit (Promega, Madison, WI, USA) as described previously [37]. Briefly, the slides were protected from direct light during experiment and Hoechst was used to stain nuclei. TUNEL positive cells in the hippocampal CA1, CA3 and dentate gyrus (DG) region were analyzed as mentioned in the immunohistochemistry staining. The density of TUNEL positive cells was calculated by dividing the number of TUNEL positive cells by the area of that brain region.

Determination of plasma S100 β

S100 β levels in the blood of mice following 6 h of anesthetic exposure were determined using Sangtec 100 ELISA kit (DiaSorin Inc, Stillwater, USA) as previously described [38]. Briefly, blood from each mouse was drawn from the left ventricle and was centrifuged for separation of plasma. Plasma (50 μ L) was placed in each well of microtiter plate and mixed with 150 μ L tracer from kit, incubated for 2 h, followed by 3,3',5,5' tetramethylbenzidine substrate and stop solution. The optical density was read at 450 nm. The sensitivity was determined by plotting the standard curve and then measuring concentrations of the samples from the standard curve.

Behavioral studies

For behavioral studies, mice were exposed to anesthetics on P7, as described above. These mice were further subjected to behavioral tests such as open-field, elevated plus-maze, Y-maze, and fear conditioning tests. The movement of each mouse was monitored and analyzed using a computer-operated video tracking system (ANY-maze video tracking system Stoelting Co., Wood Dale, USA). In tasks using an apparatus with arms, arm entry by the mouse was counted when all four legs of the animal entered each arm. All apparatus used in this study were from O'Hara & Co., Ltd (Tokyo, Japan).

Open-field test

The responses of mice to a novel environment were measured by an open-field test using 5-week-old mice, by a previously described method [39]. Activity was measured as the total distance travelled (meters) in 10 min.

Elevated plus-maze test

The elevated plus-maze test was performed as previously described by Satoh et al. [39]. This test is usually used to evaluate anxiety-related behaviour in rodents. The elevated plus-maze consisted of two open arms (25 $\times$ 5 cm) and two enclosed arms, with all arms elevated to a height of 50 cm above the floor. The behaviour of P35 mice was monitored during a 10-min test period. The percentage of time spent in the open arms was considered as an index of anxious-behaviour.

Spontaneous alternation in the Y-maze test

This study was performed as previously described [39] to assess spatial working memory. The symmetrical Y-maze made of acrylic consisted of three arms (25 × 5 cm) separated by 120° with 15 cm high transparent walls. Each P35 mice were placed in the center of the Y-maze, and the mouse was allowed to freely explore the maze for 8 min. The total number of arms entered by the mouse was recorded. The percentage of alternations was the number of triads containing entries into all three arms divided by the maximum possible number of alternations (total number of arm entries minus 2) × 100.

Fear conditioning test

This test evaluates the hippocampal-dependent and hippocampal-independent learning, as described previously [29]. Briefly, the conditioning trial for contextual and cued fear conditioning consisted of a 5 min exploration period followed by three conditioned stimulus-unconditioned stimulus pairings parted by 1 min each. Unconditioned stimulus consisted of 1 mA foot shock with 1 s duration and the conditioned stimulus is the 80-db white noise with a 20 s duration. Unconditioned stimulus was delivered during the last seconds of conditioned stimulus presentation. A contextual test was performed in the conditioning chamber for 5 min in the absence of white noise, 24 h after conditioning. A cued test (for the same set of mice) was performed by presenting a cue (80-db white noise, 3 min duration) in an alternate context with distinct visual and tactile cues. The freezing response rate (absence of movement in any part of the body during 1 s) was scored automatically and used as a measure of fear memory. Mice used for the test were 5 weeks of age. Mice subjected to the test were not used for any further testing.

Memory and learning studies-Morris water maze test

Mice were trained for 4 days (postnatal days 31-34) in the Morris water maze. A platform (10.3 cm diameter) was submerged in a circular pool (180 cm diameter, 50 cm depth) filled with warm water (23°C ± 2°C). Mice were trained in 2 sessions a day. In each of the sessions, the mice were allowed to perform four trials in

which they were released from one of the four randomly assigned release points while facing the tank wall. This enabled each mouse to have two short and two medium swims per session. Animals were allowed a time of 60 s to locate the hidden platform, and if they failed to locate in allotted time, they were guided to the platform. In either case, the mice were removed from the platform after 15 s. Training sessions were conducted until they could locate the hidden platform in less than 15 s (average time per session). All trials and swim paths were recorded with ANY-maze video tracking system (Stoelting Co., Wood Dale, 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 at the end of every test.

Cued trials

The cued trials were performed to determine any non-cognitive performance impairments as visual impairments and/or swimming difficulties. In this study, the pool was surrounded by a white cloth to hide the visual cues. The animals received 4 trials per day. In each trial, they 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) placed 20 cm above water level randomly in any of the four quadrants of the swimming pool. The mice were allotted 60 s to locate the platform and 30 s to sit on the platform after which they were taken off from the pool. If unable to locate a platform within 60 s, they were gently guided and allowed to remain there for 30 s. The time taken for each mouse 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 and the same mice were allowed to perform the place trials in order to determine the 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 mouse. The time taken to reach the platform was recorded for each trial.

Neuroprotective effect of vitamin C on anesthetics

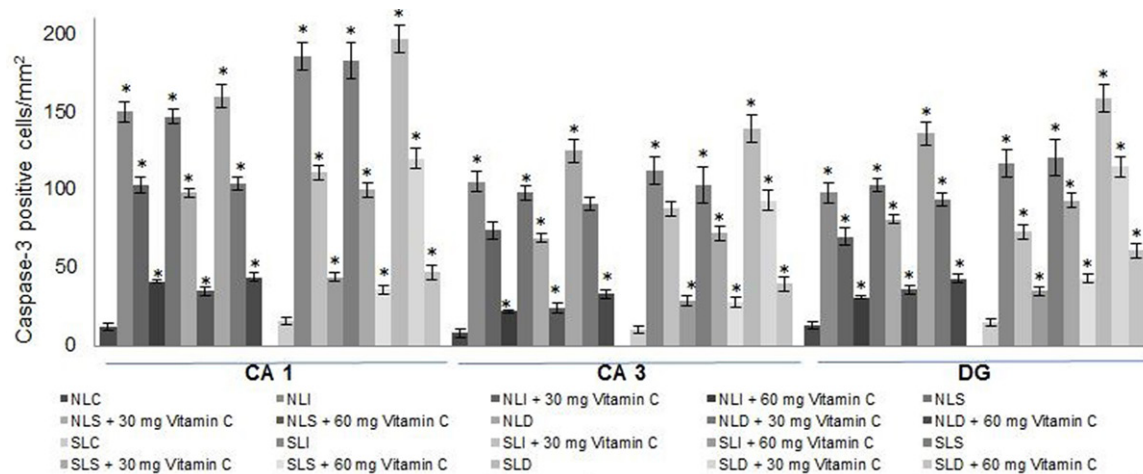


Figure 1. Vitamin C on anesthesia induced neuroapoptosis. Values are represented as mean $\pm$ SD, n = 6. *Represents statistical significance at $P < 0.05$ compared against control as determined by ANOVA.

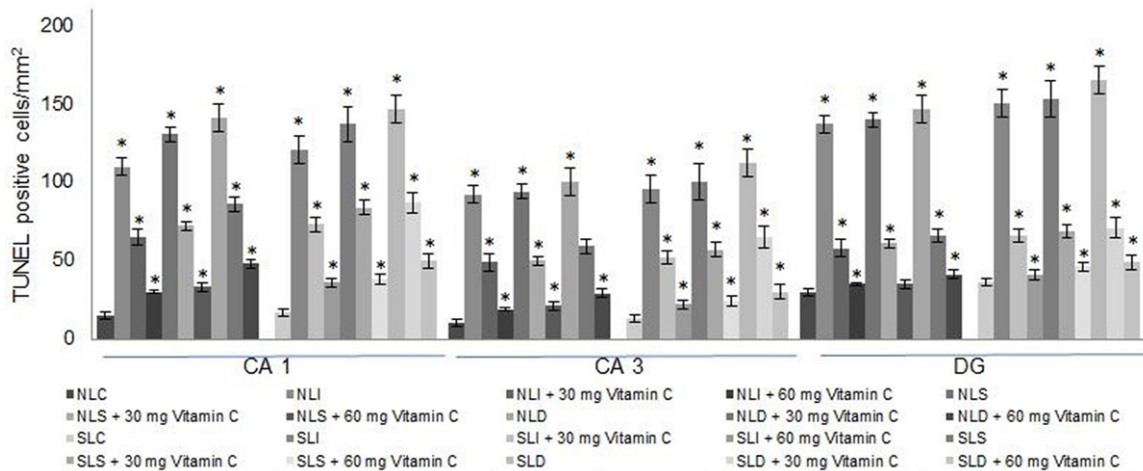


Figure 2. Influence of vitamin C on TUNEL positive cell counts following anesthesia in P7 mice. Values are represented as mean $\pm$ SD, n = 6. *Represents statistical significance at $P < 0.05$ compared against control as determined by ANOVA.

Probe trials

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

Statistical analysis

The data are represented as mean $\pm$ SD from six individual experiments and were analyzed using SPSS software, version 22.0. The differ-

ence between the means of different groups was compared by One-way Analysis of variance (ANOVA) for all tests. For behavioral studies the data were analyzed by two-way ANOVA followed by Bonferroni *post hoc* test. Differences at $P < 0.05$ were considered statistically significant.

Results

Vitamin C on inhalation anesthetic induced neuroapoptosis

To investigate neurotoxic effects on the developing brain by desflurane, sevoflurane, or isoflurane, immunohistological evaluation of acti-

Neuroprotective effect of vitamin C on anesthetics

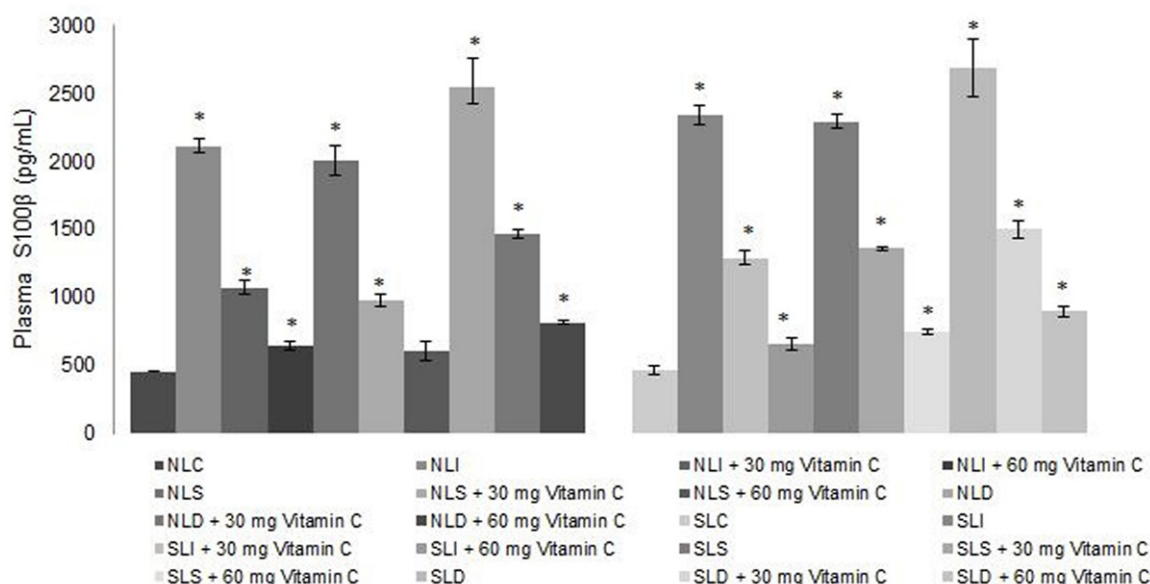


Figure 3. Plasma S100 β levels in P7 mice following anesthesia exposure. Values are represented as mean $\pm$ SD, n = 6. *Represents statistical significance at $P < 0.05$ compared against control as determined by ANOVA.

vated caspase-3 in the hippocampal CA1, CA3 and dentate gyrus (DG) were analyzed. Cleaved caspase-3 detects endogenous levels of activated caspase-3, a known and useful marker of neuronal cell death from apoptosis. Neonatal exposure to anesthetics desflurane, sevoflurane and isoflurane for 6 h presented a marked raise in the number of activated caspase-3+ cells as compared to control animals that were not exposed to anesthesia (**Figure 1**). The high fat diet fed SL mice exhibited significant multi-fold increases ($P < 0.05$) in the caspase-3+ cells higher than the NL mice. Vitamin C supplementation caused a considerable drop in the caspase-3+ cells in both SL and NL mice exposed to anesthesia; however the drop was more marked in NL mice. Vitamin C though reduced the positive cell count in anesthesia exposed mice, it had a more profound effect in sevoflurane in the order sevoflurane > isoflurane > desflurane irrespective of whether fed on high fat diet or normal chow.

Consistent to the caspase-3+ cell counts, anesthetic exposure significantly increased the amount of TUNEL positive cells. Vitamin C treatment to the mice of both SL and NL groups significantly reduced apoptotic cell counts as against anesthetic control groups that were not treated with vitamin C (**Figure 2**).

S100 proteins are a family of dimeric cytosolic calcium binding proteins made up of an alpha

and beta isomers. They are found in abundance in astroglial and Schwann cells in nervous system. S100 β , the beta isomer of S100, appears to be released into the extra-cellular space near the injured tissue and can enter into the serum from the brain through a disrupted blood brain barrier after even mild brain injury secondary to trauma, hypoxia, ischemia and neurotoxin, etc. [40]. In line with the results of TUNEL assay and caspase-3 immunohistochemistry, the plasma levels of S100 β were markedly elevated in mice exposed to anesthesia (**Figure 3**). The levels in the high fat diet-fed, SL mice were nearly two-fold higher than the NL mice fed on standard diet. The levels were observed to be more raised in the desflurane induced group against sevoflurane and isoflurane exposed mice. Vitamin C brought about a marked decline in the plasma levels of S100 β in both anesthetic exposed NL and SL mice with the higher dose of vitamin C having a more significant effect.

Vitamin C on the behaviour and memory of the mice exposed to inhalation anesthesia

To examine behavioral activity in a novel environment, mice treated with desflurane, sevoflurane, or isoflurane for 6 h on P7 were examined in an open-field test. Though there were little observable changes in the behaviour of the mice exposed to anesthesia in standard diet fed mice, however in mice fed on high fat diet substantial changes were seen compared to

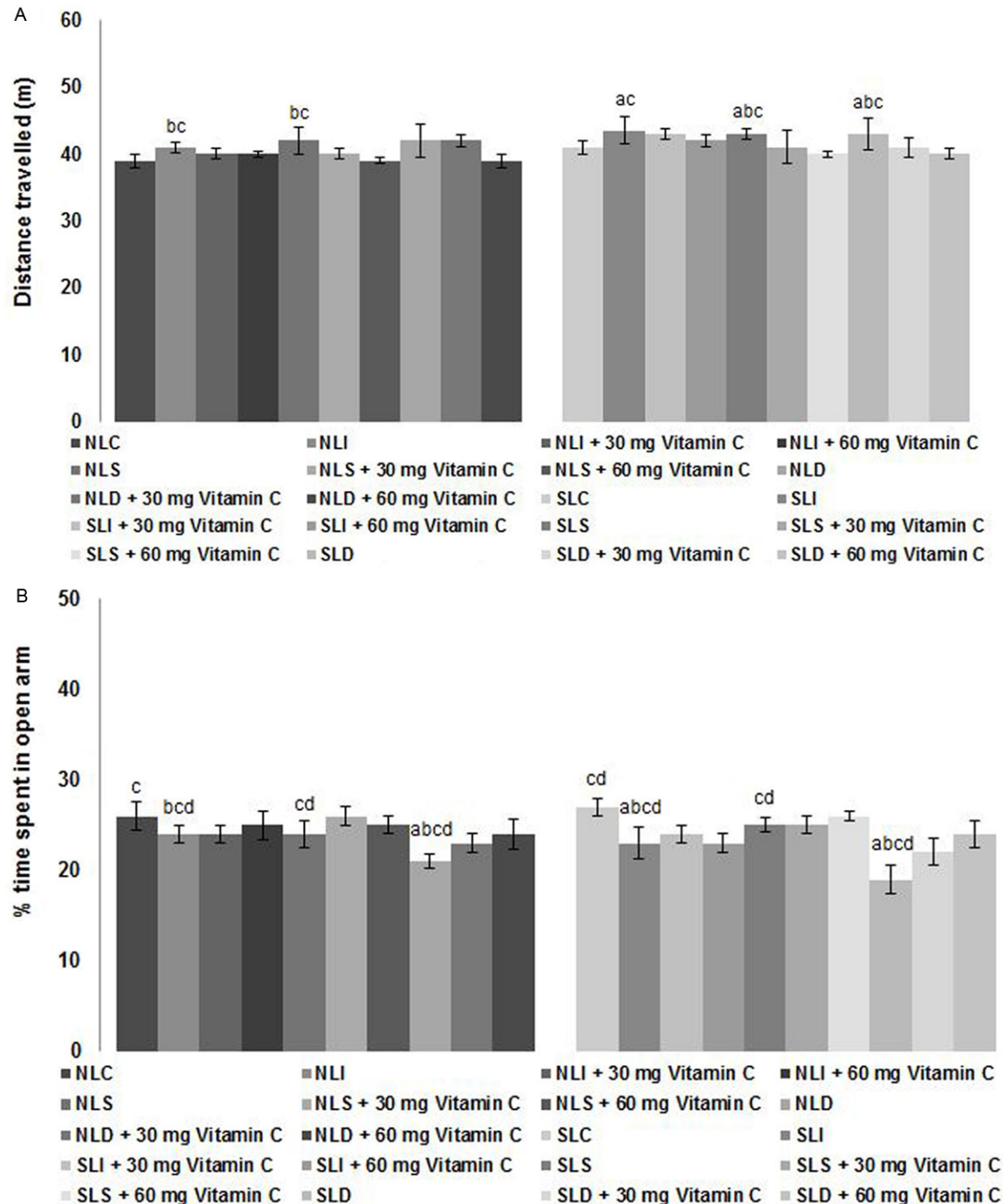


Figure 4. A. Behavioural responses of the mice following anesthesia on exposure to novel environment. Values are represented as mean $\pm$ SD, $n = 6$. a-d represent statistical difference at $P < 0.05$ determined by two-way ANOVA followed by Bonferroni *post hoc* analysis. a - statistical difference between means of different groups vs normal control; b - statistical difference between means of different groups vs 30 mg vitamin C treatment; c - statistical difference between means of different groups vs 60 mg vitamin C treatment. B. General behavior of mice in elevated maze. Substantial improvement were observed in the behavior of mice on vitamin C administration. Values are represented as mean $\pm$ SD, $n = 6$. a-d represents statistical difference at $P < 0.05$ determined by two-way ANOVA followed by Bonferroni *post hoc* analysis. a - statistical difference between means of different groups vs normal control; b - statistical difference between means of different groups vs high fat diet fed control; c - statistical difference between means of different groups vs 30 mg vitamin C treatment; d - statistical difference between means of different groups vs 60 mg vitamin C treatment.

Neuroprotective effect of vitamin C on anesthetics

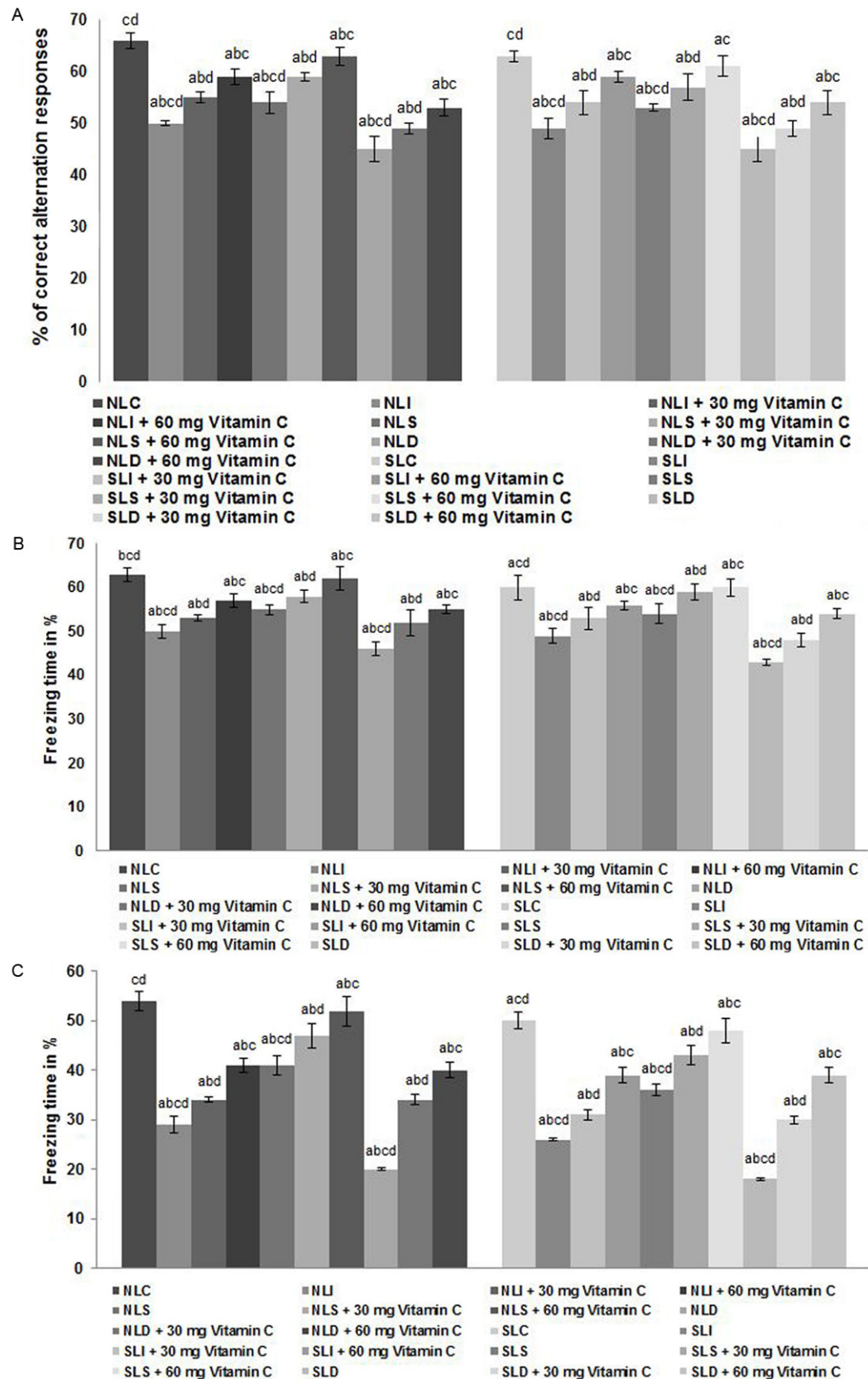


Figure 5. Influence of vitamin C on short-term spatial working memory of mice following neonatal anesthesia exposure (A) and responses upon contextual fear conditioning (B) and cued fear conditioning (C). Values are represented

as mean $\pm$ SD, $n = 6$. a-d represents statistical difference at $P < 0.05$ determined by two-way ANOVA followed by Bonferroni *post hoc* analysis. a - statistical difference between means of different groups vs normal control; b - statistical difference between means of different groups vs high fat diet fed control; c - statistical difference between means of different groups vs 30 mg vitamin C treatment; d - statistical difference between means of different groups vs 60 mg vitamin C treatment.

normal control mice (**Figure 4A**). The mice fed on standard pelleted diet and exposed to desflurane presented significant changes ($P < 0.05$) in behaviour as compared to mice exposed to isoflurane or sevoflurane, while both isoflurane and desflurane exposure caused alterations in behaviour in high fat diet fed mice. Nevertheless vitamin C supplementation improved the general behaviour of mice of both the groups with 60 mg dose presenting more prominent effects (**Figure 4B**).

Working memory could be said as the ability to hold information temporally to do complex cognitive tasks and it involves both the hippocampus and prefrontal cortex [41, 42]. In order to examine whether exposure of the developing brain to desflurane, sevoflurane, and isoflurane was associated with changes in spatial working memory, the mice were tested in a Y-maze task. This task examines whether the animals remembered the position of the arm selected in the preceding choice. By nature, rodents look for a new arm, different from that selected in the previous choice, but if working memory is impaired, the number of correct choices would be reduced in the Y-maze task.

Mice exposed to anesthetics, isoflurane or sevoflurane exhibited altered performances as against control mice. In the mice fed on high fat diet these alterations were more pronounced as compared to mice exposed to anesthesia and fed on normal diet (**Figure 5A-C**). The disturbance though noticeable in both SL and NL groups, mice exposed to 7.5% desflurane treatment had significantly impaired performance compared with that of mice exposed to isoflurane or sevoflurane irrespective of whether SL or NL groups. Vitamin C treatment at both the doses caused a marked improvement in the working memory of mice exposed to desflurane and as well on mice exposed to isoflurane or sevoflurane.

Vitamin C on the behaviour and memory of the mice exposed to inhalation anesthesia

To evaluate the effect of neonatal exposure with desflurane or sevoflurane or isoflurane on

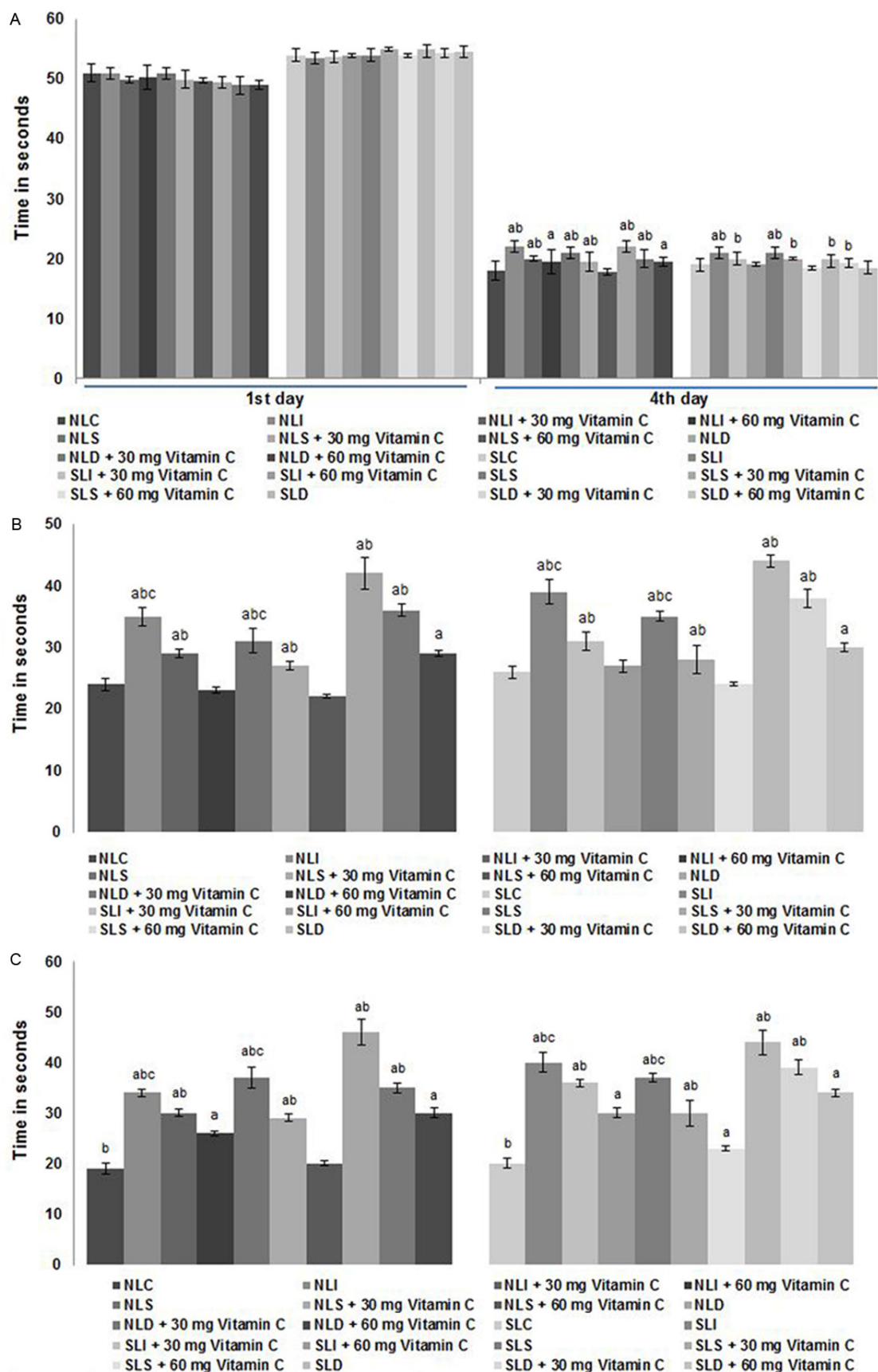
potential learning and memory deficits, we subjected the mice to Morris water maze testing. The mice were trained to explore the swimming pool and to reach on the platform. The time taken to reach the platform was noted and the duration to reach up the platform was found to decrease with each training session for all mice (**Figure 6A**).

Cued trials were conducted at postnatal day 35 to evaluate swimming and visual abilities. The mice that were exposed to anesthesia took a considerably ($P < 0.05$) longer time to reach the platform when compared to control pups that received no anesthesia. The duration was much longer in high fat diet fed mice as compared to mice that were on normal diet. Desflurane exposed mice comparatively took a much longer time than the mice exposed to isoflurane or sevoflurane. Mice that received vitamin C at both the doses were able to reach the platform much quicker as against anesthetic exposed control pups however mice that received the higher dose of vitamin C reached the platform at a lesser time as against mice that received lower dose of vitamin C (**Figure 6B**).

Furthermore, in order to evaluate the differences in visual judgments and memory after anesthetic exposure on P7, place and probe trials were performed. The trials were conducted to test the ability of the pups to learn and remember the location of a new platform (**Figure 6C**). In place trials, mice that were supplemented with vitamin C at 30 mg/kg b.wt and 60 mg/kg b.wt showed a significant improvement in performance. They were able to reach the platform in a lesser time than the anesthesia alone treated pups. Vitamin C at both the doses was more effective on mice fed on normal diet than on the mice fed on high-fat diet. However, the difference was negligible.

As demonstrated in **Figure 6D** in probe trials, mice that were exposed to isoflurane or sevoflurane or desflurane tended to spend less percentage of time in the target quadrant than mice in the control group unexposed to anesthesia. There was a significant statistical differ-

Neuroprotective effect of vitamin C on anesthetics



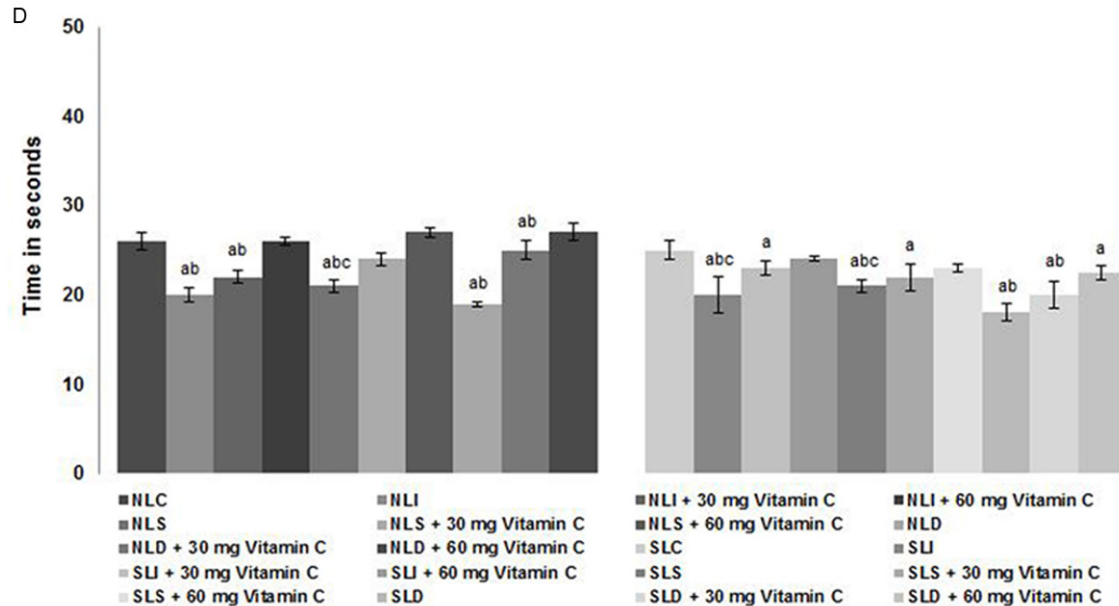


Figure 6. The learning and behavior of mice (P35/P36) that were exposed to anesthetics on P7 was evaluated using Morris water maze. (A) Represents the latency periods of mice during training in the Morris water maze. The performance of mice was assessed by (B) cued trials, (C) by place trials and (D) probe trials with the Morris water maze. The observations indicate that vitamin C supplementation considerably improved the behavior of mice. Values are represented as mean $\pm$ SD, $n = 6$. a-c represents statistical difference at $P < 0.05$ determined by two-way ANOVA followed by Bonferroni *post hoc* analysis. a - statistical difference between means of different groups vs respective controls; b - statistical difference between means of different groups vs respective groups treated with 60 mg vitamin C. c - statistical difference between desflurane alone exposed groups vs groups exposed to isoflurane and sevoflurane alone.

ence between the groups ($P < 0.05$) of normal control mice and anesthesia control mice. The over-weight mice that were on high fat diet exhibited more alterations than the mice on standard diet. The anesthetics have a higher impact on memory of the high-fat diet fed mice. Isoflurane or sevoflurane exposed mice spent considerably more time than the mice exposed to desflurane. The mice treated with vitamin spent more time in the target quadrant. Thus the treatment with vitamin C at both doses was found to have improved memory and learning ability of mice.

Discussion

Exposure to anesthesia has been demonstrated to be associated with apoptotic neurodegeneration in the developing brain of animals and as well as cause cognitive impairment [19, 20, 43]. Clinical retrospective studies have reported that anesthesia and surgery in children less than 4 years of age increase their chances of developing disabilities in reading, writing and mathematics learning as well [22, 44].

Millions of children are exposed to inhaled anesthetics every year [45]. Recent studies on various animal models demonstrate that volatile anesthetics, including isoflurane and sevoflurane, may cause neuronal death if exposed at early stages of postnatal brain development. Many of these studies reported long-term neurocognitive abnormalities [19, 29, 30, 46, 47]. These reports have led to serious concerns about the possible detrimental effects of anesthesia and sedation in the pediatric population.

In the present study, we evaluated the effect of vitamin C on the neurotoxic effects of equipotent doses of most commonly used volatile anesthetic-isoflurane, desflurane and sevoflurane. Since body weight plays a crucial role in anesthesia dosing and in surgery, the study was extended to assess vitamin C supplementation in high-fat diet fed mice.

Cell death due to apoptosis is an integral part of normal brain maturation removing 50-70% of neurons and progenitor cells throughout central nervous system development [48, 49].

However, during brain development, neuronal cell death surpassing the natural apoptotic rate can be triggered by pathologic processes such as hypoxia-ischemia, lack of neurotrophic factors and prolonged exposure to anesthetics [50, 51].

The mechanisms of inhalational anesthetic-induced neurodegeneration in the developing brain are being expansively investigated. Studies have demonstrated that inhalational anesthetics induced neuroapoptosis through the activation of both intrinsic and extrinsic apoptotic pathways [52, 53]. To further understand at the molecular level, we investigated the expression of caspase-3 in the hippocampal regions of brains of experimental mice. Activation of caspase-3 has been commonly used in these studies as a biomarker for anesthesia mediated cell death by apoptosis [18, 54].

In our study we focused on hippocampal region of the brain to assess apoptosis because previous reports in rodents have demonstrated that cell death occurs acutely in the period immediately following anesthesia [18, 30], and the thalamus and hippocampus are areas of the brain that have been reported to be susceptible to extensive neurodegeneration [18, 29, 55].

Significantly elevated levels of caspase-3 positive cells were observed in the hippocampal regions of the mice following exposure to 6 h of anesthesia. Exposure to desflurane for 6 h induced much more caspase-3 positive counts than isoflurane and sevoflurane. Neuronal apoptosis in CA1 was severe than that in CA3 and DG regions and more marked in P7 mice fed on high-fat diet. We also performed TUNEL as an independent measure of apoptotic cell death. The results of TUNEL assay were consistent with results of activated caspase-3 counts, thus indicating that desflurane caused severe neuronal apoptosis compared to isoflurane and sevoflurane. However vitamin C supplementation brought about a significant drop in apoptotic counts in both normal mice and in mice fed on high fat diet.

S100 β is a calcium binding protein in the astrocytes of the central nervous system which may be increased after even mild injury to the brains secondary to trauma, hypoxia, ischemia or neurotoxin [40]. Marked raise in the plasma S100 β

levels in mice following anesthetic exposure is suggestive of damage to brain cells that could have caused an elevation. Vitamin C supplementation resulted in a marked decline in plasma S100 β in line with the results of TUNEL assay and immunohistochemistry analysis of activated caspase-3. Similar raise in plasma S100 β levels has also been reported in previous studies on exposure to isoflurane [56].

Several previous studies reported that neonatal exposure to volatile anesthetics led to deficits in learning and memory [18, 29, 48, 57]. Our results indicated that, desflurane, sevoflurane, or isoflurane did not induce much alteration in the general behaviour of mice that were on standard diet when compared to mice on high fat diet as indicated in the open field and elevated maze test. Comparatively, desflurane had a little influence in the elevated maze test in mice on standard pelleted diet too, suggesting, the influence of diet and body weight under anesthetic exposure. Nevertheless, the spatial working memory of mice was affected on anesthetic exposure. Desflurane was observed to bring about profound alterations in the working memory of mice as evidenced in the results of Y-maze test.

Working memory refers to a cognitive function that provides concurrent temporary storage and manipulation of the information necessary to perform complex cognitive tasks [58]. Working memory is thought to be involved in higher executive functioning such as planning and sequential behavior; deficits in working memory are directly related to deficits in behavioral flexibility.

In the present study, neonatal exposure to desflurane induced the most severe alterations in spatial working memory among three halogenated ethers as given by fear conditioning and Y-maze tests. Studies have reported that isoflurane induced spatial memory deficit in neonatal rats [59], thus suggesting consistent results of working memory deficit with previous studies. Vitamin C supplementation was found to markedly improve the working memory of mice in both normal and high-fat fed over-weight mice.

The Morris water maze test was chosen to evaluate the cognitive behavior in mice as it is a reliable measure of hippocampus-dependent

spatial navigation and reference memory [60]. The mice exposed to anesthesia exhibited memory and learning impairments with the impairments being more pronounced in the mice of high fat diet fed groups. As in Y-maze test, desflurane exposed mice presented more marked impairments compared to mice induced with isoflurane or sevoflurane. Our results indicate that sevoflurane and isoflurane as well as desflurane cause deficits in memory, consistent with previous reports [29, 48, 61]. Body weight is a contributonal factor in anesthetic dosing and effects. The mice having a higher body weight exhibited more severe effects on anesthesia exposure than mice of NL.

Vitamin C supplementation to the mice markedly reduced neuronal apoptosis, learning and cognitive impairments. The mechanism of how vitamin C could offer protection is still under investigation. Vitamin C would have suppressed apoptosis via neutralising reactive oxygen species generation due to anesthetic drugs in the mitochondria, possibly by preventing caspase cascade activation. However, more research has to be made to shed light in this regard.

In summary, the current study suggests that desflurane has relatively greater neurotoxicity than sevoflurane or isoflurane, as shown by greater neuroapoptosis and additional impaired cognitive function. The order of neurotoxic potencies are given as sevoflurane < isoflurane < desflurane. Obesity and over-weight presents more vulnerability to anesthetic effects. Vitamin C was potent in protecting the brain cells from the apoptotic effect of isoflurane, sevoflurane and desflurane and as well improving memory and learning.

In conclusion, vitamin C supplementation offered protection against the neurotoxic effects of inhalation anesthetics-isoflurane, sevoflurane and desflurane.

Disclosure of conflict of interest

None.

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