Original Article

Efficacy of rutin in inhibiting neuronal apoptosis and cognitive disturbances in sevoflurane or propofol exposed neonatal mice

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Abstract: Sevoflurane and propofol are widely used in pediatric anesthesia. Neurotoxicity of sevoflurane and propofol in developing brain has been reported and these effects raise concerns on the usage of the drugs. We investigated the influence of rutin, a flavonoid on the neurodegenerative effects of sevoflurane and propofol and on memory and cognition in neonatal rodent model. Separate groups of neonatal mice (C57BL/6) were administered with rutin at 25 or 50 mg/kg body weight (b.wt) from post natal day 2 (P1) to P21. P7 mice were exposed to 2.9% sevoflurane and/or propofol (150 mg/kg b.wt). Neuroapoptosis was assessed by measuring activated caspase-3 and by Fluoro-Jade C staining. Plasma S100β levels were detected by ELISA. Morris water maze test was performed to test learning and memory impairments in the animals. General behaviour of the mice was also assessed. Anesthesia exposure caused severe neuroapoptosis and also raised the levels of plasma S100β. Neuroapoptosis, memory and cognitive deficits observed following anesthetics were comparatively more profound in mice on exposure to combined drug (sevoflurane and propofol) than in those exposed to either of the anesthetics. Rutin at both the doses was effective in reducing the apoptotic cell counts and enhanced the memory and cognitive abilities. Rutin supplementation offered significant protection against anesthetic induced neurodegeneration and learning and memory disturbances.

Keywords: Neuroapoptosis, propofol, rutin, sevoflurane

Introduction

Every year millions of children and infants undergo surgery as a part of health care. Pediatric surgeries require the administration of general anesthetics. Mounting reports have demonstrated that general anesthetics induce intense neuroapoptosis in the developing brain, and can cause long-term cognitive deficits [1-4]. The developing brain has high plasticity and the exposures to general anesthetics in young children under the age of 4 may affect learning disabilities, including reading, language and math [5].

However mechanism’s underlying neuronal apoptosis mediated by anesthetics in the developing brain is still under investigation. Many possible mechanisms have been proposed including, activation of gamma-aminobutyric acid (GABA) receptors and inhibition of N-methyl-D-aspartate (NMDA) receptors and associated impairment of synaptogenesis [1, 6-8], disruption of intracellular calcium homeostasis [9-11], activation of P75 neurotrophin receptors [12, 13] and regulation of cell cycle [14].

Sevoflurane, one of the most frequently used volatile anesthetics is especially useful for infants and children because of its properties of rapid induction and recovery together with less irritation to the airway [15, 16]. Many studies have shown neonatal exposure to sevoflurane causing learning disabilities and memory deficits [16-18]. General anesthetic, propofol, blocks NMDA receptors and potentiates GABA<sub>A</sub> receptors [19]. At clinically relevant concentrations and durations, propofol causes apoptosis in the developing brain [20, 21] and associated cognitive dysfunction as well [22, 23].
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Previous studies suggest the immature brain is more vulnerable to anesthetic-induced neurotoxicity during the period of rapid synaptogenesis [1]. Activation of extrinsic and intrinsic apoptotic pathways are involved in the anesthetic-induced neuroapoptosis [24].

These observations have raised serious concerns regarding the safe use of general anesthesia in pediatric medicine. Strategies that can possibly suppress the associated neurotoxicity of the anesthetics call for more research. Recent researches are focussing on plant products as a means of therapy for various medical conditions. Bai et al. [25] reported that reserveratrol, a phytoalexin found in grapes and other berries was able to offer protection against isoflurane-induced neurotoxicity. Pterostilbene, a flavonoid was observed to significantly improve cognitive performance of mice [26, 27]. Rutin, a bioflavonoid compound, is a glycoside derivative of quercetin, a polyphenol widely found in citrus fruits and the rinds of grapes and lime, in berries, including cranberries [28] as well as in buckwheat and asparagus [29].

Various pharmacological properties of rutin including-anticancer [30], anti-inflammatory [31] and antioxidant [32] effects have been reported. Further, rutin may also have therapeutic potential for the treatment of neurodegenerative diseases associated with oxidative stress [33]. Qu et al. [34] reported potent therapeutic effect of rutin in cognitive deficits.

Thus, considering the protective effects of rutin, the present work aimed to investigate its effect in modulating apoptosis and improving cognitive deficits in sevoflurane or propofol-induced anesthesia in neonatal rodent model.

Materials and methods

Animals

All experiments were carried out in accordance with approved institutional animal care guidelines of the University Hospital. The C57BL/6 pregnant mice (Guangdong Medical Laboratory Animal Co., China) were used in this study and were maintained in 12 h light/dark cycle at room temperature (22° ± 1°C). Mice had ad libitum access to water and food. The animals were housed individually in separate cages and monitored closely for the day of birth, which was noted as postnatal day 0 (P0). The pups (male and female) were kept in cages in a 12 h light/dark cycle with free access to water with their littermates. Separate groups of mice were administered rutin orally at 25 or 50 mg/kg b.wt from P2 to P21. On P7, the mice were further randomly assigned to different treatment groups. Control pups received no rutin or anesthesia. Treatment pups received either sevoflurane and/or propofol on P7.

Chemicals and reagents

Sevoflurane and propofol were purchased from Sigma-Aldrich, St.Louis, MO, USA. Fluoro-Jade C (0.001%) was obtained from Merck Millipore, Billerica, MA, USA. All other chemicals used in the study were of analytical grade and were obtained from Sigma-Aldrich, (St.Louis, MO, USA) unless otherwise specified.

Anaesthesia exposure

On postnatal day 7 (P7), mice were placed in a humicd chamber with manipulating gloves and exposed to anesthetics. The total gas flow was 2 L/min, using 25% O₂ as a carrier. Oxygen and anesthetic agent fractions were measured using a gas analysis system (Capnomac Ultima, GE Healthcare, Tokyo, Japan). During exposure to the anesthetic, mice were kept warm on a mat heated to 38° ± 1°C. Neonatal mice were assigned to receive 2.9% sevoflurane for 6 h in 30% oxygen [35] or a single intraperitoneal (i.p) injection of propofol at 150 mg/kg b.wt [20, 36] or a combined dose of propofol and sevoflurane. In combined dose, propofol injection (150 mg/kg) was followed by exposure to 2.9% sevoflurane for 6 h.

Determination of plasma S100β

Following anesthetic exposure, S100β levels in the blood of mice were determined using Sangtec 100 ELISA kit (DiaSorin Inc, Stillwater, MN, USA) as per manufacturer’s instructions and as previously described [37]. Briefly, blood from each mouse was drawn from the left ventricle and was centrifuged for separation of plasma 2 h after anesthesia exposure. Fifty μL plasma was placed in each well of microtiter plate and mixed with 150 μL tracer from kit, incubated for 2 h, followed by addition of 3,3',5,5' tetramethylbenzidine substrate and
stop solution. The absorbance was read at 450 nm and the concentration of S100β was measured using a standard curve.

**Evaluation of neuroapoptosis**

Apoptosis was evaluated by immunohistochemical staining for activated caspase-3 and Fluoro-Jade C staining. Five hours following exposure to anesthesia experimental treatments, mice were perfused transcardially with 0.1 M phosphate buffer containing 4% paraformaldehyde. Brain sections were prepared and processed for activated caspase-3 immunostaining using a well-established procedure for measuring neonatal apoptosis in the developing brain [7, 35]. Immunohistochemistry was performed as described previously [38]. Briefly, the brain tissue sections were paraffin-embedded (5 µm thick) and 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 secondary antibody (1:200, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for about 40 min. The sections were further incubated with avidin-biotinylated peroxidase complex (Vectostain ABC-Kit, Vector Lab, Burlingame, CA, USA) for 40 min. 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, IL, 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.

The responses of mice to a new environment were measured by an open-field test using P35 mice. The responses were recorded as the total distance travelled (meters) in 10 min. The elevated plus-maze test was used to evaluate anxiety-related behaviour. P35 mice were used in the study. The elevated plus-maze consisted of two open arms (25 × 5 cm) and two enclosed arms, with all arms elevated to a height of 50 cm above the floor. The behaviour of mice was monitored during a 10 min test period. The percentage of time the mice spend in the open arms was considered as an index of anxious-behaviour.

The Y-maze test assesses spatial working memory of mice following anesthesia exposure at P7. Symmetrical Y-maze consists of three arms (25 × 5 cm) separated by 120° with 15 cm high transparent walls. Each mouse (P35) was placed in the centre of the Y-maze and allowed to freely explore the maze for a time period of 8 min. The total number of arms entered by each mouse was recorded. The percentage of alternations in the behaviour of mice was calculated as 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. The test was performed as previously described [16]. Briefly, the P35 mice were subjected to conditioning trial for contextual and cued fear conditioning that consisted of a 5 min exploration period followed by three conditioned stimulus-unconditioned stimulus pairings parted by 60 sec time for each. The unconditioned stimulus consisted of 1 mA foot shock with 1 sec duration and an 80 db white noise is the conditioned stimulus of 20 sec duration. Unconditioned stimulus was delivered during the last seconds of conditioned stimulus. A contex-
tual 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 first second) was scored automatically and used as a measure of fear memory.

**Memory and learning studies-Morris water maze test**

To assess memory and cognitive capabilities, mice that were exposed to anesthetics on P7 were subjected to spatial reference memory and learning assessments with the Morris water maze. The trials were performed as described previously by Li et al. [38]. All trials and swim paths were recorded with ANY-maze video tracking system (Stoelting Co., Wood Dale, IL, USA).

**Escape latency**

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 during 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. Each mouse was allotted to have two short and two medium swims per session. Animals were given a time of 60 sec to locate the hidden platform. If they failed to locate in 60 sec, they were guided to the platform. In either case, the mice were removed from the platform after 15 sec. Training sessions were conducted till the mice were able to locate the hidden platform in less than 15 sec (average time per session). The trials and swim paths were recorded with ANY-maze video tracking system that measures the time taken (latency) to find the platform (s), as well as other behavioural information obtained during the spatial reference memory test.

**Cued trials**

Cued trials were performed to determine any visual impairments and/or swimming difficulties. In this study, the pool was surrounded by a white cloth to hide the visual cues. In trial (4 trials per day), the mice were placed in a fixed position of the swimming pool towards the wall and were allowed to swim to a randomly positioned platform with a rod (cue) placed 20 cm above water level in any one of the quadrants of the pool. Sixty second was allotted to locate the platform and 30 sec to sit on the platform after which the mice were removed from the pool. If unable to locate a platform within 60 sec, the mice were gently guided. The time taken for each mouse to reach the cued platform and the swim speed was recorded.

**Place trials**

After the cued trials, the white curtains and cue rod were removed. The same mice were tested for place trials to determine the ability to learn the spatial relationship between distant cues and the submerged platform that was kept in the same place for all place trials. During place trials, mice were placed in a random position in the swimming pool, facing the wall, and time taken to reach the submerged platform positioned in the pool was recorded.

**Probe trials**

Probe trials were conducted to evaluate memory retention following 24 h after place trials. During the trial, submerged platform was removed and mice were placed in quadrant diagonally opposite from the previous platform location. Swimming time spent by each mice in the target quadrant (probe time) and the number of times animal crossed the original position of the platform (platform-cross) were recorded.

**Statistical analysis**

All the values are represented as mean ± standard deviation (SD). Values at $P < 0.05$ are considered significant as determined by One-way Analysis of variance (ANOVA). The values were analyzed using SPSS software (version 17.0).

**Results**

**Rutin reduces the intensive apoptotic neurodegeneration due to neonatal anesthesia**

Caspase-3 is the main cell death marker and executioner enzyme of the apoptotic cell death cascade [40, 41]. The most vulnerable brain region, the hippocampus, reveals neural degeneration even on the exposure to the lowest sevoflurane concentration (1%) [18]. Caspase-3...
positive cells were detected in the CA1, CA3 areas of hippocampus and in the dentate gyrus (DG) of the mice exposed to anesthetics. Sevoflurane at 2.9% and propofol at 150 mg caused intense apoptosis. The number of caspase-3 positive cell counts and Fluoro-Jade C positive cells were strikingly higher in the animals that received combined dose of sevoflurane and propofol than in the animals that were exposed to either one of the anesthetics (Figure 1). Further the anesthetics induced more marked apoptosis in CA1 region than CA3 and DG irrespective of whether given as a single drug or combined with sevoflurane, exhibiting a higher percentage of apoptotic counts than propofol. Rutin caused considerable reduction in the apoptotic cell counts at both the doses. Rutin at 50 mg was more effective in markedly reducing apoptosis positive cells in pups whether exposed to sevoflurane and/or propofol. Rutin exhibited more efficiency against propofol exposure.

**Plasma S100β levels in pups exposed to anesthesia on P7**

S100β has been demonstrated as a useful biomarker for the detection of anesthetic-mediated neurodegeneration [37, 42]. S100β, the β
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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. [43]. Consistent with the apoptotic cell counts observed, anesthetic exposure caused a multi-fold raise in plasma S100β levels in the order sevoflurane + propofol > sevoflurane > propofol (Figure 2). Though plasma S100β were higher following propofol exposure, the raise was not much significant as compared to control pups that were not exposed to anesthesia. Nevertheless, rutin supplementation to neonatal mice significantly (*P < 0.05) reduced the levels of S100β with higher rutin dose exhibiting more efficiency. Rutin however showed more potent effects against propofol exposure than sevoflurane, as propofol > sevoflurane > propofol + sevoflurane. Rutin brought the levels of S100β to almost near to control levels.

Influence of rutin supplementation on the behaviour of neonatal mice following sevoflurane and propofol exposure on P7

To examine behavioral activity of the mice treated with sevoflurane and/or propofol on P7, an open-field test was performed on P35. There were observable changes in the behaviour of the mice exposed to propofol and/or sevoflurane as compared against control mice not exposed to anesthesia (Figure 3A). Rutin administration caused negligible changes in the behaviour of mice. Similar results as in open field test were observed in elevated-maze test (Figure 3B). Sevoflurane showed alteration as compared to control mice, however no significant changes were found in mice induced with propofol alone. Combined exposure to sevoflurane and propofol caused pronounced alterations than sevoflurane or propofol given as a single drug. Rutin at both the doses (25 mg and 50 mg) was able to effectively prevent the behavioural changes induced by anesthesia.

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 [44, 45]. In order to examine whether exposure of the developing brain to, sevoflurane and/or propofol was associated with changes in spatial working memory, the mice were tested in a Y-maze task. The experiment examines whether the mice were able to remember the position of the arm selected in the preceding choice. By nature, rodents normally look out for a new arm, differ-

Figure 2. Plasma S100β levels in P7 mice following anesthesia exposure. Values are represented as mean ± SD, n = 6. * represents statistical significance at *P < 0.05 compared against control as determined by one-way ANOVA.
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Mice exposed to anesthetics, propofol and/or sevoflurane exhibited altered performances as against control mice. In the mice that were supplemented with rutin, these alterations were not significant as compared to anesthesia exposure without rutin (Figure 3C). The disturbances were more noticeable in mice exposed to 2.9% sevoflurane + 150 mg propofol. The results indicate that anesthesia exposure had significantly impaired performance of the mice irrespective of whether given alone or as combined drugs. Rutin treatment at both the doses caused a marked improvement in the working memory of mice.

The P36 mice were examined in a contextual/cued fear conditioning test to assess memory following conditioning. The freezing responses of mice exposed to sevoflurane and/or propofol were significantly reduced in the contextual test compared with those of controls (Figure 3D). The
startled freezing responses showed by the mice that were supplemented with rutin were considerably higher than the percentage of responses expressed by the mice that were exposed to anesthesia but not rutin administered.

**Influence of rutin supplementation on learning and memory**

The mice exposed to anesthesia were subjected to Morris Water Maze (MWM) testing to evaluate the effect of neonatal exposure with sevoflurane and/or propofol on potential learning and memory deficits. MWM is a reliable measure of hippocampus-dependent spatial navigation and reference memory [46].

The P35 mice that were exposed to anesthesia on P7 were trained to explore the swimming pool and to reach on the platform. The escape latency of the mice was recorded as the time taken to reach the platform. With the training sessions, escape latency of the mice was found to gradually decrease for all the mice irrespective of whether exposed to anesthesia alone or were treated with rutin. However the mice exposed to anesthesia without rutin were found to take a longer time to reach the platform. Rutin significantly reduced the escape latency in both sevoflurane and propofol treatment mice and in mice exposed to sevoflurane and propofol (Figure 4).

Cued trials were conducted on P35 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 sevoflurane and propofol exposed mice as compared to mice that were exposed to either sevoflurane or propofol. The mice that received rutin at both the doses were able to reach the platform much quicker. However, mice that received higher dose of rutin reached the platform at a lesser time as against those which received lower dose (Figure 5).

Place and probe trials were conducted to evaluate the differences in visual judgments and memory. The trials assess the ability of mice to learn and remember the location of a new platform (Figure 5). Rutin supplementation to the neonatal mice showed a significant improvement in performance and the mice were able to reach the platform in a lesser time than the anesthesia alone treated mice. Rutin at both doses was observed to be more effective on
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Figure 5. Learning and memory of mice following anesthesia exposure on P7 as determined by cued, place and probe trials with Morris Water maze. Values are represented as mean ± SD, n = 6. *represents statistical significance at P < 0.05 compared against control as determined by one-way ANOVA.

propropofol than on sevoflurane alone or in combination with propofol. However, the differences were negligible in place trials.

As illustrated in Figure 5, in probe trials the mice that were exposed to propofol and/or sevoflurane tend to spend less percentage of time in the target quadrant than mice in the control group unexposed to anesthesia. There was a statistically significant difference between the groups (P < 0.05) of normal control mice and anesthesia control mice. Mice that were exposed to sevoflurane and propofol exhibited more alterations in the swim speed and swim path than those treated with sevoflurane or propofol as single anesthetic. Combined anesthetics exhibited to have a higher impact on memory. Rutin at 25 mg or 50 mg recorded a higher probe time as-propofol + rutin > sevoflurane + rutin > sevoflurane + propofol+ rutin. Thus the treatment with rutin at both the doses was found to have improved the memory and learning ability of mice.

Discussion

Exposure to general anesthetics has been demonstrated to cause apoptotic neurodegeneration in the developing brains and subsequent cognitive dysfunctions [47-50]. Clinical retrospective studies have reported that anesthesia and surgery in children increase the risk of developing cognitive disabilities [51, 52]. Research in animal models has demonstrated that volatile anesthetics including isoflurane and sevoflurane could cause neuronal death if exposed at early stages of postnatal brain development [16, 48, 53]. Many of these studies reported long-term neurocognitive abnormalities [35, 47, 48]. These observations lead to further research in the use of anesthetics in pediatric surgeries. The present study evaluates the effectiveness of rutin in neonatal mice exposed to sevoflurane and propofol anesthesia.

Cell death due to apoptosis is a vital part of normal brain maturation, removing about 50-70% of neurons and progenitor cells [55, 56]. However, during brain development, neuroapoptosis exceeding the natural apoptotic rate can be triggered by various pathologic processes as hypoxia-ischemia, lack of neurotrophic factors, or due to prolonged exposure to anesthetics [57, 58].
Neuroapoptosis following exposure to sevoflurane and/or propofol presented significant increase in caspase-3 positive cells. Caspase-3, a member of the caspase family, plays a central role in execution of apoptosis cascade and is well accepted as a biomarker for cell death by apoptosis [36, 59]. Previous studies indicate that sevoflurane [16, 53] and propofol [36, 58] could cause neurodegeneration in the developing brains of neonatal rodent models. In our investigation, rutin (25 mg and 50 mg) effectively lowered caspase-3 positive cell counts in the hippocampal CA1 and CA3 and in DG regions of the brain. Although anesthetic-induced neurodegeneration has been found in many brain regions, our study focused on hippocampus, as previous reports have demonstrated that neonatal rats show normal short-term memory, a function predominantly involving the prefrontal cortex with severe hippocampal lesion [60]. Robust neurogenesis ensures hippocampal learning [61], whereas decreased neurogenesis impairs it [62, 63].

The levels of neuroapoptosis correlated with the levels of plasma S100β, a neurodegenerative biomarker in blood. Previous studies have shown similar elevations following anesthesia [37, 42]. Marked decreases in apoptotic cell counts and plasma S100β in rutin administration suggest that rutin was able to efficiently protect the neurons against anesthetic insult.

In addition to neuroapoptosis, sevoflurane and/or propofol administration produced neurocognitive deficits in mice at 5 weeks of age. Long-term memory and working memory were impaired. MWM test was used to evaluate long-term spatial learning/memory that involves a sequence of specific molecular processes in the hippocampal CA1 region. The results suggest impaired working memory and learning. It is widely recognized that the effects of anesthetics on subsequent spatial learning/memory are associated at least partially with damage to the hippocampal region [58]. Earlier reports also demonstrated that sevoflurane and as well as propofol exposure can induce neuronal apoptosis and also decrease cognition in mice [17, 58, 64].

Working memory refers to cognitive functions that provide concurrent temporary storage and manipulation of the informations that are vital and are required to perform complex cognitive tasks [65]. Working memory is involved in higher cognitive functioning as planning and sequential execution of tasks.

Neurogenesis in the brain proceeds throughout adulthood and impaired adult neurogenesis has been suggested to be associated with deficits in hippocampal-dependent memory including working memory [66-68]. In this study, it is notable that sevoflurane and propofol induced neuroapoptosis could be possibly attributed to be responsible for learning and memory deficits. Anesthetic exposure also affected the general behaviour of mice in open field tests, elevated and Y-maze tests. The observed improvements in the memory and behaviour of mice may possibly be due to the reduction in neuroapoptosis as observed in rutin administration. The exact mechanisms through which rutin offers neuroprotection and improves cognition and memory have to be unravelled, however, possible means could be by interfering with the caspase cascade.

Conclusion

The current study suggests that combination of sevoflurane and propofol drugs presented higher neurotoxicity than sevoflurane or propofol when administered alone. Rutin exhibited potential neuroprotective effects against the anesthetics in the order-propofol > sevoflurane > sevoflurane + propofol. Rutin could be further investigated for the molecular events involved in neuroprotection.

Disclosure of conflict of interest

None.

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