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Protective effects of green tea polyphenol, epigallocatechin-3-gallate against sevoflurane-induced neuronal apoptosis involves regulation of CREB –BDNF-Trk-B and PI3K/Akt/mTOR signalling pathways in neonatal mice

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Abstract
Epigallocatechin-3-gallate (EGCG), a polyphenol in green tea is an effective antioxidant and possesses neuroprotective effects. For neurogenesis and synaptic plasticity, brain-derived neurotrophic factor (BDNF) and cyclic AMP response element-binding protein (CREB) are crucially involved. In this study we aimed to assess protective effects of EGCG against sevoflurane-induced neurotoxicity in neonatal mice. Distinct groups of C57BL/6 mice were given EGCG (25, 50 or 75 mg/kg b.wt) from postnatal day 3 (P3) to P21 and were subjected to sevoflurane (3 %; 6 h) exposure on P7. EGCG significantly inhibited sevoflurane-induced neuroapoptosis as determined by FluroJade B staining and TUNEL assay. Increased levels of cleaved caspase-3, down-regulated Bad and Bax with significantly enhanced Bcl-2, Bcl-xL, xIAP,c-IAP-1 and survivin expression were observed. EGCG induced activation of PI3K/Akt pathway as evidenced by increased Akt, phospho-Akt, GSK-3β, phospho- GSK-3β and mTORc1 levels. Sevoflurane-mediated down-regulation of cAMP/CREB and BDNF-TrkB signalling were inhibited by EGCG. RT-PCR analysis revealed enhanced BDNF and TrkB mRNA levels on EGCG administration. Improved performance of mice in Morris water maze tests suggests enhanced learning and memory. The study indicates that EGCG was able to effectively inhibit sevoflurane-induced neurodegeneration and improve learning and memory retention of mice via activation of CREB-BDNF/TrkB - PI3K/Akt signalling.

Keywords: brain derived neurotrophic factor, cAMP-response element-binding protein, epigallocatechin-3- gallate, neurodegeneration, phosphatidylinositol 3-kinase signalling, sevoflurane
Introduction

Green tea (*Camellia sinensis*) is consumed widely for its flavour and potential health benefits. Experimental and epidemiologic studies have proved that, regular consumption of green tea reduces the risk of cancer, diabetes and obesity (Higdon and Frei 2003; Crespy and Williamson 2004; Yang and Lambert 2011). (-)-epigallocatechin-3-gallate (EGCG) is the key polyphenol in green tea (Yang and Wang, 1993). EGCG possesses several biological activities such as anti-oxidant (Shin et al. 2016; Yin et al. 2008), anticancer (Crespy and Williamson 2004; Fang et al. 2015), anti-inflammatory (Dona et al. 2003), anti-arthritic (Ahmed et al. 2004; Roy et al. 2012) and neuroprotective effects (Wang et al. 2015).

Sevoflurane is a frequently used anesthetic for general anesthesia during surgeries especially in pediatric patients because of their speedy induction and recovery (Lerman and Johr 2009; Singh et al. 2009). However, accumulating experimental data has demonstrated that early exposure to sevoflurane causes widespread neurodegeneration and also leads cognitive impairments (Satomoto et al. 2009; Istanaphous et al. 2011). EGCG has been reported to exhibit protective effects against bupivacaine-induced neurotoxicity (Wang et al. 2015). We investigated the ability of EGCG to reduce sevoflurane-induced neurodegeneration and further assessed its influence on major pathways of neurogenesis and survival - CREB-BDNF/TrkB and PI3K/Akt pathways in the developing brains of neonatal mice.

The downstream transcription factor of cAMP and Ca^{2+} signal transduction pathways is CREB and phosphorylation at serine 133 (S133) by either cAMP/protein kinase A (PKA) or Ca^{2+}/calmodulin-dependent kinase IV (CaMKIV) activates CREB (Chrivia et al. 1993; Gonzalez et al. 1989; Gonzalez and Montminy, 1989). CREB is involved in hippocampal neurogenesis, proliferation, differentiation and survival (Ao et al. 2006; Fujioka et al. 2004; Nakagawa et al. 2002). Activated CREB leads to the activation of CREB-mediated
transcription of downstream target genes such as c-fos, BDNF etc., there by regulating neurogenesis (Chrivia et al. 1993; Kida and Serita 2014). Studies have shown that inhalation anesthetics isoflurane and sevoflurane down-regulated cAMP/CREB signalling (Xiong et al. 2013; Zhang et al. 2014).

Brain-derived neurotrophic factor (BDNF), a significant growth factor of the neurotrophin family, plays a significant role in neuronal development, synaptogenesis, learning and memory (Bibel and Barde 2000; Minichiello 2009; Park and Poo 2013). BDNF-mediated signal transduction involves binding to cell surface receptors- Trk and p75 neurotrophic receptor (p75NTR) (Huang and Reichardt 2003). Nevertheless, phosphorylation and activation of TrkB was found to be crucial for cell survival-promoting effects of BDNF (Reichardt 2006). BDNF-TrkB signal activates many intracellular signalling pathways such as - the mitogen activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K)/Akt, and phospholipase C (PLC)-γ1cascades thus affecting the development and function of the nervous system (Patapoutian and Reichardt 2001). PI3K/Akt signal in particular is vital in survival of neurons (Brunet et al. 2001) and is essential in many circumstances for trophic-factor-induced cell survival (Miller et al. 1997; Yao and Cooper 1995). Further, impaired BDNF-TrkB signalling has been recently proposed in anesthetic-induced neurotoxicity (Pontén et al. 2011). This study assesses the influence of EGCG on vital pathways of neuronal survival CREB–BDNF-TrkB-PI3K/Akt signalling.
Materials and methods

Antibodies and chemicals

From Sigma-Aldrich (St. Louis, MO, USA) EGCG and sevoflurane were obtained. Antibodies against CREB, p-CREB, cleaved caspase-3, Bcl-2, Bad, Bcl-xL, Bax, survivin, β-actin, phosphatase and tensin homolog (PTEN) and mammalian target of rapamycin complex 1 (mTORc1) were procured from Cell Signalling Technology (Beverly, MA, USA). Akt, p-Akt, GSK-3β, p-GSK-3β, BDNF, TrkB and p-TrkB were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Phospho diesterase 4 (PDE4), Protein kinase A (PKA) and Ca^{2+}/calmodulin-dependent kinase IV (CaMKIV) were used for expression analysis and were purchased from Abcam. Other reagents and chemicals were obtained from Sigma-Aldrich, unless otherwise are specified.

Study animals

Pregnant C57BL/6 mice were procured from the animal centre of Shandong Jining No.1 People’s Hospital, China. All experiments were reviewed and approved by Animal Investigation Ethics Committee of the Hospital and all the procedures were done in accordance with the Guidelines for the Care and Use of Laboratory Animals from the National Institutes of Health, USA and China. The mice were retained under normal environmental surrounding (22 - 24°C; 12-h light/dark cycle; 55% - 65% humidity levels) and were given access to water and standard pellet. Mice were observed carefully for the birth of pups and were recorded as post-natal day 0 (P0). The pups were maintained in sterile cages under constant in house conditions as mentioned above.
Animal grouping and anesthetic exposure

Orally EGCG (25, 50 or 75 mg/kg b.wt) was administered regularly from day P3 and continued till P21 along with standard pelleted diet. On P7, the pups were exposed to 3% sevoflurane for 6 h in 60% oxygen or air (temperature 33 - 35°C) (Satomoto et al. 2009). P7 pups were chosen for the study based on previous experiments suggesting that one week after birth in rats and mice is the rapid period of brain development and is susceptible to anesthesia-induced neuronal injury (Yuede et al. 2010). Separate group of pups that received no anesthesia or EGCG served as normal control pups. At the end of 6 h of sevoflurane exposure, the pups of different groups (n = 6) were sacrificed and the brain tissues were excised immediately and used for assessment of apoptosis, protein expression analysis by western blotting and RT-PCR. To determine CREB and BDNF-TrkB expression at P45, mice on postnatal day 45 (n = 6) were sacrificed following behaviour tests. The pups that were exposed only to sevoflurane served as anesthetic control.

Analysis of neuroapoptosis by TUNEL fluorescent assay

Following 6 h of exposure to sevoflurane, the animals were suffused transcardially with saline (ice-cold) and followed with 4% paraformaldehyde in 0.1 M phosphate buffer, brain tissues were excised and neuroapoptosis in the hippocampal regions was assessed by TUNEL studies. Hippocampi were sliced to sections of 5 µm thickness that were about 200 µm apart. The analysis was carried out as previously described (Li et al. 2013) using Promega’s TUNEL system kit (DeadEnd TM fluorometric TUNEL system) (Promega, Madision, WI, USA) as per manufacturer’s instructions. The apoptotic cells (TUNEL positive cells) were observed in the hippocampal CA1, CA3 and Dentate gyrus (DG) regions and were subjected to further analysis using NIS-Elements BR imaging processing and analysis software (Nikon Corporation, Japan).
Fluoro-Jade B staining

To further assess neurodegeneration, the hippocampal tissues were subjected to Fluoro-Jade B (FJB) staining. Hippocampal sections (30 µm thickness) were fixed on gelatin-coated slides and were allowed to dry in room temperature (overnight). Slides were then rehydrated and further incubated in potassium permanganate solution (0.06%) for about 15 min and were rinsed using distilled H₂O and finally stained with FJB. The sections were then incubated with 0.1% acetic acid for 30 min and observed under microscope (DM IRB; Leica).

Immunohistochemistry staining

Neuronal apoptosis was assessed by immunohistochemical staining for activated caspase-3. Caspase-3 expression is considered as a marker for apoptosis. Briefly, 4 µm hippocampal sections were implanted in paraffin-wax blocks and incubated in PBS-Triton (PBST) with 0.1% H₂O₂ for blocking endogenous peroxidase action. The slides were then washed with PBST and incubated with anti-cleaved caspase-3 primary antibody at 4°C, overnight and further incubated for 40 min with secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and with avidin-biotinylated peroxidase complex (Vectostain ABC-Kit, Vector Lab, Burlingame, CA, USA) for another 40 min. The sections were then treated with diaminobenzidine. With NIS-Elements BR imaging processing and analysis software (Nikon Corporation, Japan) the Cleaved caspase-3 positive cells in the hippocampal CA1, CA3 and dentate gyrus (DG) were analyzed.
Determination of cyclic AMP

Cyclic AMP (cAMP) levels in the hippocampi were determined using cAMP Complete ELISA kit (Abcam, Shanghai, China) according to manufacturer’s procedure. The cAMP levels were expressed as pmol/mL.

RT-PCR analysis

RT-PCR was performed to evaluate the effect of EGCG on BDNF and TrkB gene expression in the hippocampal tissues. Using Trizol (Invitrogen) total RNA was isolated and concentration was determined (Nanodrop spectrophotometer –ND-1000, Bio-Rad, USA). Using Revert Aid First Strand cDNA Synthesis Kit (Fermentas Company, USA) the first strand of complementary DNA was synthesized. PCR were performed as per the PCR kit protocol (Fermentas Company, USA). The primer sequences used were as follows -
BDNF  -  Forward: 5’-CGAAGAGCTGGATGGAG-3’, Reverse: 5’-ATGGGATTACACTTGGTCTCG-3’
TrkB  -  Forward: 5’-CCTCCACCGATGTGGTCTGA-3’, Reverse: 5’-GGCTGTGTTGGTGATACCGAAGTA-3’. GAPDH was used as the internal control. GADPH primer sequences were - Forward: 5’-CCGTATCGGACGCCTGGTTA-3’, Reverse: 5’-GGCTGTGTTGGTGATACCGAAGTA-3. PCR products were then subjected to electrophoresis on 1% agarose gel and were visualised using 0.05% ethidium bromide. The band intensities of the products were analysed using Bio-Gel imagery apparatus (Bio-Rad, USA).

Immunoblotting

The harvested hippocampal tissues were subjected to western blotting to assess protein expression following sevoflurane exposure. In brief, the hippocampal tissues were homogenized using cell lysis buffer (2 mM EDTA, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl,
0.5% Nonidet P-40) with protease inhibitors - pepstatin A, aprotinin and leupeptin at 1 mg/mL concentration for protein extraction. The protein concentration of extracts was estimated by means of BCA Protein Assay Reagent Kit (Novagen, Madison, WI, USA). Equivalent quantities of proteins (50 µg) were separated by SDS-PAGE and the separated bands were blotted and transferred to PVDF. The membranes were then incubated with blocking solution (0.1% TBST and 5% non-fat milk) for 2 h and were then incubated overnight (at 4°C) with primary antibodies. Following washing of the membranes with TBST, membranes were incubated with horseradish peroxidase-conjugated-secondary antibodies for 1 h (Cell Signalling Technology). Positive bands were visualized using an ECL detection kit (GE health care) and analysed using Image J software (NIH Image, USA). Expression of the proteins was normalised using β-actin expression.

Morris water maze test

Using Morris water maze test, memory and spatial learning of mice were tested as described earlier (Li et al. 2007). The circular fiberglass pool of 60 cm in height and 150 cm in diameter was filled with water leaving a 1.5 cm height from the rim. About 10 cm beneath the rim, a transparent platform of diameter 15 cm was placed at one quadrant of the pool in such a way that about 1.5 cm water remained over the surface of the platform. The pool was placed in a room with several constant visual cues that were unchanged during the studies. The P41 (n = 12) mice exposed to sevoflurane on P7 and/or administered EGCG were trained in the pool for 4 consecutive days with 2 training sessions a day. The mice were permitted to swim liberally and explore the pool and reached the platform, if unable to find the platform in 60 sec, the mice were directed and allowed to stay for 30 sec. The swim paths of the animals were automatically recorded using ANY-maze video tracking system.
(Stoelting Co., Wood Dale, IL, USA). The time taken by each animal to locate the submerged platform (latency time) was recorded.

After trial sessions (4 days) on P45, cued trials were conducted. The test aims in assessing any non-cognitive defects such as visual impairments. The circular pool was blinded by surrounding with a white cloth to hide any visual cues. Mice were trained (4 trials/day) to locate the submerged platform with the help of the cued rod attached to platform. The rod was placed few centimetres above the water level. Time taken by the mice to locate the cued platform was recorded.

For place trials, cloth surrounding the pool and the cue rod were removed. Visual cues were made visible to the mice. The animals were positioned at random points and the time taken to find out the platform without the rod was noted. To assess spatial memory retention, after 24 h of place trials, the probe trials were conducted. During the probe trials, the platform was placed in a different quadrant other than the target quadrant (refers to the quadrant where the submerged platform was kept during the cued and place trials). Time spent by the mice in the target quadrant searching for the submerged platform was noted.

**Statistical analysis**

Data were evaluated by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) post-hoc test. For comparison of the effect of 75 mg EGCG treatment on protein expressions at P7 and P45, t-test was performed. Statistical analysis was done using software package for statistics (SPSS for Windows, V. 22.0, Chicago, USA). The results were presented as mean ± standard deviation (SD); n = 6; p values < 0.05 were observed as significant statistically.
Results

EGCG reduced sevoflurane-induced neuroapoptosis

Earlier reports have indicated extensive apoptotic neurodegeneration of the growing brains following neonatal sevoflurane exposure (Fang et al. 2012; Lei et al. 2013; Zheng et al. 2013). Neuronal apoptosis in the hippocampal regions of P7 pups was measured following 6 h of sevoflurane exposure. Increase in TUNEL positive cell counts ($p < 0.05$) was observed in CA1, CA3 and DG regions of pups (Fig. 1a). Further, FJB staining was also performed to detect neuronal degeneration. FJ is an anionic X fluorescein derivative that specifically stains degenerating neurons (Schmued et al. 1997). Sevoflurane exposure presented significantly raised apoptotic cell counts (Fig. 1b). Significantly raised apoptotic counts were observed in sevoflurane exposed pups in comparison with normal controls. Interestingly, EGCG administration significantly ($p < 0.05$) reduced the number of apoptotic cells in a dose-dependent way, where 75 mg EGCG showed significant reduction.

EGCG reduced Cleaved caspase-3 expression levels

Immunohistochemistry revealed significant ($p < 0.05$) increase in number of cells expressing cleaved caspase-3 (Fig. 2). Immunoblotting disclosed noticeable increase in the protein levels of cleaved caspase-3 following sevoflurane exposure compared with control (Fig. 3 a-c). The results of western blotting analysis revealed that EGCG pre-treatment down-regulated activated caspase-3 expression, in line with reduced number of cleaved caspase-3 positive cell counts as noticed in IHC. EGCG (75 mg) more effectively suppressed cleaved caspase-3 expression compared to lower doses. These observations suggest that decreased apoptotic cell counts observed on EGCG treatment could have in part be due to down-regulated caspase-3 activation.
EGCG modulated proteins of apoptotic pathway

To further investigate the molecular events related with anti-apoptotic effects of EGCG, the apoptotic cascade proteins expression were assessed by immunoblotting. Sevoflurane exposure caused significant up-regulation ($p < 0.05$) in the levels of Bad and Bax while down-regulating Bcl-xL and Bcl-2. Expression of inhibitors of apoptosis proteins - IAPs (xIAP, c-IAP-1) and survivin were found to be reduced (Fig. 3 a-c). Prior treatment with EGCG effectively up-regulated the expression of cell survival proteins - Bcl-xL, Bcl-2, xIAP, c-IAP-1 and survivin while suppressing Bad and Bax. The down-regulated expression of Bax and Bad was found to be in line with activated caspase-3 levels. These observations indicate that EGCG effectively promoted cell survival and inhibited sevoflurane-induced neuronal degeneration.

EGCG up-regulates CREB activation

CREB is an important downstream transcription factor of the cAMP and Ca$^{2+}$ signal transduction pathways that is critically involved in neurogenesis and expression of genes involved in memory consolidation (Kida and Serita 2014; Sakamoto et al. 2011). The influence of EGCG on CREB expression and activation was assessed where in suppressed expression of CREB, PKA and CaMKIV was observed following sevoflurane exposure on P7 (Fig. 4 a-c) along with reduced phosphorylated levels of CREB. Expression of the enzyme PDE4 was up-regulated. Nevertheless, EGCG inhibited PDE4 levels and enhanced phosphorylation of CREB. Expression levels of total CREB, PKA and CaMKIV were found raised ($p < 0.05$), suggesting activation of CREB and improved CREB signalling. Further, the expression of CREB, PKA and CaMKIV were noticeably higher at P45 as compared to levels at P7, suggesting that EGCG administration till P21 more effectively improved CREB signalling. At both the time periods, 75 mg EGCG was more effective than the lower doses.
Influence of EGCG on cAMP levels

Accumulation of cAMP activates PKA that eventually activates CREB signalling (Carlezon et al. 2005). We assessed the levels of cAMP on P7 and P45. Multi-fold decrease in cAMP levels were observed following sevoflurane exposure, both on days P7 and P45 (Fig. 5), indicating that this decrease could have also contributed to the decrease in phosphorylation of CREB. EGCG administration increased cAMP levels and the cAMP levels on P45 were almost near to control levels. These observations indicate that EGCG-mediated increase in p-CREB levels in part could be due to increase in cAMP levels thus causing up-regulation of the cAMP/CREB signalling.

Effect of EGCG on BDNF-TrkB expression

BDNF-TrkB signalling activates many intracellular signalling cascades involved in neurogenesis and cell survival (Yamashima 2012). Sevoflurane was found to cause a significant ($p < 0.05$) down-regulation in the expression of BDNF (Fig. 6 a-d) and TrkB. Phosphorylation and activation of TrkB was observed to be reduced following exposure to sevoflurane. At P45, the expression was found to be noticeably enhanced than at P7. Further, significant ($p < 0.05$) up-regulation of BDNF and TrkB expression both at mRNA and protein levels was noticed in the hippocampal tissues of mice administered with EGCG prior to anesthetic exposure. The expression was more at P45 as compared to P7, suggesting the effect of EGCG in improving the expression.

EGCG modulated PI3K/Akt signalling

Following up-regulation of BDNF-TrkB, we also assessed influence of EGCG in activating the pathway. As expected, a significant ($p < 0.05$) suppression in the levels of Akt,
GSK-3β and mTORc1 (Fig. 7 a and b) on P7 was noticed with significant \( p < 0.05 \) increase in PTEN levels on sevoflurane exposure, indicating down-regulation of the pathway. Further, marked up-regulation in the phosphorylated forms of Akt and GSK-3β was noticed on EGCG supplementation. The levels of mTORc1 were also enhanced while that of PTEN was found to reduce. These observations indicate that EGCG was able to activate PI3K/Akt signalling and aid in neuronal survival. The effects of EGCG observed could be either direct and/or through the activation of BDNF-TrkB signalling.

**EGCG improved spatial learning and memory**

Learning and memory are the most important features of cognition. Anesthetic-induced cognitive deficits are well documented. We assessed learning and memory of P45 mice using Morris water maze. The experimental data revealed negligible changes in the escape latencies of mice of different test groups on the 1\(^{st}\) day of training period (Fig. 8 a and b). Nevertheless, on day 2, slight changes were noticed in the latency time between the mice that were exposed to sevoflurane-alone compared to other groups. The differences in the latency time were observed to be considerably longer \( p < 0.05 \) on day 3 and 4, between the sevoflurane-alone exposed mice and mice that were administered with EGCG. Mice treated with EGCG exhibited latency time similar to that of normal controls.

Cued and place trials were performed to test spatial navigation and memory, by measuring the time taken to identify the submerged platform. Longer escape latencies \( p < 0.05 \) were exhibited by mice exposed to anesthetic sevoflurane-alone than the other groups, which is indicative of sevoflurane-induced disturbances in spatial learning and memory. EGCG treatment was found to significantly \( p < 0.05 \) improve the performance of mice (Fig. 8 a and b). The animals treated with EGCG took much lesser time to reach the platform even while in the absence of visual cues. Nevertheless, in probe trials, the sevoflurane-alone
treated mice spent much lesser time in the target quadrant looking for the platform, suggesting an impaired memory. EGCG administered mice spent extended time in the target quadrant looking out for the platform. These observations reveal the enhanced memory of mice suggesting that EGCG supplementation significantly improved learning and memory.
Discussion

EGCG is the major green tea catechin, representing 50% to 80% of total green tea catechins (Sang et al. 2011). Green tea catechins and epicatechin have been reported to pass the blood brain barrier and accumulate in brain (Wu et al. 2012) as EGCG itself and its metabolites in free and protein-bound forms (Suganuma et al. 1998). Studies have shown that EGCG counteracts microglial activation in neurodegenerative diseases as Parkinson’s (Le et al. 2001) and Alzheimer’s diseases (Rezai-Zadeh et al. 2005) and amyotrophic lateral sclerosis (Xu et al. 2006). Neuroprotective effects of EGCG were assessed in the present study against sevoflurane-induced neurotoxic effects and the mechanism of action of EGCG on sevoflurane toxicity is presented as simplified scheme (Fig. 9).

Sevoflurane is one of the most frequently employed anesthetics in surgeries especially in children and infants due to its properties of less irritation of the airway, rapid induction and recovery (Lerman et al. 1994; Satomoto et al. 2009). Nevertheless sevoflurane-induced widespread neurodegeneration and cognitive impairments in developing brains have been well documented (Lei et al. 2013; Satomoto et al. 2009; Zheng et al. 2013).

Neuronal degeneration triggered by sevoflurane has been reported to involve activation of caspase cascade via Bax-dependent intrinsic pathway (Yang et al. 2015; Zheng et al. 2013). Sevoflurane-induced apoptosis in the hippocampi was assessed by TUNEL and FJB staining. In line with the previous reports, robust apoptosis was noticed in the CA1, CA3 and the DG regions following 6 h of sevoflurane exposure. It is well known that increased expression and activation of caspase enzymes contributes to neuronal death (Rupinder et al. 2007). Both immunohistochemistry and western blot studies showed that sevoflurane exposure caused enhanced expression of cleaved caspase-3. Caspase-3, an executioner caspase is a crucial member in apoptosis (Gown and Willingham 2002). Caspase-3 activation is reflected as a marker in anesthetic injury (Istaphanous et al. 2011; Kong et al. 2011).
EGCG reduced the number of cleaved caspase-3 positive cells and cleaved caspase-3 expression as well. TUNEL and FJB positive cell counts in the hippocampal tissues were also significantly reduced.

Western blot analysis revealed up-regulation of Bad and Bax proteins with reduced levels of Bcl-xL and Bcl-2 after 6 h of sevoflurane exposure. Bax translocates from the cytosol to mitochondria in response to various apoptotic stimuli leading to the release of cytochrome C, subsequently activating the caspase cascade (Dohare et al. 2008). The anti-apoptotic proteins - Bcl-2 and Bcl-xL block the translocation of Bax, maintain mitochondrial membrane potential and prevent activation of caspase pathway (Hsu et al. 1997). EGCG mediated down-regulation of Bax and Bad proteins and up-regulation of Bcl-2 and Bcl-xL is suggestive of inhibition of apoptosis. Expression of survivin and xIAP and cIAP-1 was also elevated upon EGCG pre-treatment.

Growth factors which activates PI3K also aids in cell survival via activation of Akt (Cantley 2002). Activated PI3K/Akt pathway stimulates cell survival and suppresses caspases involved in promoting apoptosis (Cardone et al. 1998; Manning and Cantley 2007). In this study, sevoflurane exposure has down-regulated the expression of p-Akt. Further, expression of downstream target molecules of Akt - GSK-3β and mTORc1 were also reduced, indicating inhibition of PI3K/Akt-mediated signalling. Akt crucially regulates varied pathological and physiological processes in the brain that includes neuronal survival, differentiation, proliferation and synaptic plasticity (Dummler and Hemmings 2007). Thus, suppression of Akt by sevoflurane could have also contributed to the elevated apoptosis levels. EGCG pretreatment significantly up-regulated phosphorylation of Akt, as evidenced by raised p-Akt levels. EGCG also enhanced expression of mTORc1 and GSK-3β, indicating activation of PI3K/Akt signalling.
BDNF exerts pivotal role in neuron growth, differentiation and survival (Huang and Reichardt 2001). BDNF-TrkB signalling is crucial in synaptic plasticity and mediates learning memory and behaviour (Lee et al. 2012; Lee and Son 2009; Numakawa et al. 2010; Yamada and Nabeshima 2003). Sevoflurane exposure has significantly suppressed both BDNF and TrkB at mRNA and protein levels. Immunoblotting analysis revealed reduced phosphorylated-TrkB levels. EGCG supplementation prior sevoflurane exposure remarkably reduced sevoflurane-induced suppression of BDNF and TrkB expression. The effects were more prominently found on P7 than on P45. The 75 mg dose effectively enhanced BDNF and TrkB levels to almost near control levels on P45 demonstrating the effectiveness of EGCG. Further, BDNF signalling has been reported to activate PI3K/Akt cascade, a major cell survival pathway in neurons via activation of Akt (Nguyen et al. 2010). Sevoflurane-induced down-regulation of BDNF-TrkB signalling could have in part contributed to the suppression of PI3K/Akt pathway. Thus, EGCG-mediated activation of BDNF-TrkB probably could have induced PI3K/Akt signalling.

Involvement of CREB in neuroprotection, synaptic plasticity and long term memory is well documented (Fujioka et al. 2004; Nakagawa et al. 2002; Xiong et al. 2013). Phosphorylation is required for activation of CREB. Phosphorylation of CREB has been found to be essential in neurotrophin-mediated neuronal survival (Bonni et al. 1999). PKA, CaMKIV and p38MAPK are the chief kinases involved in phosphorylation and activation of CREB (Josselyn and Nguyen 2005). We observed significant down-regulation of CREB expression along with reduced phosphorylation of CREB on sevoflurane exposure both on P7 and on P45, nevertheless the expression on P45 were slightly higher than on P7. This increase could have been probably due to growth of new cells. While the expression level of enzymes PKA and CaMKIV was suppressed, PDE4 expression was re up-regulated on sevoflurane exposure. PDE4 catalyses cAMP hydrolysis and reduces the levels of cAMP available for
activation of PKA (Li et al. 2009). Thus, reduced cAMP levels observed could be due to raised PDE4 expression. EGCG effectively improved cAMP levels and as well enhanced PKA and CaMKIV expression. As expected, p-CREB expression was also up-regulated by EGCG and on P45 the expression levels were almost near normal.

Further, CaMKII, protein kinase C (PKC), CaMKIV, and calcineurin have also been found to regulate synaptic plasticity (Meffert et al. 1991; Soderling 2000; Winder and Sweatt 2001). Morris water maze tests were conducted to assess the influence of EGCG on sevoflurane-induced cognitive and memory deficits. In the Morris water maze, mice were trained to locate a submerged platform in a pool of opaque water. Hippocampal lesions have been reported to block spatial learning in the water maze (Morris et al. 1982; Sutherland et al. 1982). P45 mice exposed to sevoflurane-alone on P7 took a longer time to locate the platform following training sessions in the cued and place trials that reflects sevoflurane-induced cognitive and memory impairments. Down-regulated CREB and BDNF signalling observed could be responsible for memory and learning deficits. EGCG administered mice reached the platform at a significantly lesser time. This shorter latency time indicates better performance of the mice that is suggestive of better learning and memory. In probe trials, the EGCG treated mice spent considerably more time in the target quadrant, looking out for the platform. These observations indicate improved memory retention in EGCG administration. EGCG-mediated inhibition of apoptosis may possibly have contributed to the inhibition of hippocampal lesions thus aiding in improved memory and cognition. Enhanced cAMP/CREB and BDNF-TrkB signalling observed on EGCG treatment in part be responsible for improved learning and enhanced spatial memory.
Conclusion

EGCG administration was found to inhibit sevoflurane-induced neuroapoptosis. EGCG effectively activated cAMP/CREB and BDNF/TrkB –PI3K/Akt signalling, the major pathways involved in neuroprotection, learning and synaptic plasticity. The study suggests that EGCG could be further explored as a valuable compound in the therapy of anesthetic-induced neurotoxicities.

Conflict of interest

The authors declare that there is no conflict of interest associated with this work.

Acknowledgements

Nil
References


Figure legends

Figure 1. Influence of EGCG on Sevoflurane-induced neuroapoptosis

EGCG administration prior to sevoflurane exposure, markedly reduced neuroapoptosis as determined by TUNEL assay (a) FluroJade B staining (b)

Data are presented as mean ± SD, n = 6. a represents p < 0.05 between the means of control vs all other groups; b-e refers to mean values of different treatment groups that differ at p < 0.05 as attained by one-way ANOVA followed by DMRT analysis.

Figure 2. EGCG reduces sevoflurane-induced activation of caspase-3

EGCG dose-dependently reduced cleaved caspase-3 positive cell counts in the hippocampal regions

Data are presented as mean ± SD, n = 6. a represents p < 0.05 between the means of control vs all other groups; b-f refers to mean values of different treatment groups that differ at p < 0.05 as attained by one-way ANOVA followed by DMRT analysis.

Figure 3. EGCG modulates the expression of apoptosis pathway proteins

EGCG significantly reduced the raised expressions of pro-apoptotic proteins while it dose-dependently enhanced the expressions of proteins contributing to cell survival (a-c)

(L1-Control; L2-Sevoflurane; L3-Sevoflurane + 25 mg EGCG; L4-Sevoflurane + 50 mg EGCG; L5-Sevoflurane + 75 mg EGCG)

Data are presented as mean ± SD, n = 6. a represents p < 0.05 between the means of control vs all other groups; b-e refers to mean values of different treatment groups that differ at p < 0.05 as attained by one-way ANOVA followed by DMRT analysis
**Figure 4.** Effect of EGCG on CREB signalling

EGCG significantly enhanced activation of CREB and also modulated enzymes that regulate pCREB expression (a-c)

(L1-Control; L2-Sevoflurane; L3-Sevoflurane + 25 mg EGCG; L4-Sevoflurane + 50 mg EGCG; L5-Sevoflurane + 75 mg EGCG)

Data are presented as mean ± SD, n = 6. * represents p < 0.05 between the means of control vs all other groups; b-f refers to mean values of different treatment groups that differ at p < 0.05 as attained by one-way ANOVA followed by DMRT analysis. # represents statistical significance at p < 0.05 between the means of 75mg EGCG treated groups at P7 and P45 determined by t-test.

**Figure 5.** EGCG up-regulates CREB expression

Data are presented as mean ± SD, n = 6. * represents p < 0.05 between the means of control vs all other groups; b-f refers to mean values of different treatment groups that differ at p < 0.05 as attained by one-way ANOVA followed by DMRT analysis. # represents statistical significance at p < 0.05 between the means of 75mg EGCG treated groups at P7 and P45 determined by t-test.

**Figure 6.** EGCG up-regulated BDNF-TrkB activation

EGCG at the tested doses markedly improved BDNF and TrkB mRNA levels (a-d)

(L1-Control; L2-Sevoflurane; L3-Sevoflurane + 25 mg EGCG; L4-Sevoflurane + 50 mg EGCG; L5-Sevoflurane + 75 mg EGCG)

Data are presented as mean ± SD, n = 6. * represents p < 0.05 between the means of control vs all other groups; b-f refers to mean values of different treatment groups that differ at p < 0.05 as attained by one-way ANOVA followed by DMRT analysis. # represents statistical
significance at \( p < 0.05 \) between the means of 75mg EGCG treated groups at P7 and P45 determined by \( t \)–test.

**Figure 7.** EGCG activated PI3K/Akt pathway

Treatment with EGCG significantly up-regulated PI3K/Akt signalling (a,b)

(L1-Control; L2-Sevoflurane; L3-Sevoflurane + 25 mg EGCG; L4-Sevoflurane + 50 mg EGCG; L5-Sevoflurane + 75 mg EGCG)

Data are presented as mean ± SD, \( n = 6 \). \(^a\) represents \( p < 0.05 \) between the means of control vs all other groups; \(^{b-f}\) refers to mean values of different treatment groups that differ at \( p < 0.05 \) as attained by one-way ANOVA followed by DMRT analysis.

**Figure 8.** Performance of P45 mice in MWM test

EGCG improved the escape Latency and enhanced learning and memory of P45 mice following anesthesia exposure on P7 as determined by cued, place and probe trials with Morris water maze (a,b)

Data are presented as mean ± SD, \( n = 6 \). \(^a\) represents \( p < 0.05 \) between the means of control vs all other groups; \(^{b-f}\) refers to mean values of different treatment groups that differ at \( p < 0.05 \) as attained by one-way ANOVA followed by DMRT analysis.

**Figure 9.** Mechanism of action of EGCG on sevoflurane toxicity

Effects of the green tea polyphenol, EGCG (epigallocatechin-3-gallate) against sevoflurane-induced neuronal apoptosis is showed in a simplified scheme.
Figure 1. Influence of EGCG on Sevoflurane-induced neuroapoptosis

EGCG administration prior to sevoflurane exposure, markedly reduced neuroapoptosis as determined by TUNEL assay (a) FluroJade B staining (b)

Data are presented as mean ± SD, n = 6. a represents p < 0.05 between the means of control vs all other groups; b-e refers to mean values of different treatment groups that differ at p < 0.05 as attained by one-way ANOVA followed by DMRT analysis.
Figure 2. EGCG reduces sevoflurane-induced activation of caspase-3
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groups; b-f refers to mean values of different treatment groups that differ at p < 0.05 as attained by one-
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Figure 3. EGCG modulates the expression of apoptosis pathway proteins

EGCG significantly reduced the raised expressions of pro-apoptotic proteins while it dose-dependently enhanced the expressions of proteins contributing to cell survival (a-c)

(L1-Control; L2-Sevoflurane; L3-Sevoflurane + 25 mg EGCG; L4-Sevoflurane + 50 mg EGCG; L5-Sevoflurane + 75 mg EGCG)

Data are presented as mean ± SD, n = 6. a represents p < 0.05 between the means of control vs all other groups; b-e refers to mean values of different treatment groups that differ at p < 0.05 as attained by one-way ANOVA followed by DMRT analysis

396x254mm (300 x 300 DPI)
Figure 4. Effect of EGCG on CREB signalling
EGCG significantly enhanced activation of CREB and also modulated enzymes that regulate pCREB expression (a-c)
(L1-Control; L2-Sevoflurane; L3-Sevoflurane + 25 mg EGCG; L4-Sevoflurane + 50 mg EGCG; L5-Sevoflurane + 75 mg EGCG)
Data are presented as mean ± SD, n = 6. a represents p < 0.05 between the means of control vs all other groups; b-f refers to mean values of different treatment groups that differ at p < 0.05 as attained by one-way ANOVA followed by DMRT analysis. # represents statistical significance at p < 0.05 between the means of 75mg EGCG treated groups at P7 and P45 determined by t-test.
Figure 5. EGCG up-regulates CREB expression

Data are presented as mean ± SD, n = 6. a represents p < 0.05 between the means of control vs all other groups; b-f refers to mean values of different treatment groups that differ at p < 0.05 as attained by one-way ANOVA followed by DMRT analysis. # represents statistical significance at p < 0.05 between the means of 75mg EGCG treated groups at P7 and P45 determined by t-test.
Figure 6. EGCG up-regulated BDNF-TrkB activation

EGCG at the tested doses markedly improved BDNF and TrkB mRNA levels (a-d)
(L1-Control; L2-Sevoflurane; L3-Sevoflurane + 25 mg EGCG; L4-Sevoflurane + 50 mg EGCG; L5-
Sevoflurane + 75 mg EGCG)

Data are presented as mean ± SD, n = 6. a represents p < 0.05 between the means of control vs all other
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way ANOVA followed by DMRT analysis. # represents statistical significance at p < 0.05 between the means
of 75mg EGCG treated groups at P7 and P45 determined by t –test.

330x254mm (300 x 300 DPI)
Treatment with EGCG significantly up-regulated PI3K/Akt signalling (a,b) (L1-Control; L2-Sevoflurane; L3-Sevoflurane + 25 mg EGCG; L4-Sevoflurane + 50 mg EGCG; L5-Sevoflurane + 75 mg EGCG)

Data are presented as mean ± SD, n = 6. a represents p < 0.05 between the means of control vs all other groups; b-f refers to mean values of different treatment groups that differ at p < 0.05 as attained by one-way ANOVA followed by DMRT analysis.
Figure 8. Performance of P45 mice in MWM test
EGCG improved the escape latency and enhanced learning and memory of P45 mice following anesthesia exposure on P7 as determined by cued, place and probe trials with Morris water maze (a, b).
Data are presented as mean ± SD, n = 6. a represents p < 0.05 between the means of control vs all other groups; b-f refers to mean values of different treatment groups that differ at p < 0.05 as attained by one-way ANOVA followed by DMRT analysis.
Figure 9. Mechanism of action of EGCG on sevoflurane toxicity
Effects of the green tea polyphenol, EGCG (epigallocatechin-3-gallate) against sevoflurane-induced neuronal apoptosis is showed in a simplified scheme.

176x84mm (300 x 300 DPI)