(-)Epigallocatechin-3-gallate attenuates anesthesia-induced memory deficit in young mice via modulation of nitric oxide expression

LI DING, XIANG GAO, JIANLEI HU and SHENGHUI YU

Department of Anesthesiology, The People's Hospital of Yinzhou, Ningbo, Zhejiang 315040, P.R. China

Abstract. (-)Epigallocatechin-3-gallate (EGCG) is a type of polyphenol monomer and is the predominant component of catechin compounds extractable from green tea. Previous studies have demonstrated that EGCG exhibits numerous bioactivities both *in vitro* and *in vivo*, including antitumor, antioxidant and anti-inflammatory activities, as well as lowering blood lipid levels and protecting against radiation. The present study aimed to investigate whether administration of EGCG may attenuate anesthesia-induced memory deficit in young mice and to reveal the associated underlying mechanisms. The present study revealed that EGCG administration significantly attenuated memory deficit, oxidative stress and cell apoptosis exhibited by anesthesia-induced mice, as determined by Morris water maze testing and ELISA analysis. Furthermore, the results of ELISA and western blot analysis demonstrated that EGCG administration restored acetylcholinesterase activity and modulated the expression levels of neuronal nitric oxide synthase (nNOS), β-amyloid and amyloid precursor protein in anesthesia-induced mice. The present study also employed L-arginine as an nNOS substrate and 7-nitroindazole as an nNOS inhibitor, which were demonstrated to inhibit or potentiate the effects of EGCG, respectively, on anesthesia-induced memory deficit in mice. Therefore, the present study demonstrated that the administration of EGCG attenuated anesthesia-induced memory deficit in young mice, potentially via the modulation of nitric oxide expression and oxidative stress.

Introduction

Postoperative cognitive dysfunction (POCD) refers to alterations in cognitive abilities experienced by patients following anesthesia, affecting functions such as orientation, memory, attention and insight (1). Furthermore, changes in personality, social skills and other cognitive functions may have a negative impact upon patient recovery, such as extension of hospital stays and regression of quality of life (2). POCD is a common central nervous system complication affecting elderly people following surgery and presents as a mild cognitive dysfunction (2).

Learning and memory are closely associated with the central nervous system, particularly with the function of the hippocampus. The hippocampus is a major component of the limbic system and is an important structure for learning, memory and the regulation of behavior (3). Neuronal nitric oxide synthase (nNOS) in the hippocampus is a key enzyme involved in the synthesis of the neurotransmitter nitric oxide (NO), which participates in mechanisms of learning and memory (4). Furthermore, the long-term potentiation of synaptic transmission efficiency in the hippocampus is recognized as the synaptic plasticity model of memory, which underpins the neuronal mechanisms responsible for behavior, learning and memory (5).

Oxidative stress refers to the process in which the body has been exposed to harmful stimuli and an imbalance between antioxidative and oxidative systems in the body develops, thus predisposing the body towards oxidation (3). Oxidative stress results in the infiltration of neutrophils, increased secretion of proteases and the production of a large number of oxide intermediates, including reactive nitrogen species (RNS) and reactive oxygen species (ROS) (6). Furthermore, excessive levels of oxidation intermediates have been reported to cause tissue damage, which is considered to be an important factor leading to aging and the development of disease (7). RNS include NO, nitrogen dioxide and peroxide nitride, while ROS include the ultra-oxygen anion, oxygen free radicals and hydrogen peroxide (8).

Highly enriched in green tea, polyphenols account for ~22-30% of the dry weight of tea leaves and are primarily composed of four components: Catechin, flavonoids, phenolic acids and flower pigment composition. (-)Epigallocatechin-3-gallate (EGCG) is an antioxidant that protects the adenosine triphosphate enzyme in erythrocytes from oxidative damage (9). Previous studies have indicated that when used as a food additive, EGCG may attenuate the effects of POCD. Green tea leaves contain numerous natural

Correspondence to: Dr Li Ding or Mr. Shenghui Yu, Department of Anesthesiology, The People's Hospital of Yinzhou, 251 Baizhang East Road, Ningbo, Zhejiang 315040, P.R. China
E-mail: difangzhui53939@163.com
E-mail: ylpace4340305@126.com

active compounds (9,10). For example, as the predominant catechin in green tea in terms of content, EGCG exhibits important physiological and pharmacological effects (11). As a result, further research is required in order to investigate the therapeutic potential of EGCG (11). Furthermore, numerous plant polyphenols with diverse bioactivities require further investigation in order to determine their therapeutic potential (11). Considering this, the present method of investigation into the effects of EGCG administration may represent a valuable reference for future research aiming to investigate the therapeutic potentials of other polyphenols. The present study investigated whether EGCG administration may attenuate anesthesia-induced memory deficit in young mice, as well the potential underlying mechanisms.

Materials and methods

Experimental animals. All experiments were performed with the approval of the Ethics Committee of Yinzhou People's Hospital (Ningbo, China). Following the purchasing at post-natal day 14, C57BL/6J mice (male; weight, 4-7 g, n=110) were purchased from Animal laboratory of Zhejiang University, housed in polypropylene cages at 22-24°C and 55-60% humidity with free access to food and water on a 12 h light/dark cycle. A total of 110 mice were used as follows: 30 in the first set of experiments followed by 40 (n=10 each for control, model, EGCG treatment and EGCG + L-arginine treatment) and 40 (n=10 each for control, model, EGCG treatment and EGCG + 7-nitroindazole treatment) in the second set of experiments.

A total of 30 mice were randomly divided into three groups (n=10 per group): Control group, anesthesia model group and EGCG treatment group. In the anesthesia model and EGCG treatment groups, mice were administered 2% sevoflurane delivered via humidified 30% O_2 carrier gas for 5 h. Following this, mice belonging to the EGCG treatment group were intra-gastrically administered EGCG (2 mg/kg/day, Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for a total of 4 weeks. The chemical structure of EGCG is presented in Fig. 1A.

Following this, a total of 40 mice were randomly divided into four groups (n=10 per group): Control group, anesthesia model group, EGCG treatment group (2 mg/kg/day of EGCG) and EGCG + L-arginine (Sigma-Aldrich; Merck KGaA) group (50 mg/kg once a week administered intragastrically; L-arginine for a total of 4 weeks).

Following this, a total of 40 mice were randomly divided into four groups (n=10 per group): Control group, anesthesia model group, EGCG treatment group (2 mg/kg/day of EGCG) and EGCG + L-arginine (Sigma-Aldrich; Merck KGaA) or 7-nitroindazole (Sigma-Aldrich; Merck KGaA) group (50 mg/kg once a week administered intragastrically; 7-nitro-indazole administered for a total of 4 weeks). The chemical structures of 7-nitroindazole and L-arginine are presented in Fig. 1B and C, respectively.

Morris water maze. A round pool (90x50 cm) was filled with warm water (25-27°C) and painted black in order to appear opaque. An escape platform (10 cm diameter) was fixed 0.5 cm below the water line in one of four quadrants of the round pool. Mice (n=3/group) were placed in a fixed position of one quadrant facing the wall of the pool. Mice were allowed 120 sec to swim and discover the hidden platform in order to escape. Mice that discovered the hidden platform were permitted to remain on the platform for a further 30 sec. The duration of the escape latencies (finding the hidden platform) was recorded for each mouse and the experiment was repeated three times for each mouse. The mean time was considered to represent the result for any given mouse for that day. Following this, mice were wiped dry before being returned to their cages. On the fifth day of experimentation, the platform was removed and mice were permitted to swim in the pool for a 1 min time period. The number of times mice crossed the original platform site and the time spent in the target quadrant were recorded.

ELISA. Brain tissue was homogenized using a radioimmu-noprecipitation assay (Beyotime Institute of Biotechnology, Haimen, China) and protein concentrations of the homogenates were determined using a bicinchoninic assay (Beyotime Institute of Biotechnology). A total of 5 μg protein samples were analyzed for the detection of oxidative stress via determination of malondialdehyde (MDA; cat. no. A003-1), superoxide dismutase (SOD; cat. no. A001-1-1) and glutathione (GSH; cat. no. A006-2) expression levels, while caspase-3/9 activities (cat. nos. G015/G018) were analyzed to determine apoptosis and acetylcholinesterase (AChE; cat. no. A024) activity was also measured, using ELISA kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Western blot analysis. Brain tissue was homogenized using a radioimmunoprecipitation assay. Total protein was determined using a bicinchoninic assay. A total of 50 μg protein was subjected to 8-12% SDS-PAGE analysis and transferred to nitrocellulose membranes. Membranes were blocked using 5% skimmed milk powder in TBS with 0.1% Tween-20 for 1 h at room temperature and then incubated at 4°C overnight with the following primary antibodies: nNOS (cat. no. c-17825, 1:1,000, Santa Cruz Biotechnology, Inc., Dallas, TX, USA), β-amyloid (Aβ; cat. no. 8243, 1:2,000, Cell Signaling Technology, Inc.), amyloid precursor protein (APP; cat. no. sc-374527, 1:5,000, Santa Cruz Biotechnology, Inc.) and GAPDH (cat. no. sc-51631, 1:5,000, Santa Cruz Biotechnology, Inc.). Following this, membranes were incubated at 37°C for 1 h with corresponding horseradish peroxidase-conjugated secondary antibodies (cat. no. sc-2004, 1:2,000; Santa Cruz Biotechnology, Inc.). Membranes were visualized using the ECL Plus western blotting detection system (Beyotime Institute of Biotechnology), developed on a C-DiGit Blot Scanner (LI-COR Biosciences, Lincoln, NE, USA) and quantified using Image Lab 3.0 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. Data are presented as the mean ± standard error (n=3) and were analyzed by one-way analysis of variance followed by Tukey's post-hoc tests using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

EGCG administration attenuates memory deficit in anesthesia-induced mice. After EGCG treatment, mice from each group were subjected to Morris water maze analysis in
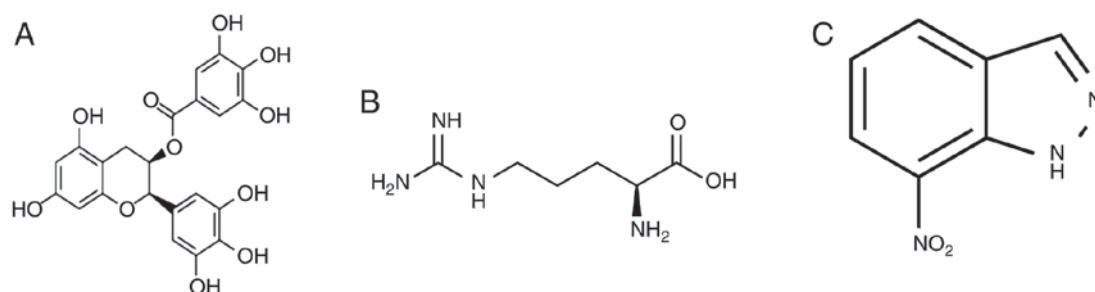
Figure 1. Chemical structure of EGCG and a neuronal nitric oxide synthase inhibitor and substrate. Chemical structures are presented for (A) EGCG, (B) 7-nitroindazole and (C) L-arginine. EGCG, (-)epigallocatechin-3-gallate.
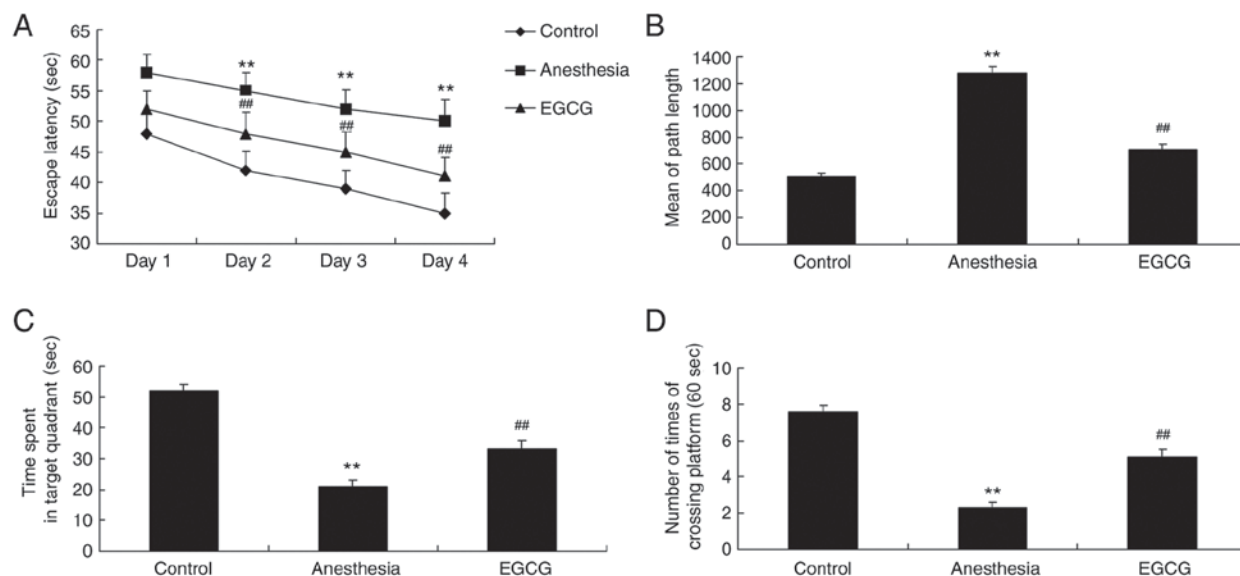

Figure 2. Effects of EGCG on memory deficit in anesthesia-induced mice. The effects of EGCG on the duration of escape latency. Results are presented as (A) time of escape latency, (B) mean path length, (C) duration of time spent in target quadrant and (D) number of times crossing platform. **$P < 0.01$ vs. control group; ##$P < 0.01$ vs. anesthesia model group. EGCG, (-)epigallocatechin-3-gallate.

order to investigate the effects of EGCG administration. As revealed in Fig. 2A and B, the duration of escape latency and the mean path length (on day 5) exhibited by the anesthesia model group were significantly increased compared with the control group. In addition, the duration of time spent in the target quadrant and the number of times crossing the platform exhibited by the anesthesia model group were significantly decreased compared with the control group (Fig. 2C and D). However, treatment with EGCG was demonstrated to significantly decrease the escape latency duration and the mean path length, and significantly increase the time spent in the target quadrant and the number of times crossing the platform, compared with the anesthesia model group (Fig. 2).

EGCG administration attenuates oxidative stress in anesthesia-induced mice. In order to investigate the effect of EGCG administration on oxidative stress levels in mice, samples were subjected to ELISA analysis in order to determine the expression levels of MDA, SOD and GSH. The results demonstrated that there was a significant increase in the expression of MDA, and a significant suppression of SOD and GSH expression, in the anesthesia model group compared with the control group (Fig. 3). However, administration of EGCG

was revealed to significantly suppress the expression of MDA, and increase the expression of SOD and GSH, compared with the anesthesia model group (Fig. 3).

EGCG administration attenuates apoptosis in anesthesia-induced mice. The effects of EGCG on apoptosis in anesthesia-induced mice were investigated by ELISA. It was demonstrated that caspase-3 and caspase-9 activities were significantly enhanced in the anesthesia model group compared with the control group (Fig. 4). However, treatment with EGCG significantly suppressed both caspase-3 and caspase-9 activity compared with the anesthesia model group (Fig. 4).

EGCG administration suppresses nNOS, Aβ and APP expression and AChE activity in anesthesia-induced mice. In order to investigate the biological alterations underlying cognitive impairment induced by anesthesia, nNOS, Aβ and APP expression, as well as AChE activity, were analyzed by western blotting and ELISA, respectively. Notably, in the anesthesia model group, nNOS, Aβ and APP protein expression levels, as well as AChE activity, were enhanced compared with the control group (Fig. 5). However, EGCG administration significantly suppressed nNOS, Aβ and APP protein expression
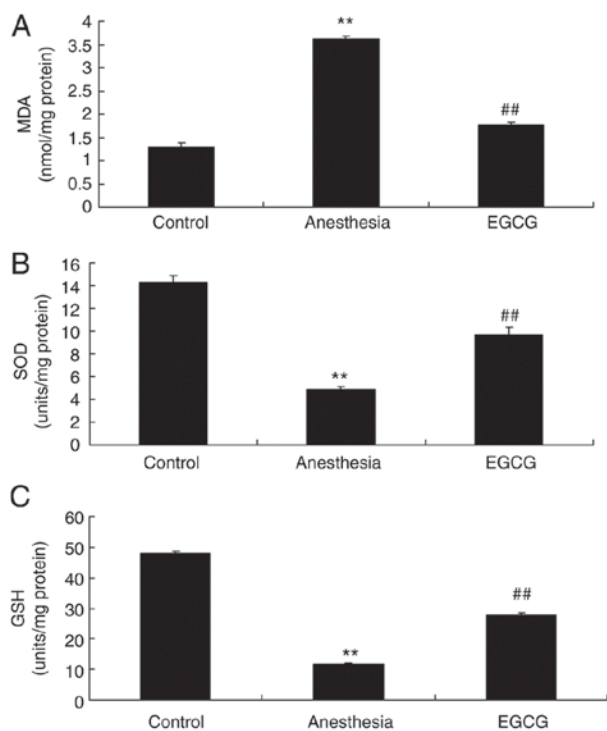
Figure 3. Effects of EGCG on oxidative stress in anesthesia-induced mice. Effects of EGCG on (A) MDA, (B) SOD and (C) GSH levels. **P<0.01 vs. control group; ##P<0.01 vs. anesthesia model group. EGCG, (-)epigallocatechin-3-gallate; MDA, malondialdehyde; SOD, superoxide dismutase; GSH, glutathione.
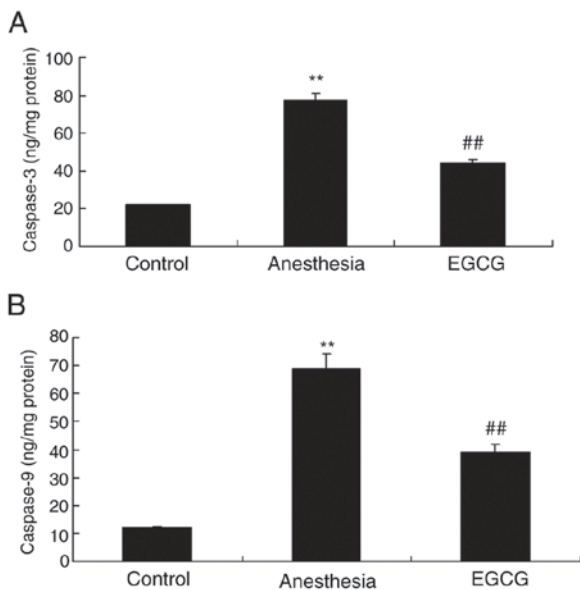

Figure 4. Effects of EGCG on apoptosis in anesthesia-induced mice. Effects of EGCG administration on (A) casapse-3 and (B) caspase-9 activity. **P<0.01 vs. control group; ##P<0.01 vs. anesthesia-induced model. EGCG, (-)epigallocatechin-3-gallate.

levels, as well as AChE activity, compared with the anesthesia model group (Fig. 5).

L-arginine administration reverses the protective effects of EGCG on memory deficit in anesthesia-induced mice. The role of nNOS expression in ECGC-induced attenuation of memory deficit was investigated via administration of L-arginine (an nNOS substrate) alongside EGCG. L-arginine was revealed to significantly enhance nNOS protein expression in anesthesia-induced mice following EGCG treatment, compared with the EGCG-only treatment group (Fig. 6A and B). Furthermore, following treatment with L-arginine, the effects of EGCG treatment on the results of the Morris water maze experiment for the investigation of memory deficit in anesthesia-induced mice were significantly attenuated (Fig. 6C-F).

Inhibition of nNOS with 7-nitroindazole enhances the effects of EGCG administration with regards to memory deficit. The effect of the inhibition of nNOS on memory deficit exhibited by anesthesia-induced mice treated with EGCG was also investigated. 7-nitroindazole was employed as an nNOS inhibitor, and the results of western blotting demonstrated that 7-nitroindazole significantly inhibited nNOS protein expression in anesthesia-induced mice treated with EGCG (Fig. 7A and B). However, the inhibition of nNOS via administration of 7-nitroindiazole significantly enhanced the effects of EGCG treatment with regards to memory deficit in anesthesia-induced mice, as measured by the Morris water maze tests (Fig. 7C-F).

Discussion

The elderly population represents a high-risk group for the development of POCD following clinical anesthesia (12). As a result, ketamine increases the incidence of POCD in elderly people, thus limiting the clinical application of ketamine for anesthetic purposes with regards to elderly patients (12). Sevoflurane is a frequently used intravenous anesthetic in clinical practice. Previous studies have also demonstrated that a combinatory administration of propofol and ketamine reduces the required dosage of ketamine as well as the associated adverse side effects, such as mental and cardiovascular excitation (13,14). In the present study, it was demonstrated that EGCG administration attenuated memory deficit in anesthesia-induced mice. He *et al* (15) revealed that EGCG administration attenuated acrylamide-induced apoptosis and astrogliosis in the cerebral cortex of rats (12). These results indicate that EGCG may represent a novel therapeutic agent for the treatment of anesthesia-induced memory deficit; however, determining the exact molecular mechanism underlying this process requires further investigation.

Sevoflurane has previously been demonstrated to attenuate cell apoptosis mediated by tumor necrosis factor through the suppression of caspase-3 activity and inhibition of apoptosis via regulation of apoptosis-associated protein expression, such as affecting the Bcl-2/Bcl-2-associated X protein ratio (16). The caspase family refers to a group of regulator genes closely associated with apoptosis, and caspase-3 represents an important apoptotic protein responsible for the induction and execution of cell apoptosis in mammals (17). Hippocampal neurons are the most responsive to total cerebral ischemia and thus are the most susceptible to apoptotic induction (18). The present study revealed that treatment with EGCG significantly suppressed caspase-3 and caspase-9 activity in anesthesia-induced mice.
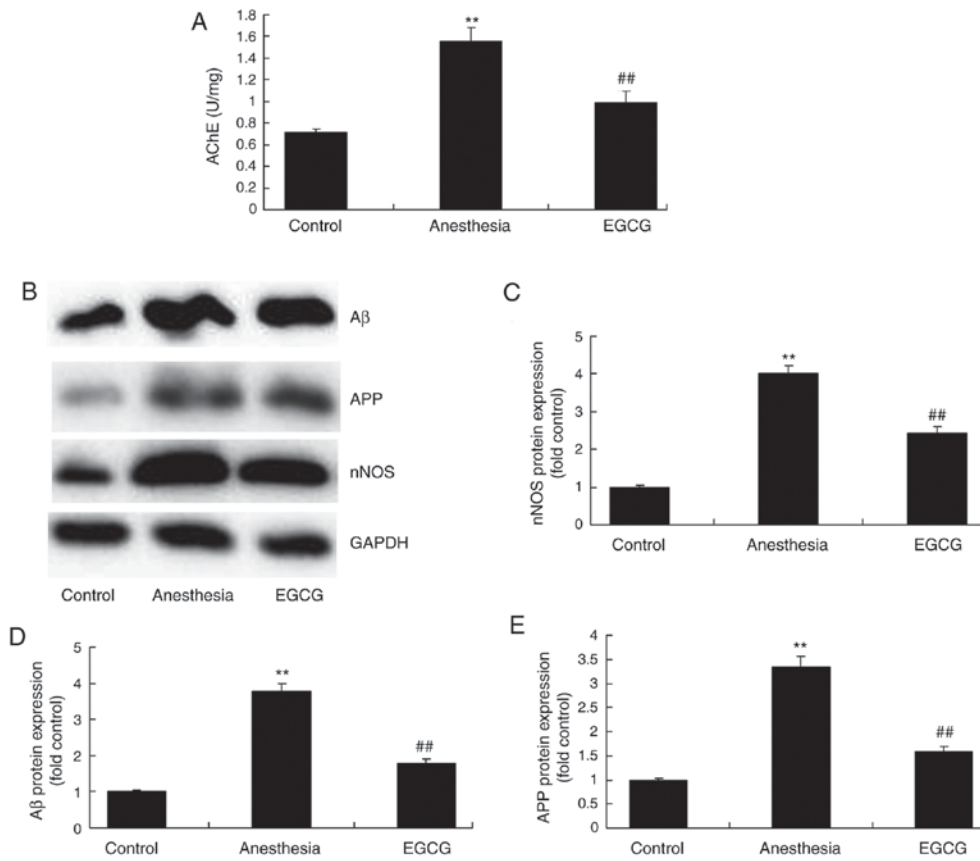
Figure 5. Effects of EGCG administration on the activity of AChE, and nNOS, Aβ and APP protein expression, in anesthesia-induced mice. (A) ELISA was performed to determine the effect of EGCG administration on AChE activity in anesthesia-induced mice. (B) Representative protein bands for nNOS, Aβ and APP protein expression by western blotting analysis. Densitometric analysis of western blotting results was performed to quantify and statistically analyze the protein expression of (C) nNOS, (D) Aβ and (E) APP. **$P<0.01$ vs. control group; ##$P<0.01$ vs. anesthesia model group. EGCG, (-)epigallocatechin-3-gallate; AChE, acetylcholinesterase; nNOS, nitric oxide synthase; Aβ, β-amyloid; APP, amyloid precursor protein.
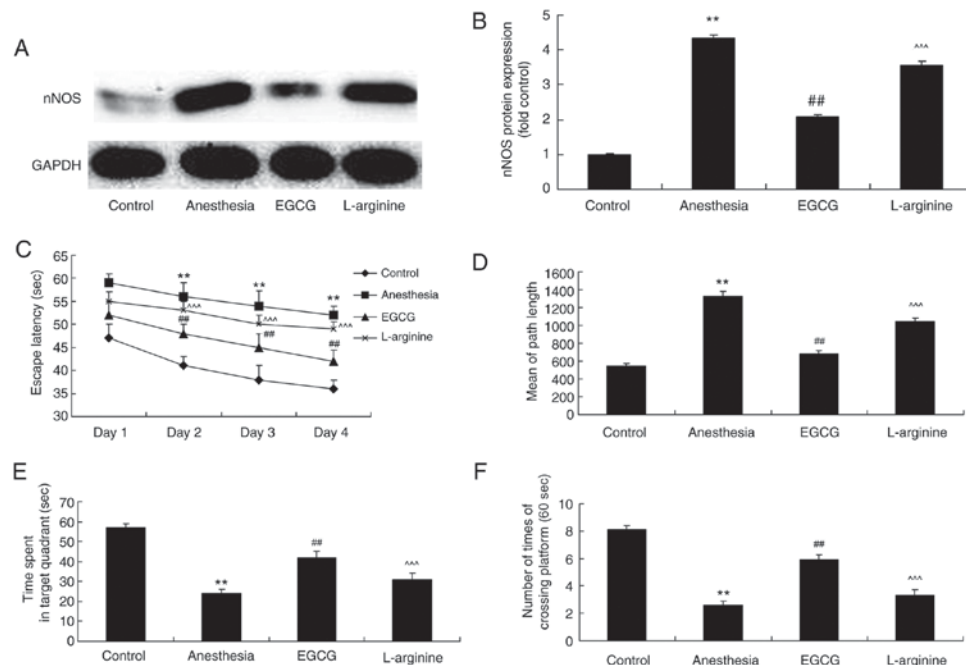

Figure 6. nNOS substrate influence on the effects of EGCG administration on memory deficit. (A) Effect of L-arginine, an nNOS substrate, on nNOS protein expression in EGCG-treated anesthetized mice was revealed by western blotting analysis. (B) Densitometric analysis was performed to quantify and statistically analyze nNOS protein expression. A Morris water maze was employed to determine the effects of L-arginine on (C) time of escape latency, (D) mean path length, (E) time spent in target quadrant and (F) number of times crossing platform in EGCG-treated anesthetized mice. **$P<0.01$ vs. control group; ##$P<0.01$ vs. anesthesia-induced model; ^^^$P<0.01$ vs. EGCG-treated anesthetized mice. nNOS, nitric oxide synthase; EGCG, (-)epigallocatechin-3-gallate.
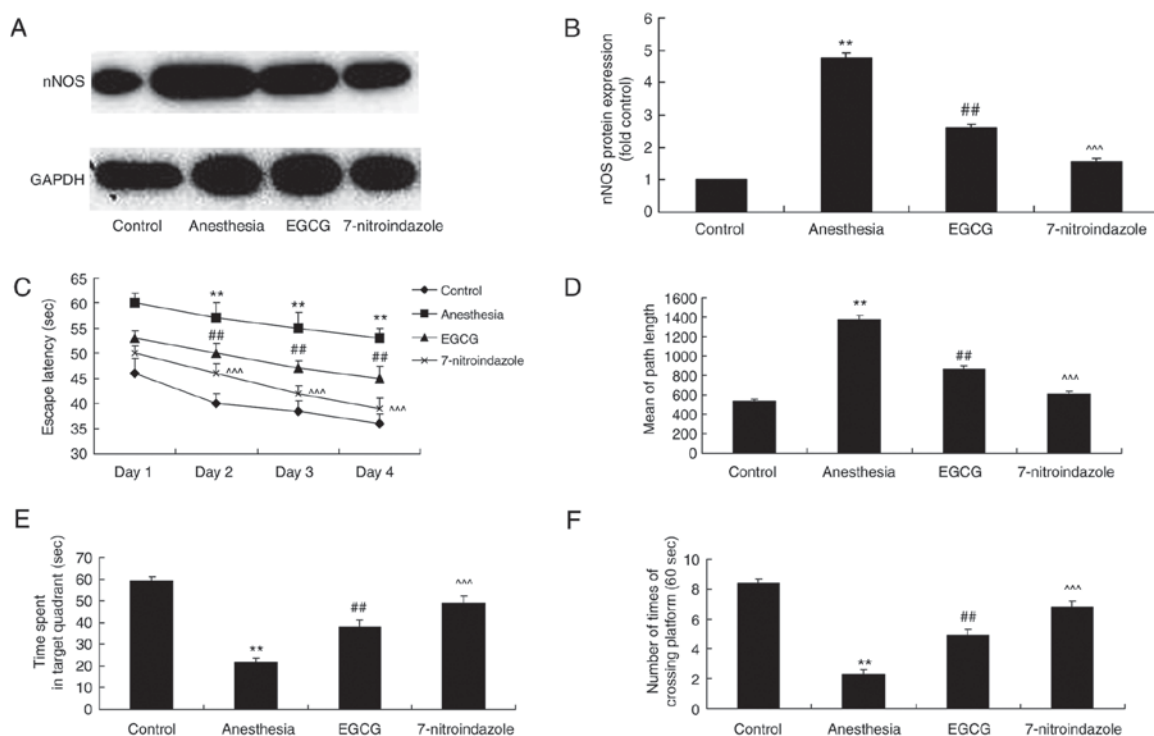
Figure 7. nNOS inhibitor influence on the effects of EGCG administration on memory deficit. (A) Effect of 7-nitroindazole, an nNOS inhibitor, on nNOS protein expression in EGCG-treated anesthetized mice was revealed by western blotting analysis. (B) Densitometric analysis was performed to quantify and statistically analyze nNOS protein expression. A Morris water maze was employed to determine the effects of 7-nitroindazole on (C) time of escape latency (D) mean path length, (E) time spent in target quadrant and (F) number of times crossing platform in EGCG-treated anesthetized mice. **P<0.01 vs. control group; ##P<0.01 vs. anesthesia-induced model; ^^^P<0.01 vs. EGCG-treated anesthetized mice. nNOS, nitric oxide synthase; EGCG, (-)epigallocatechin-3-gallate.

Previous studies have demonstrated that isoflurane administration affects the formation and degradation process of APP, which may lead to alterations in the secondary structure of Aβ (19,20). Following this, Aβ aggregates form oligomers and eventually plaque formation surrounding brain neurons (19). Furthermore, Aβ sediments were reported to trigger immune inflammatory responses and activate neurotoxic pathways, resulting in the degeneration and induction of apoptosis in neurons (20). In addition, Aβ polypeptides inhibit the release of endogenous acetylcholine (19). Based on the aforementioned factors, it may be hypothesized that the use of isoflurane in anesthesia may damage hippocampal cholinergic neurons, inhibit the release of acetylcholine in the hippocampus and induce the development of POCD (21). The present study demonstrated that EGCG treatment significantly inhibited the activity of AChE and the protein expression of Aβ and APP in mice exposed to anesthesia. In the present study, the regulation of AChE activity by EGCG, as well as the modulation of Aβ and APP expression, was a potentially important mechanism for the attenuation of anesthesia-induced memory deficit in mice. Furthermore, we will investigate the underlying regulatory mechanisms of EGCG administration in anesthesia-induced mice with regards to AChE activity, as well as Aβ and APP expression, in a future study.

Oxidative stress is a predominant factor involved in the aging process. In 1956, a study demonstrated that free radicals were implicated in the aging process of the body (22). Numerous studies have since demonstrated that free radical damage represents part of the aging process, and oxidative stress

causes damage to cells and may induce numerous diseases, including brain damage, arteriosclerosis, cognitive dysfunction and dementia (3). As oxidative stress has been reported to accelerate neurodegeneration in Alzheimer's disease, it may be hypothesized that oxidative stress represents part of the neural pathological process associated with POCD (23). Therefore, oxidative stress may represent a predominant source of the damage experienced by neurons and ROS may be considered an important causal factor for the development of POCD and numerous other neuropathies (23). In addition, the present study revealed that treatment with EGCG significantly suppressed MDA levels and significantly enhanced SOD and GSH levels in anesthesia-induced mice. Furthermore, He *et al* (24) demonstrated that EGCG administration inhibited acrylamide-induced oxidative stress and apoptosis in PC12 cells via the reduction of oxidative stress.

As a relatively recently identified neurotransmitter, NO administration is effective in POCD to a certain extent as it aids learning and memory processing via the regulation of synaptic transmission as well as the induction and maintenance of long-term potentiation (25). Despite this, a dual role for NO in POCD has been reported. A low concentration of NO was reported to boost nerve growth and protect against apoptosis, while a high concentration of NO exerted neurotoxic effects via the induction of mitochondrial dysfunction and the activation of apoptotic pathways (26). However, other studies have indicated that different types of NOS have different effects on POCD (27). The present study demonstrated that EGCG treatment significantly suppressed

nNOS expression in anesthesia-induced mice. In addition, the present study also demonstrated that treatment with L-arginine, an nNOS substrate, and 7-nitroindazole, an nNOS inhibitor, inhibited and potentiated the effects of EGCG, respectively, on anesthesia-induced memory deficit in mice. Furthermore, Wei et al (28) revealed that EGCG attenuated NADPH-diaphorase/nNOS expression in the motor neurons of rats. In addition, the present study demonstrated that EGCG administration modulated nNOS levels in brain tissues and attenuated memory deficit in anesthesia-induced mice. However, the underlying therapeutic mechanisms of EGCG administration on nNOS levels have not yet been determined and require further investigation.

In conclusion, the results of the present study demonstrated that EGCG administration attenuated memory deficit, and suppressed apoptosis and oxidative stress, in anesthesia-induced mice. Furthermore, it was revealed that the mechanism underlying this process may involve the regulation of nNOS expression. Thus, the protective effects of EGCG, functioning as a memory deficit suppressor during anesthesia, may represent a novel therapeutic agent for the treatment of POCD.

Availability of data and materials

The analyzed data sets generated during the present study are available from the corresponding author on reasonable request.

Authors' contributions

SY designed the study; LD, XG and JH performed the experiments; SY analyzed the data; LD, XG and JH wrote the manuscript.

Ethics approval and consent to participate

All experiments were performed with the approval of the Ethics Committee of Yinzhou People's Hospital (Ningbo, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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