



# Edaravone at high concentrations attenuates cognitive dysfunctions induced by abdominal surgery under general anesthesia in aged mice

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## Abstract

Postoperative cognitive dysfunction (POCD) is a common neurological disease affecting the elderly patients after surgery. Unfortunately, no effective treatment for this disease has been discovered. Edaravone, a clinical-used free radical scavenger, at 3 mg/kg has been reported to prevent neuroinflammation induced by the combination of surgery and lipopolysaccharide in adult rodents. However, we found that edaravone at such low concentration could not inhibit POCD in aged mice. Instead, edaravone at 33.2 mg/kg significantly prevented recognition and spatial cognitive dysfunctions in 14 month aged mice after abdominal surgery under general anesthesia with isoflurane. Furthermore, edaravone significantly prevented the increase of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ) and interleukin-6 (IL-6) induced by abdominal surgery in aged mice. Edaravone could also decrease glial fibrillary acidic protein (GFAP) and ionized calcium binding adaptor molecule-1 (Iba-1) positive areas in the hippocampal regions of surgery mice, suggesting that edaravone might inhibit surgery-induced over-activation of microglia and astrocytes. Moreover, edaravone substantially increased the expression of PSD-95 and pSer9-glycogen synthase kinase-3 $\beta$  (pSer9-GSK3 $\beta$ ) as demonstrated by Western blotting assay. Furthermore, the activity of acetylcholinesterase (AChE) is decreased in the mice in edaravone group. All these results suggested that edaravone at high concentrations could inhibit surgery-induced cognitive impairments in aged animals, possibly via the attenuation of neuroinflammation, the increase of synaptic proteins, and the elevation of cholinergic transmission, providing a further support that edaravone might be developed as a treatment of POCD.

**Keywords** Edaravone · Post-operative cognitive dysfunction · GFAP · Iba-1 · PSD-95 · GSK3 $\beta$

## Abbreviations

AChE	Acetylcholinesterase
ANOVA	Analysis of variance
DAPI	4',6-diamidino-2-phenylindole
ELISA	Enzyme linked immunosorbent assay
GFAP	Glial fibrillary acidic protein
GSK3 $\beta$	Glycogen synthase kinase-3 $\beta$

Iba-1	Ionized calcium binding adaptor molecule-1
IHC	Immunohistochemical
IL-1 $\beta$	Interleukin-1 $\beta$
IL-6	Interleukin-6
LPS	Lipopolysaccharide
OD	Optical density
PBS	Phosphate buffer solution
POCD	Post-operative cognitive dysfunction
SD	Standard deviation
TNF- $\alpha$	Tumor necrosis factor- $\alpha$

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## Introduction

Postoperative cognitive dysfunction (POCD) is a common neurological disease in elderly patients after surgery, with an incidence reaching about 25% in Western counties (Ballard

et al. 2012). POCD largely prolonged hospitalization, impaired postoperative quality of life, and increased mortality and costs in elderly patients. Therefore, it is urgent to look for the effective treatments for this disease (Bilotta et al. 2013; Moller et al. 1998; Vlisides and Mashour 2016). Mounting evidence suggests that neuroinflammation, oxidative stress, and the down-regulation of cholinergic transmission play important roles at the onset of POCD (Zhao et al. 2017). It is suggested that the combination of anesthesia and surgery could lead to excessive neuroinflammation, further exacerbating cognitive impairments (Rundshagen 2014). High levels of pro-inflammatory cytokines significantly contribute to surgery-induced cognitive deficits (Cao et al. 2010). Moreover, during inflammatory process, glycogen synthase kinase-3 $\beta$  (GSK3 $\beta$ ), a key signaling transduction molecule, could be regulated via activating astrocytes and microglia (Jope et al. 2007). Phosphorylated GSK3 $\beta$  at serine 9 site (pSer9-GSK3 $\beta$ ) could decrease the activity of GSK3 $\beta$ , and decrease the expression of pro-inflammatory cytokines. Moreover, increased level of oxidative stress, especially in the hippocampal region, may be involved in the pathogenesis of POCD (An et al. 2013). In addition, in the post-transcriptional phase, acetylcholine could attenuate the production of pro-inflammatory cytokines, such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ) and interleukin-6 (IL-6) (Kalb et al. 2013; Rosas-Ballina and Tracey 2009). Acetylcholinesterase (AChE), a key enzyme responsible for the breakdown of acetylcholine in the synapses, could lead to the reduction of synaptic transmission in the brain. Therefore, drugs, which could inhibit neuroinflammation, AChE and scavenge oxygen free radicals, might possess neuroprotective effects against POCD (Yatin et al. 2000).

Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one) is a powerful free radical scavenger (Kikuchi et al. 2013). Edaravone is clinically utilized to treat ischemia stroke (Dohare et al. 2014). Edaravone at a concentration of 3 mg/kg could reduce the combination of surgery and lipopolysaccharide (LPS)-induced neuroinflammation in 2 month old rats, and prevent hippocampal neuronal apoptosis induced by abdominal surgery under local bupivacaine anesthesia in 14 month old mice, indicating that edaravone might have benefits for the treatment of POCD (Tian et al. 2017). However, in our preliminary study, we found that edaravone at 3 mg/kg could not inhibit cognitive impairments induced by abdominal surgery under general isoflurane anesthesia in aged mice (unpublished data). It is also published that edaravone at around 40 mg/kg could significantly attenuate cognitive deficits in 12 month old APP/PS1 mice, and ameliorate autoimmune thyroiditis in rats (Jiao et al. 2015; Li et al. 2018). Therefore, we speculated that the higher concentrations of edaravone might be required in our POCD model.

In this study, we explored whether edaravone at high concentrations (8.3–33.2 mg/kg) is effective in attenuating surgery-induced cognitive impairments in our model. Moreover, we investigated how edaravone attenuated cognitive impairments, particularly focusing on the inhibition of neuroinflammation and the prevention of cholinergic transmission impairments.

## Material and methods

### Chemicals and reagents

Isoflurane was supplied by RWD Life Science Co., Ltd. (Shenzhen, China). Edaravone was obtained from Shanghai Aladdin Biochemical Technology Co., Ltd. (China).

### Animal experiments

14 month-year-old male ICR mice weighted about 30–40 g were supplied by Zhejiang Academy of Medical Sciences (Hangzhou, China). The environment is 12 h light/dark cycle under controlled humidity ( $50 \pm 10\%$ ) and temperature ( $22 \pm 2$  °C). Sufficient animal food (Shanghai Slac Laboratory Animal Co. LTD, Shanghai, China) and water were given to mice. Every process was executed according to the regulation proposed by the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23, revised 1996) and certified by the Animal Care and Use Committee of Ningbo University.

Seventy-five mice were randomly divided into five groups with each group of 15 mice: control (Con), surgery plus saline, surgery plus low edaravone (8.3 mg/kg), medium edaravone (16.6 mg/kg), and high edaravone (33.2 mg/kg). Mice in the control group were not received surgery. Mice in the surgery + saline group were administered with normal saline (solvent of edaravone). Drugs or saline were administered once daily for 16 days consecutively by *i.p.* injection.

### Anesthesia and surgical procedure

The mice were anesthetized in a chamber (RWD Life Science Co., Ltd) prefilled with 1.5% isoflurane. The anesthetic effects were determined by the blink reflexes. All operations were carried out using a standard process (Chen et al. 2018). Firstly, the fur in the surgical site of the mice was scratched. Abdominal exploration was executed using a 1.5 cm median incision. The small intestine (5 cm) was taken away from the peritoneal cavity, laid over with clean and wet gauze. The small intestine was taken away from the peritoneal cavity for 5 min, and the sutures were used to close the abdominal wall. All the process lasted about 10 min.

## Novel object recognition test

The novel object recognition test was carried out in an open-field box (30 × 30 × 30 cm), which is made of plywood, acrylic and polyvinyl chloride (Bevins and Besheer 2006). The test is consisted of adaptation, training, and examination 3 days in a row. On the first day, the mice were adapted to the experimental site for 5 min without external disturbance. On the second day, the mice explored two same objects (black square stone, 5 × 5 × 5 cm) for 5 min. On the third day, a new objects with different shape and color replace one of the objects (gray triangular pyramid, 5 × 5 × 7 cm), and the mice were allowed to explore the area for 5 min once again. The box was cleaned with 5% ethanol solution, and dried up with cloth after the test. The mice explored the objects by touching or sniffing with their nose and/or within 2 cm around. Sitting on the objects was not counted in exploration time. The exploratory behavior was observed by a video camera. Then the videos were analyzed by an observer who is blinded to all the procedure. The sum of the exploration time of two objects is the total exploration time. The recognition index was used to measure the cognitive function, which is the ratio of the exploration time of one of the two objects (training session) or the exploration time of the novel object (examination session) to the total time.

## Morris water maze

The Morris water maze was used to test spatial memory as mentioned previously (Chen et al. 2017). The water maze is a circular pool with a diameter of 110 cm filled with water at 23 ± 2 °C to overpass the platform. The platform was placed in the middle of the northwest quadrant except the testing day. A video camera linked to a computer-based image system was used to record swimming track. The test of learning was performed for four consecutive days. During the four-day training period, each mouse was trained to recognize and locate the platform. The time spent looking for a platform hidden under the water was measured as the escape latency. On the testing day, the platform was removed to perform a probe trial, and let the mice to swim 90 s to locate it. The swim speed was automatically measured from the swimming track by the software of Morris water maze (Duoyi, Shanghai, China). Swimming time in the four quadrants of the water maze was separately evaluated from the swimming track by the software of Morris water maze. The percentage of the time spend in target quadrant was determined as the proportion of the time swimming in the quadrant where the platform placed previously (northwest quadrant) within 90 s.

## Brain tissue harvest

Mice were anesthetized and performed cardiac perfusion with ice-cold saline. Brains were collected in a short time. Proteins

in the hippocampus were exacted for Western blotting analysis (3 mice per group) and enzyme linked immunosorbent assay (ELISA, 4 mice per group). The proteins were stored at –80 °C before use. Other brain samples were used for the measurements of acetylcholinesterase (AChE) activity (5 mice per group) or the performance of immunohistochemical (IHC) staining (3 mice per group).

## Western blotting analysis

Western blotting analysis was performed as described previously (Cui et al. 2014). Firstly, brain tissue in the hippocampal region was extracted at 4 °C for 1 min by using a lysis buffer, and centrifuged at 16000 rpm for 10 min. The protein levels in the supernatant were assessed by Bradford assay, followed by SDS-PAGE of tissue samples (40 µg), and transfer to polyvinylidene fluoride membrane. The membranes were blocked with 5% non-fat milk in TBST for 2 h, and incubated overnight at 4 °C with primary antibodies against, PSD-95, GSK3β, pSer9-GSK3β and β-actin (Cell Signaling Technology, Beverly, USA). After washing the samples three times with TBST, the membranes were incubated with a secondary antibody. Blots were developed using enhanced chemiluminescence as instructed by the manufacturer (Amersham Bioscience, Aylesbury, UK). All data were representative of three independent experiments. Data were expressed as ratios of optical density (OD) compared with controls for statistical analyses.

## ELISA assay

The levels of TNF-α, IL-1β and IL-6 in the hippocampal region of mice were evaluated from freshly prepared hippocampal tissues. Brain samples in the hippocampal region were homogenized in 0.1 M phosphate buffer solution (PBS). After sonication, the homogenizations were centrifuged at 2000 rpm for 15 min at 4 °C. The levels of TNF-α, IL-1β and IL-6 were determined by ELISA kits (Excell bio, Shanghai, China) according to the manufacturer's protocol. The microplate reader was used to measure the absorbance.

## IHC staining

Brains were removed and incubated with 4% paraformaldehyde for a day. After washing with 0.1 M PBS, the brain tissues were dehydrated in 30% glucose solution for 2 days until sinking to the bottom. Then the samples were cut into 20 µm sections by a freezing microtome (CM1950, Leica, Buffalo Grove, IL, USA). The sections were transferred into multi-well plate containing 1–2 mL PBS and incubated with 1:100 diluted primary GFAP (Abcam) or Iba-1 (Abcam) antibodies at 4 °C overnight. The specimens were washed and incubated with fluorescence secondary antibodies (Solarbio, Beijing, China)

for 60 min in dark. The sections were labeled with 4',6-diamidino-2-phenylindole (DAPI), and then observed by a fluorescence microscope. ImageJ was used to enhance the contrast of the image, applying a threshold to distinguish positively stained features from the background, and then applying a size exclusion threshold to identify and distinguish GFAP and Iba-1 positive areas. The protocol was imitated according to a previous publication (Chen et al. 2018).

### Measurement of AChE activity

The measurement of AChE activity was performed according to a previous study (Huang et al. 2016). Briefly, brains of mice were used as the source of AChE. Weighing the brain and adding 10 times volume of lysis buffer [10 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid, pH 7.5, 1 mM ethylene diamine tetra-acetic acid, 1 mM ethylene glycol-bis(2-aminoethyl ether)-N,N,N',N'-tetra-acetic acid, 150 mM NaCl and 0.5% Triton X-100]. The homogenization was performed on ice for 15 min. The homogenates were extracted by centrifugation for 15 min at 3000 rpm at 4 °C. The assay medium contained 0.1 M Na<sub>2</sub>HPO<sub>4</sub> (pH 7.5), 10 mM 5,5'-dithiobis-2-nitrobenzoic acid and 1 mM acetylthiocholine iodide. The brain lysate was incubated with 0.1 mM ethopropazine hydrochloride for 5 min to inhibit butyrylcholinesterase activity. The microplate reader was used to read the absorbance of the plates to determine the activity after incubation at 37 °C for 30 min.

### Data analysis and statistics

Data were expressed as means ± standard deviation (SD). One-way analysis of variance (ANOVA) was used to determine statistical significance, and Tukey's test was used for post hoc multiple comparison.  $P < 0.05$  was considered as statistical significance.

## Results

The schedule of surgery and arrangement of animal experiments is shown in Fig. 1. Edaravone was *i.p.* injected daily for 16 consecutive days. During the day 7–15 post-surgery, the effects of edaravone on cognition were tested by behavioral analysis.

### Edaravone at high concentrations significantly attenuates cognitive dysfunctions induced by abdominal surgery under general anesthesia in aged mice

The effectiveness of edaravone in improving recognition in animals was evaluated by novel object recognition tests.

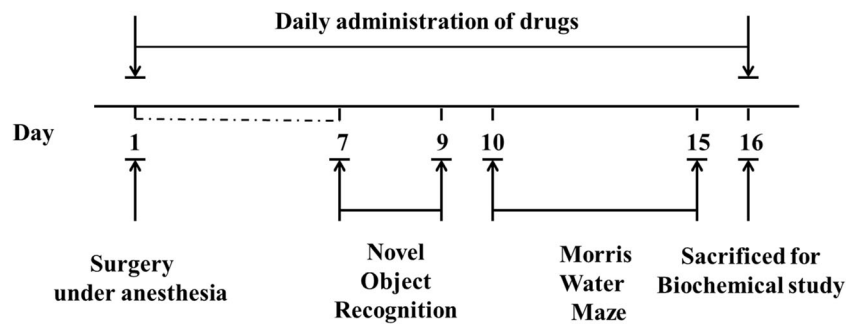
During the training session, the total exploration time and the recognition indexes in various groups were similar (one-way ANOVA and Tukey's test, for total exploration time,  $F(4, 70) = 0.307$ ,  $p > 0.05$ , Fig. 2a; for the recognition index,  $F(4, 70) = 0.755$ ,  $p > 0.05$ , Fig. 2b). And in the retention session, the total exploration time in various groups were similar (one-way ANOVA and Tukey's test,  $F(4, 70) = 0.502$ ,  $p > 0.05$ , Fig. 2c). However, the recognition index varied in different groups (one way ANOVA and Tukey's test,  $F(4, 70) = 4.654$ ,  $p < 0.01$ , Fig. 2d). Furthermore, in the retention session, the control group showed a significantly higher recognition index than the surgery + saline group (one way ANOVA and Tukey's test,  $p < 0.01$ , Fig. 2d). Edaravone at 33.2 mg/kg significantly prevented the decrease of the recognition index induced by the abdominal surgery when compared to the surgery + saline group (one way ANOVA and Tukey's test,  $p < 0.05$ , Fig. 2d). However, during the retention session, the recognition index in 8.3 mg/kg and 16.6 mg/kg edaravone groups and the surgery + saline group did not have significant difference.

### Edaravone at high concentrations significantly attenuates spatial learning and memory impairments induced by abdominal surgery under general anesthesia in aged mice

The role of edaravone in improving spatial cognition in aged mice was determined by Morris water maze tests. At the fourth day of the training session, compared with the control group (no surgery), surgery + saline group took longer time to locate the platform (one way ANOVA,  $F(4, 70) = 2.296$ ,  $p < 0.01$ , Fig. 3a, d). Edaravone at 8.3–33.2 mg/kg significantly decreased the escape latency, indicating that edaravone could prevent impairments in spatial memory induced by abdominal surgery under general anesthesia (one way ANOVA and Tukey's test,  $p < 0.05$ , Fig. 3a, d). During the probe trial, the spatial memory was tested. The swim speed in various groups was not significantly different (one way ANOVA,  $F(4, 70) = 0.505$ ,  $p > 0.05$ , Fig. 3b). Moreover, different groups have significantly different percentage of the time in target quadrant (one way ANOVA,  $F(4, 70) = 3.383$ ,  $p < 0.01$ , Fig. 3c, e). Edaravone at 33.2 mg/kg significantly reduced the percentage of the time in target quadrant, suggesting that edaravone could attenuate spatial learning and memory induced by abdominal surgery under general anesthesia in aged mice (one way ANOVA and Tukey's test,  $p < 0.05$ , Fig. 3c, e).

### Edaravone decreases the increase of TNF- $\alpha$ , IL-1 $\beta$ and IL-6 expressions induced by abdominal surgery under general anesthesia in aged mice

We further applied ELISA to evaluate the production of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in aged mice. All three parameters



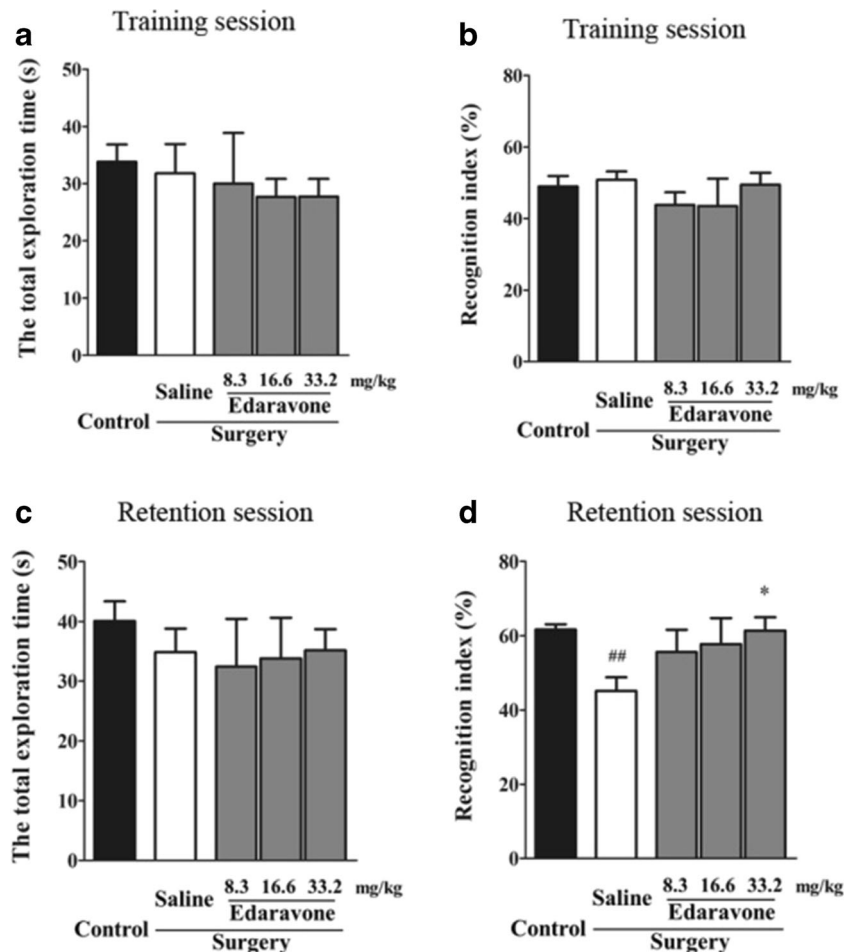
**Fig. 1** Experiment design. At day1, aged mice were anesthetized in a chamber prefilled with 1.5% isoflurane, and underwent 10 min abdominal exploration. Edaravone was further administered *i.p.* daily for 16 days. After surgery, mice were allowed to recover for 6 days.

Novel object recognition and Morris water maze analysis were used to evaluate the effects of edaravone on cognition. At day 16, animals were sacrificed for biochemical study

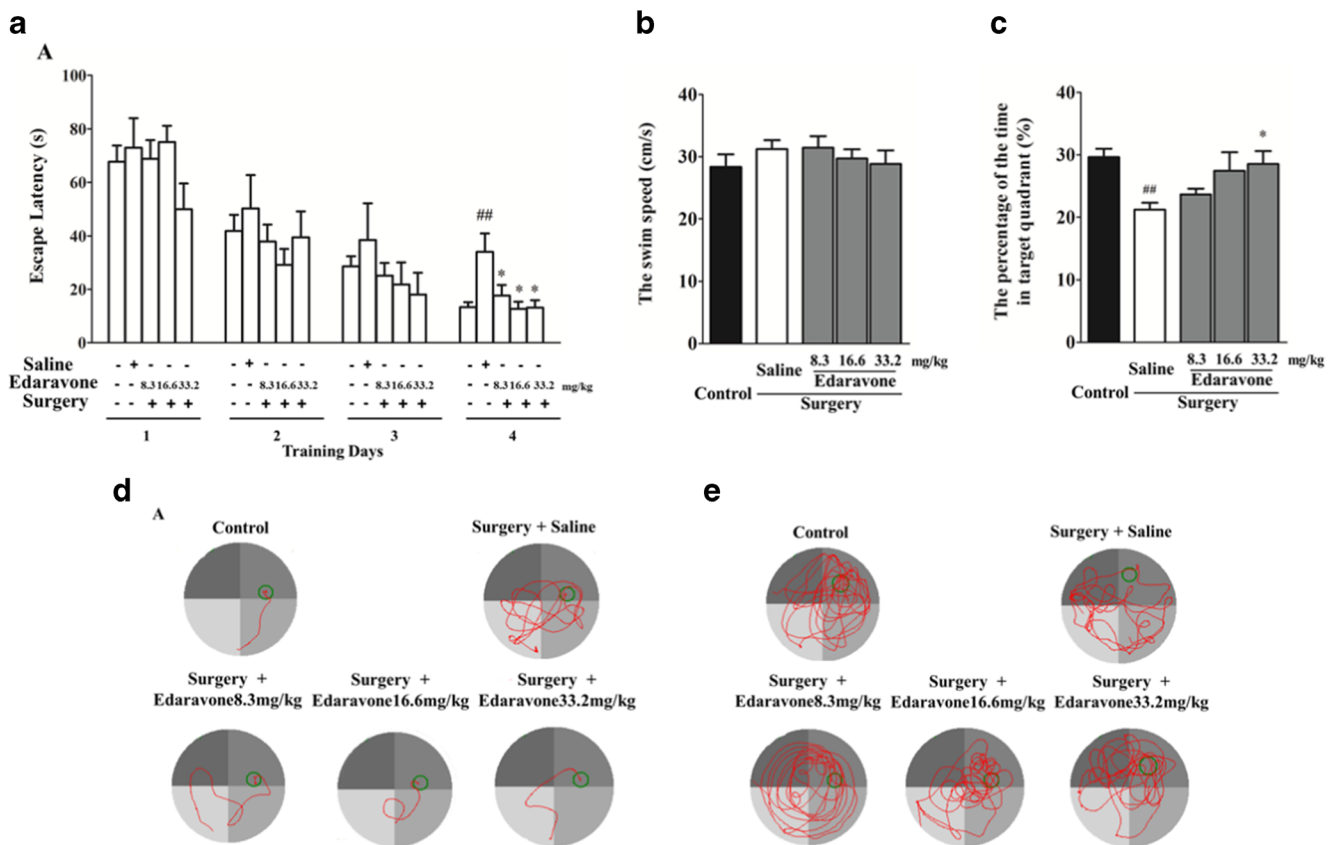
were significantly altered among groups [for TNF- $\alpha$ , one way ANOVA,  $F(4, 15) = 28.495, p < 0.01$ ; for IL-1 $\beta$ , one way ANOVA,  $F(4, 15) = 37.434, p < 0.01$ ; for IL-6, one way ANOVA,  $F(4, 15) = 46.857, p < 0.01$ , Fig. 4]. In the surgery + saline group, the levels of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 in the hippocampal region of mice were significantly higher than those in the control group without surgery (one way ANOVA and Tukey’s test,  $p < 0.01$ ,

Fig. 4). Moreover, edaravone at 8.3–33.2 mg/kg significantly decreased the production of TNF- $\alpha$  (one way ANOVA and Tukey’s test,  $p < 0.01$ , Fig. 4a). Edaravone at 16.6 mg/kg significantly decreased the production of IL-1 $\beta$  (one way ANOVA and Tukey’s test,  $p < 0.05$ , Fig. 4b). Edaravone at 33.2 mg/kg significantly decreased the production of IL-6 (one way ANOVA and Tukey’s test,  $p < 0.01$ , Fig. 4c).

**Fig. 2** Edaravone attenuates the recognition impairments induced by abdominal surgery under general anesthesia in aged mice. On days 8–9 after surgery, novel object recognition tests were performed. **a** During the training session of novel object recognition, all the groups exhibited similar total exploration time. **b** During the training session of novel object recognition, all the groups exhibited similar recognition index. **c** During the retention session of novel object recognition, all the groups exhibited similar total exploration time. **d** During the retention session of novel object recognition, edaravone at 33.2 mg/kg significantly attenuated the decrease in recognition index. Data represent the mean  $\pm$  SD ( $n = 15$ ). ##  $p < 0.01$  vs. control group without surgery, \*  $p < 0.05$  vs. surgery + saline group (one way ANOVA and Tukey’s test)







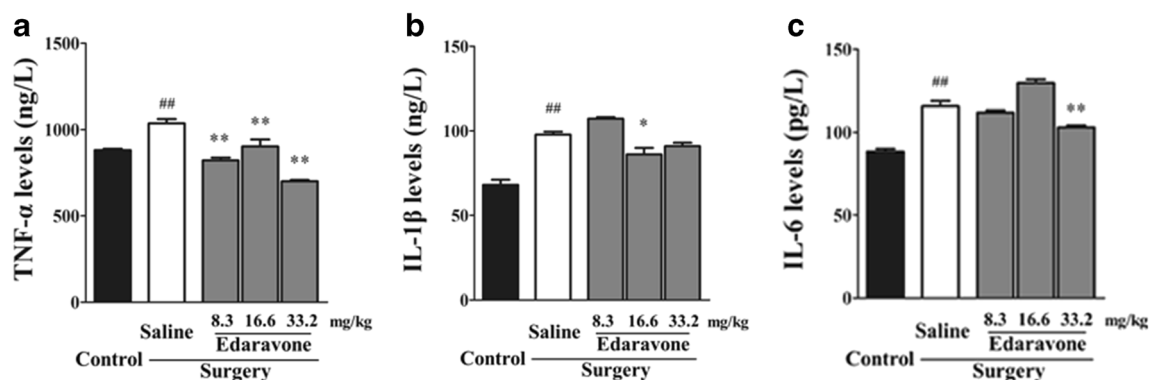
**Fig. 3** Edaravone attenuates the impairments in spatial learning and memory induced by abdominal surgery under general anesthesia in aged mice. The Morris water maze tests were performed on days 10 to 15 post-surgery. **a** During the training periods, edaravone significantly decreased the escape latency at the last day of training. **b** During the probe trial, all the groups exhibited similar swim speed. **c** During the probe trial,

edaravone at 33.2 mg/kg significantly increased the percentage of the time in target quadrant. **d** Representative path on the last day of various groups in the training periods. **e** Representative path of various groups in the probe trial. Data represent the mean  $\pm$  SD ( $n = 15$ ).  $^{##}p < 0.01$  vs. control group without surgery,  $^{*}p < 0.05$  vs. surgery + saline group (one-way ANOVA and Tukey's test)

### Edaravone prevents the decrease of PSD-95 expression induced by abdominal surgery under general anesthesia in aged mice

PSD-95 is a representative post-synaptic protein. We assessed the expression of this protein in the

hippocampal region of aged mice by Western blotting assay. The expression of PSD-95 was significantly lower in the surgery + saline group than those in the control + saline group (one way ANOVA and Tukey's test,  $p < 0.01$ , Fig. 5). Moreover, when compared with the surgery + saline group, the expression



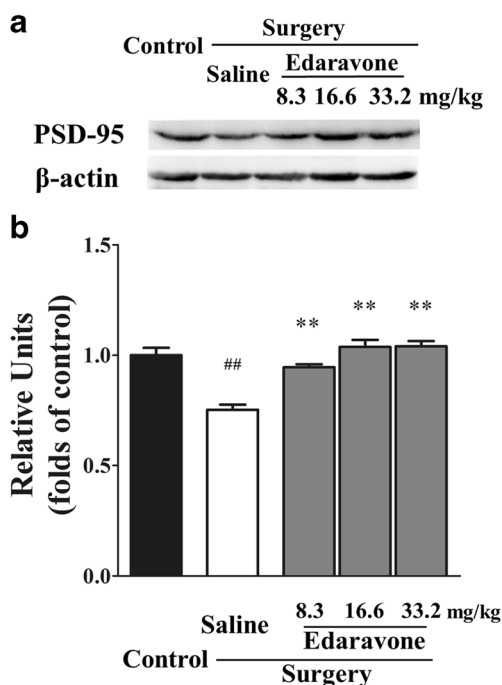
**Fig. 4** Edaravone attenuates the increase of the TNF- $\alpha$ , IL-1 $\beta$  and IL-6 expression in the hippocampus induced by abdominal surgery under general anesthesia in aged mice. Mice were sacrificed at day 16 post-surgery. The expressions of **a** TNF- $\alpha$ , **b** IL-1 $\beta$  and **c** IL-6 were evaluated by

ELISA assay in the hippocampal extraction of the aged mice. Data represent the mean  $\pm$  SD ( $n = 4$ );  $^{##}p < 0.01$  vs. control group without surgery,  $^{*}p < 0.05$  and  $^{**}p < 0.01$  vs. surgery + saline group (one-way ANOVA and Tukey's test)

of PSD-95 in 8.3–33.2 mg/kg edaravone group significantly increased (one way ANOVA and Tukey's test,  $p < 0.01$ , Fig. 5b). These results indicated that edaravone may prevent the decrease of synaptic protein induced by abdominal surgery under general anesthesia in aged mice.

### Edaravone prevents the decrease of pSer9-GSK3 $\beta$ expression induced by abdominal surgery under general anesthesia in aged mice

We used Western blotting assay to evaluate the expression of pSer9-GSK3 $\beta$ . The results showed that the expression of pSer9-GSK3 $\beta$  is significantly decreased in the surgery + saline group compared with the control group without surgery (one way ANOVA and Tukey's test,  $p < 0.01$ , Fig. 6). Furthermore, the expression of pSer9-GSK3 $\beta$  in the 33.2 mg/kg edaravone group is significantly higher than the surgery + saline group (one way ANOVA and Tukey's test,  $p < 0.05$ , Fig. 6b). These results indicated that high dose of edaravone may promote the expression of pSer9-GSK3 $\beta$ , leading to the reduction of pro-inflammatory cytokines in aged mice.



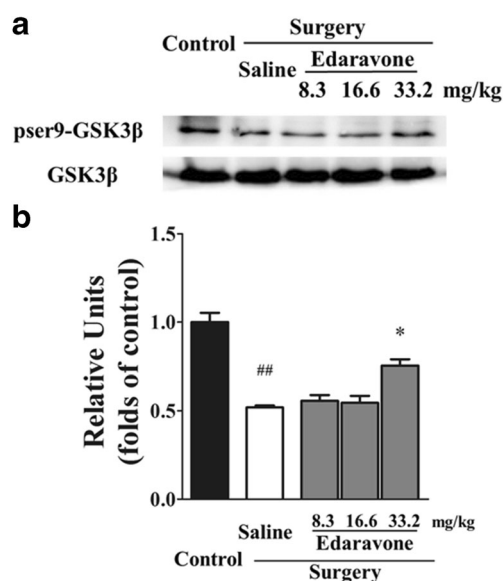
**Fig. 5** Edaravone attenuates the decreased expression of PSD-95 induced by abdominal surgery under general anesthesia in aged mice. At day 16 post-surgery, mice were sacrificed. **a** Western blotting analysis were used to evaluate the expressions of PSD-95 and  $\beta$ -actin. The quantitative analysis of levels of PSD-95 was shown in (b). Data represent the mean  $\pm$  SD ( $n = 3$ ); ### $p < 0.01$  vs. control group without surgery, \*\* $p < 0.01$  vs. surgery + saline group (one-way ANOVA and Tukey's test)

### Edaravone attenuates the increase of GFAP-positive and Iba-1-positive areas induced by abdominal surgery under general anesthesia in aged mice

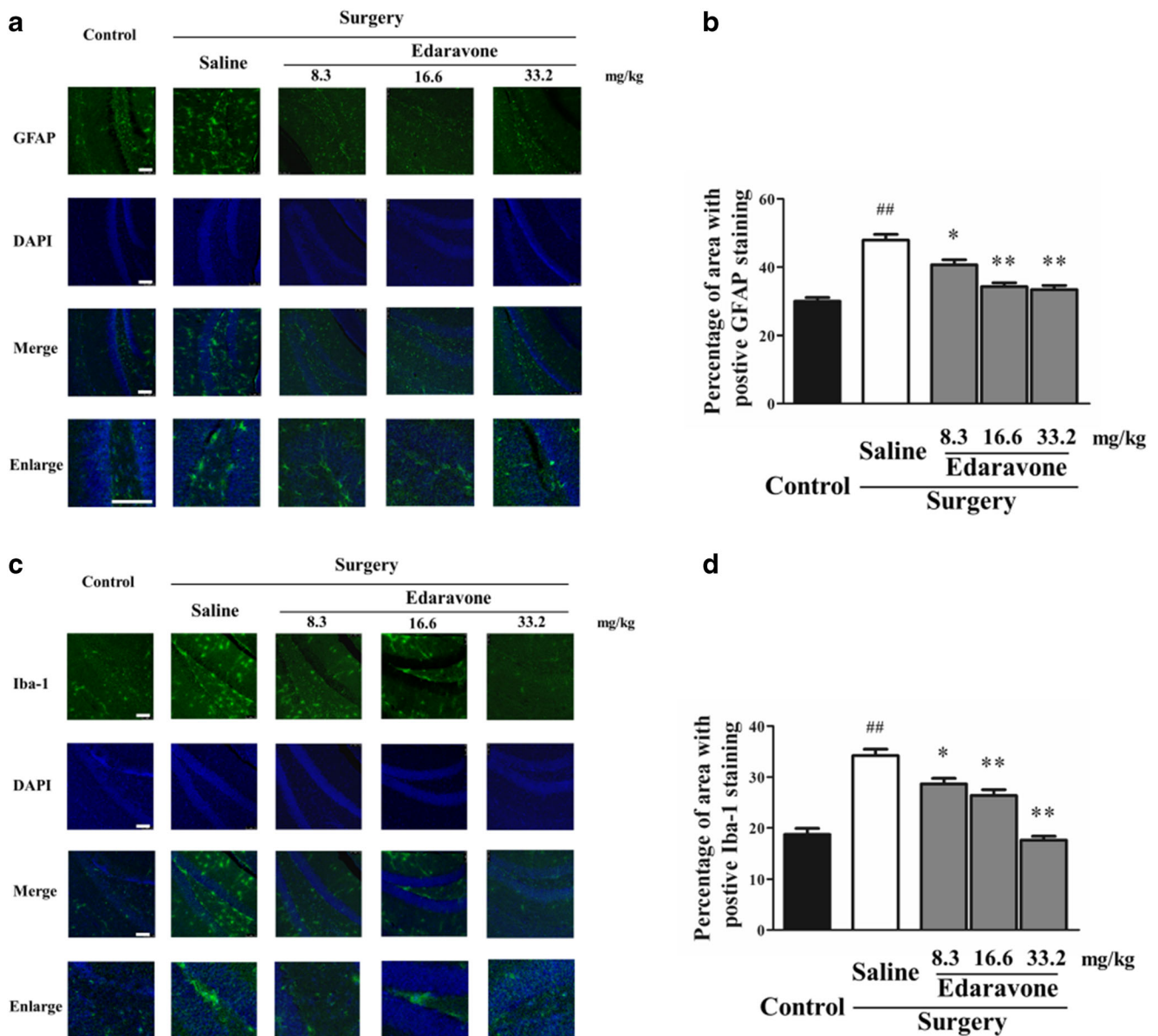
To explore the activation of astrocytes and microglia, we used IHC staining to evaluate the GFAP-positive and the Iba-1-positive areas, respectively, in the hippocampal region of mice (Fig. 7). GFAP-positive and Iba-1-positive areas were significantly changed among groups (one way ANOVA and Tukey's test, for GFAP-positive area,  $F(4, 10) = 27.146$ ,  $p < 0.01$ , Fig. 7b; for Iba-1 positive area,  $F(4, 10) = 39.375$ ,  $p < 0.01$ , Fig. 7d). GFAP-positive and Iba-1-positive areas in the surgery + saline group was significantly increased compared with the control group without surgery (one way ANOVA and Tukey's test,  $p < 0.01$ , Fig. 7b, d). Furthermore, edaravone at 8.3–33.2 mg/kg significantly decreased the increase of GFAP-positive and Iba-1-positive area induced by abdominal surgery under general anesthesia, indicating that edaravone might attenuate the over-activation of astrocytes and microglia in aged mice (one way ANOVA and Tukey's test,  $p < 0.05$ , Fig. 7b, d).

### Edaravone decreases the increase of AChE activity induced by abdominal surgery under general anesthesia in aged mice

The activities of AChE in the brain were analyzed ex vivo. The AChE activities were varied among different groups (one



**Fig. 6** Edaravone attenuates the decreased expression of pSer9-GSK3 $\beta$  in the hippocampal region induced by abdominal surgery under general anesthesia in aged mice. Mice were sacrificed at day 16 post-surgery. **a** Western blotting analysis was used to evaluate the expressions of pSer9-GSK3 $\beta$  and GSK3 $\beta$  in the hippocampus. The quantitative analysis of levels of pSer9-GSK3 $\beta$  was shown in (b). Data represent the mean  $\pm$  SD ( $n = 3$ ); ## $p < 0.01$  vs. control group without surgery, \* $p < 0.05$  vs. surgery + saline group (one-way ANOVA and Tukey's test)



**Fig. 7** Edaravone attenuates the increase of GFAP-positive and Iba-1-positive areas induced by abdominal surgery under general anesthesia in aged mice. At day 16 post-surgery, mice were sacrificed. **a** The expression of GFAP in the hippocampus of mice was analyzed by IHC staining (scale bar = 75  $\mu$ m). The quantitative analysis of GFAP-positive area was

way ANOVA,  $F(4, 20) = 11.531$ ,  $p < 0.05$ , Fig. 8). Edaravone at 33.2 mg/kg could prevent the increase of AChE activity induced by abdominal surgery under general anesthesia *ex vivo*, suggesting that edaravone might reverse the impairments of cholinergic transmissions (one way ANOVA and Tukey's test,  $p < 0.05$ , Fig. 8).

## Discussion

We have found that edaravone at a high concentration (33.2 mg/kg) could attenuate cognitive dysfunctions induced

shown in (b). **c** The expression of Iba-1 in the hippocampus of mice was analyzed by IHC staining (scale bar = 75  $\mu$ m). The quantitative analysis of Iba-1-positive area was shown in (d). Data represent the mean  $\pm$  SD ( $n = 3$ ); <sup>##</sup> $p < 0.01$  vs. control group without surgery, <sup>\*</sup> $p < 0.05$  and <sup>\*\*</sup> $p < 0.01$  vs. surgery + saline group (one-way ANOVA and Tukey's test)

by abdominal surgery under general anesthesia in aged mice. We have also discovered that edaravone could prevent the increase of neuroinflammation, the decrease of synaptic proteins, and the reduction of pSer9-GSK3 $\beta$  in the hippocampal region of aged mice, which might contribute to the cognitive enhancement effects of edaravone.

POCD is common following surgery in elderly patients. However, the pathogenic mechanisms of this disease remain unknown. Clinically, most POCD were occurred in elderly, but not young people, after surgery and general anesthesia (Berger et al. 2018). And some anesthetics could even promote, but not decrease, cognition when used in the adult



animals (Su et al. 2012). Furthermore, the combination of surgery and anesthesia could induce POCD in aged people. Therefore, the cognitive impairments in POCD might be caused by the combination of multiple factors, including aging, surgery and general anesthesia, but not only one factor. It is speculated that aging could cause the fragility of the brain in the elderly, leading it to be easily attacked by the impacts (e.g. neuroinflammation and nociceptive stimulation) of surgery and general anesthesia combination (Peng et al. 2013).

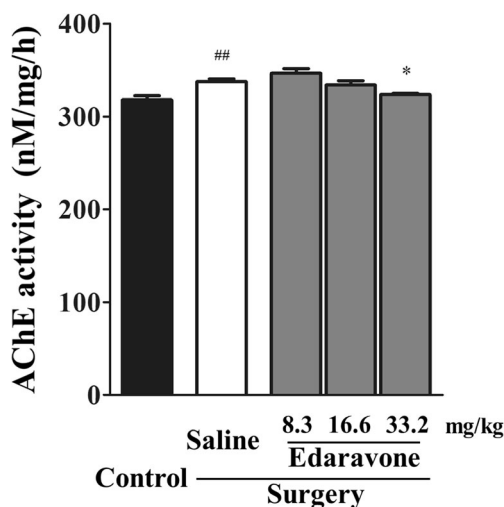
Exploratory laparotomy in animals could simulate abdominal surgery in humans, and is commonly performed to induce POCD in rodents (Zhang et al. 2016). In addition, abdominal surgery is widely used to establish the POCD model because it is easy to operate, and has high modeling success rate (Zhang et al. 2016). Moreover, abdominal surgery in old animals could induce the release of pro-inflammatory cytokines through trauma and stress, regulate neuronal and glial activity, and affect cognition (Quan et al. 2019). Abdominal surgery could also induce the increase in intestinal permeability, leading to the elevation of pro-inflammatory cytokine levels, which is harmful to cognitive functions of animals (Quan et al. 2019). Therefore, we used this surgery to establish our POCD model.

Isoflurane is a representative inhalant general anesthesia used in clinical (Tsukamoto et al. 2015). Previous studies using appendectomy after general isoflurane anesthesia demonstrated that such surgery could activate microglia, and release large amounts of pro-inflammatory cytokines, leading to spatial learning and memory deficits (Wang et al. 2015). Therefore, we combined exploratory laparotomy and isoflurane general

anesthesia to establish the model of POCD in aged mice. Our study showed that comparing to the mice in control group without surgery, the cognitive ability of the surgery + saline group was decreased, suggesting that abdominal surgery under general isoflurane anesthesia could indeed cause cognitive impairments in aged mice, which is consistent to a previous study (Wang et al. 2016a). In this study, we used male mice because 1) female mice have a 2–3 day estrous cycle, which is difficult to control (Ghimire et al. 2019; Szpak et al. 2018); 2) female mice could produce estrogen which may be protective to many insults (Farmer et al. 2014); and 3) the wide application of male animals allows the male animals more accessible (Zucker and Beery 2010).

Edaravone could easily pass the blood-brain barrier, and remove free radicals in the brain, alleviating the deterioration of post-surgical cognitive imperilments in patients undergoing carotid endarterectomy (Wang et al. 2016b). However, the mechanism by which edaravone acts on POCD is not clear (Tian et al. 2017). It has been demonstrated that edaravone at 3 mg/kg can prevent the activation of microglia after the combination of surgery and LPS administration in adult rodents (Wang et al. 2016b). Moreover, edaravone at 3 mg/kg could prevent apoptosis induced by abdominal surgery under local anesthesia in mice (Tian et al. 2017). These results indicated that edaravone might be useful to prevent cognitive impairments in age-related post-surgical administration. In the preliminary experiments, we found that 3 mg/kg edaravone was not effective in treating cognitive impairments induced by abdominal surgery under general isoflurane anesthesia in aged mice. These discrepant results might be due to different types of surgery and anesthesia used in two studies. The large amount of free radicals and pro-inflammatory cytokines retained in the hippocampus of aged animals, resulting the intensity of inflammatory response of microglia and astrocytes far exceeds that of adult animals (Jin et al. 2014). Moreover, it is reported that general but not local anesthesia might cause severe injury in aged brain, which is crucial for POCD (Li et al. 2014; Sanders et al. 2009). Therefore, we speculated that general isoflurane anesthesia might cause more severe damage than local anesthesia in aged mice, leading to the higher concentration of edaravone required in the prevention of POCD in our model.

What is the main mechanism of edaravone to attenuate postoperative cognitive impairments in our model? Surgery stimulates the delivery of pro-inflammatory cytokines in aged mice. After surgery, over-activation of microglia and astrocytes could lead to the excess release of pro-inflammatory cytokines such as nitric oxide, TNF- $\alpha$  and IL-1 $\beta$ , leading to neuronal impairments and synaptic damage (Rappold et al. 2016; Yuan et al. 2014). We found that edaravone could not only inhibit the over-activation of microglia and astrocytes,



**Fig. 8** Edaravone significantly prevented the inhibition of AChE activity induced by abdominal surgery under general anesthesia in aged mice. AChE activity was measured ex vivo. Data represent the mean  $\pm$  SD ( $n = 5$ ); ## $p < 0.01$  vs. control group without surgery, \* $p < 0.05$  vs. surgery + saline group (one-way ANOVA and Tukey's test)

but also decrease the expression of pro-inflammatory cytokines, indicating that edaravone can alleviate surgery-induced neuroinflammation in aged animals. Moreover, previous experiments have shown that general isoflurane anesthesia could enlarge the synaptic gap, and significantly reduce post-synaptic density area (Kong et al. 2013). PSD-95 is a marker of post-synaptic which provided essential scaffolds for post-synaptic receptors and ion channels (Savioz et al. 2014). Our findings that edaravone significantly attenuated surgery-induced decrease of PSD-95 expression, indicated that edaravone might also prevent synaptic impairments. GSK3 $\beta$  regulates many important cellular processes in the brain, and is important in the process of inflammation activation (Jope et al. 2007). GSK3 $\beta$  could enhance mRNA expressions of pro-inflammatory cytokines, and thus produce such proteins (Rodionova et al. 2007). In our study, we found that edaravone up-regulated the expression of pSer-GSK3 $\beta$ , the inactive form of GSK3 $\beta$ , in the hippocampus, further providing a support that edaravone might reduce neuroinflammation in aged mice.

Cholinergic transmission is important for cognition. Increased levels of acetylcholine in the cholinergic synapses could alleviate the symptoms associated with the cognitive dysfunctions (Ragab et al. 2019; Saeed et al. 2014). AChE is mainly responsible for the breakdown of acetylcholine, leading to the decrease of cholinergic transmission. A previous study has shown that edaravone could reduce the increase of AChE induced by A $\beta$  injection in the brain, indicating that edaravone might act on the cholinergic transmission (He et al. 2014). Therefore, our results that surgery-induced increase of AChE could be alleviated by edaravone, further suggested that the cognitive enhancement effects of edaravone might be partially through the activation of cholinergic system.

In conclusion, we found that significant cognitive impairments and neuroinflammation activation were induced in aged mice by receiving general isoflurane anesthesia and abdominal exploration. Edaravone, a clinically used free radical scavenger, at a high concentration could reduce surgery-induced cognitive impairments in this model. The cognitive enhancement effects of edaravone might be due to the inhibition of neuroinflammation, the increase of synaptic protein, and the elevation of cholinergic transmission. Therefore, we anticipated that edaravone might be developed as a treatment of POCD.

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