# Edaravone improves spatial memory and modulates endoplasmic reticulum stress-mediated apoptosis after abdominal surgery in mice

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**Abstract.** Patients who receive major surgery often develop postoperative cognitive dysfunction (POCD); however, there is a lack of effective management as the pathogenesis of this disorder has not been fully elucidated. The neuroprotective effects of edaravone have been characterized in both *in vitro* cultured cells and in experimental animal models. The present study aimed to determine the potential role of edaravone in surgery-induced cognitive decline in mice. Animals were assigned to three groups: Control group (n=32), where mice received local anesthesia; surgery group (n=32), where mice underwent abdominal surgery under anesthesia; and edaravone group (n=32), where mice received abdominal surgery and were administered with edaravone (3 mg/kg). Morris water maze and T-maze tests demonstrated that edaravone attenuated surgery-induced cognitive impairment. Nissl staining indicated that edaravone prevented neuronal loss in the hippocampus of mice that underwent surgery. Furthermore, treatment with edaravone mitigated the surgery-induced upregulation of glucose-regulated protein 78 and CCAAT-enhancer-binding homologous protein and reduced the number of terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling-positive nuclei in mice hippocampi. In conclusion, edaravone may prevent POCD-induced neuronal apoptosis through attenuating endoplasmic reticulum stress.

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*Abbreviations:* POCD, postoperative cognitive dysfunction; GRP, glucose-regulated proteins; CHOP, CCAAT-enhancer-binding homologous protein; ERS, endoplasmic reticulum stress; MWM, Morris water maze

*Key words:* edaravone, cognition, apoptosis, endoplasmic reticulum stress

## Introduction

Postoperative cognitive dysfunction (POCD) is an illness characterized by cognitive decline in patients who have had to undergo surgery. Patients with POCD may have attention defects, poor concentration, language impairments or spatial memory loss (1,2). Accumulative clinical evidence indicates that patients undergoing surgery in the absence of general anesthesia are also at risk of developing POCD (3,4). Due to the fact that no influence from anesthesia has been identified in association with POCD, attention has shifted to focus on the association of the surgical intervention itself with POCD. Nevertheless, the pathogenesis of POCD remains poorly understood and there is a lack of effective management for this disorder.

Previous studies have demonstrated that surgery may lead to the development of POCD by inducing neuroinflammation. One study identified increased production of proinflammatory cytokines, such as TNF-α, after surgery (5). In addition, major surgery results in the activation of microglia in the brain (6). Despite the fact that surgical intervention is commonly accompanied by inflammation, only a minor proportion of the patients receiving major surgery suffer from POCD (7). The association between neuroinflammation and cognitive decline in patients after major surgery remains largely unclear.

Endoplasmic reticulum stress (ERS) is involved in various cellular processes, such as cell growth, differentiation and apoptosis. Upon stimulation, the initiation of ERS results in impaired protein synthesis and the upregulation of molecular chaperones that promote correct protein folding and cell survival under harmful conditions (8); however, sustained ERS contributes to the activation of the apoptotic cascade (9). It is understood that ERS is capable of inducing mitochondria- and death receptor-independent apoptosis (10). In these cases, the upregulation of ERS-related transcription factors, including glucose-regulated protein (GRP)78 and CCAAT-enhancer-binding homologous protein (CHOP) have been identified (11,12). The involvement of ERS has been widely observed in the brain pathogenesis of neurological disorders and neurodegenerative diseases, including cerebral ischemia, Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (9,13,14). Nonetheless, to the best of

our knowledge, the regulatory role of ERS in the brain damage of POCD has not been described.

Edaravone, a free radical scavenger, exerts neuroprotective effects (15). It has been reported that edaravone is able to prevent hypoxia- and ischemia-induced ERS and improve neurological status in mice (16). The present study aimed to investigate whether treatment with edaravone attenuates surgery-induced cognitive decline and to determine the involvement of ERS in apoptotic neuronal injury in mice after surgery.

## Materials and methods

*Ethics statement*. All animal experiments were approved by the Animal Care and Use Committee of the China Medical University (Shenyang, China) and were conducted according to the guidelines for care and use of laboratory animals outlined by the Chinese Academy of Science (Beijing, China).

*Animals*. A total of 96 14-month old C57BL/6 female mice (38±2 g) were used in the present study. Mice were housed in a pathogen-free environment under a 12-h light-dark cycle at 23˚C and 40-70% humidity with free access to food and water.

*Surgical procedures and treatment protocol*. C57BL/6 mice were randomly assigned to three groups, with 32 mice in each group. The groups included a control group (group C), a surgery group (group S) and an edaravone group (group E). Half of the mice in each group (n=16) were used in Morris water maze (MVM) and T-maze tests, while the remaining mice were used for pathological examinations. In the surgery group, mice received local anesthesia by subcutaneous injection of 0.5% bupivacaine (0.1 ml; Shanghai Fuxing Chaohui Pharmaceutical Co., Ltd., Shanghai, China) into the abdominal area. A 2.5-cm incision was subsequently made in the middle of the abdomen, and the abdominal cavity was opened and subsequently closed back up. The entire procedure lasted ~5 min. The day on which mice received surgery was defined as day 0. On postoperative days 1 and 2, mice were administered with 2.5% lidocaine (Shanghai Fuxing Chaohui Pharmaceutical Co., Ltd., ) to relieve pain. In the control group, mice received the same anesthetic treatment as the surgery group; however, the incision intervention was not performed. In the edaravone group, mice underwent abdominal surgery followed by daily administration of 3 mg/kg edaravone (Xiansheng Pharmaceutical Corp., Nanjing, China) continued until the end of the experiment.

*MWM test*. In order to elucidate the effects of edaravone on the spatial learning ability of mice, the MWM test was performed (17). Mice were tested daily, with three trials per day, for 7 consecutive days after surgery. After each trial of the MWM test, the wound of each mouse was immediately dried to avoid infection, as described previously (18). In each trial, the animal was placed in a different starting quadrant and allowed to swim. The overall swimming distance, swimming speed and escape latency to the hidden platform were recorded by a video camera. Data were analyzed using HVS image water maze 2020 software (HVS Image Software Ltd, Buckingham, UK). Additionally, probe trials were conducted on postoperative days 1, 3 and 7, in order to evaluate the retention memory of the mice. In the probe trials, the platform was removed from the water and the time spent in the target quadrant that previously contained the submerged platform was recorded during a time period of 90 sec.

*T-maze tests*. The T-maze test is a widely-used behavioral test that enables the evaluation of the cognitive ability of rodents (19). During testing, mice were subjected to a restricted feeding schedule of 85% of their free-feeding weight. Mice were placed into a T-shaped maze and were left to collect reward foods (small sugar cubes). Each test trial consisted of a sample run and a choice run with an interval of 10 sec between the sample and choice runs. In the sample run, animals were placed into the starting arm and were forced to visit one particular arm to get a reward, while the other arm was blocked. In the choice run, the blocked door was removed and the mice were allowed a free choice of either arm. If mice entered the previously non-visited arm, they were rewarded. The time interval between the sample and choice runs was further increased to 90 and 180 sec. Each daily session included five trials, and mice participated in one trial at a time with an intra-trial interval of 10 min.

*Pathological examination*. On postoperative day 3, the hippocampus was removed from 16 mice in each group and tissues were fixed in 10% formalin solution at room temperature for 24 h. Samples were subsequently embedded in paraffin and sectioned. Sections of 6 $\mu$m in thickness were used for Nissl staining and the number of survived hippocampal neurons per 1 mm was counted under a Nikon Eclipse E800 microscope (Nikon Corporation, Tokyo, Japan). The average number was calculated from three sections of bilateral hippocampal slices.

*Immunohistochemistry*. For immunohistochemistry, 5-$\mu$m thick hippocampal sections were blocked in 2% normal goat serum (Beijing Solarbio Science & Technology Co., Ltd. Beijing, China). Following blockage, samples were incubated with primary rabbit monoclonal anti-GRP78 (sc:376878; 1:20) or polyclonal anti-CHOP antibodies (15204-1-AP; 1:100; both from Santa Cruz Biotechnology, Inc., Dallas, TX, USA) overnight at 4˚C. Following three washes with phosphate-buffered saline (PBS), sections were probed with a biotin-conjugated anti-rabbit secondary antibody (SE205; 1:100) at 37˚C for 30 min. Negative control samples were incubated with PBS in the absence of a primary antibody. Following three washes with PBS, the reaction product was visualized using diaminobenzidine (DAB). The integrated optical density (OD) of GRP78- or CHOP-positive staining in the hippocampal region was evaluated using a MetaMorph 2.0 software system (Molecular Devices, LLC, Sunnyvale, CA, USA).

*Terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) staining*. To determine cell apoptosis in the hippocampus, TUNEL staining was conducted according to the manufacturer's instructions of a TUNEL assay kit (KGA702; Kaiji, Nanjing, China). In brief, the hippocampal sections from the different experimental groups were exposed to the TUNEL reaction mixture at 37˚C for 60 min. Following this, samples were incubated with horseradish peroxidase-conjugated

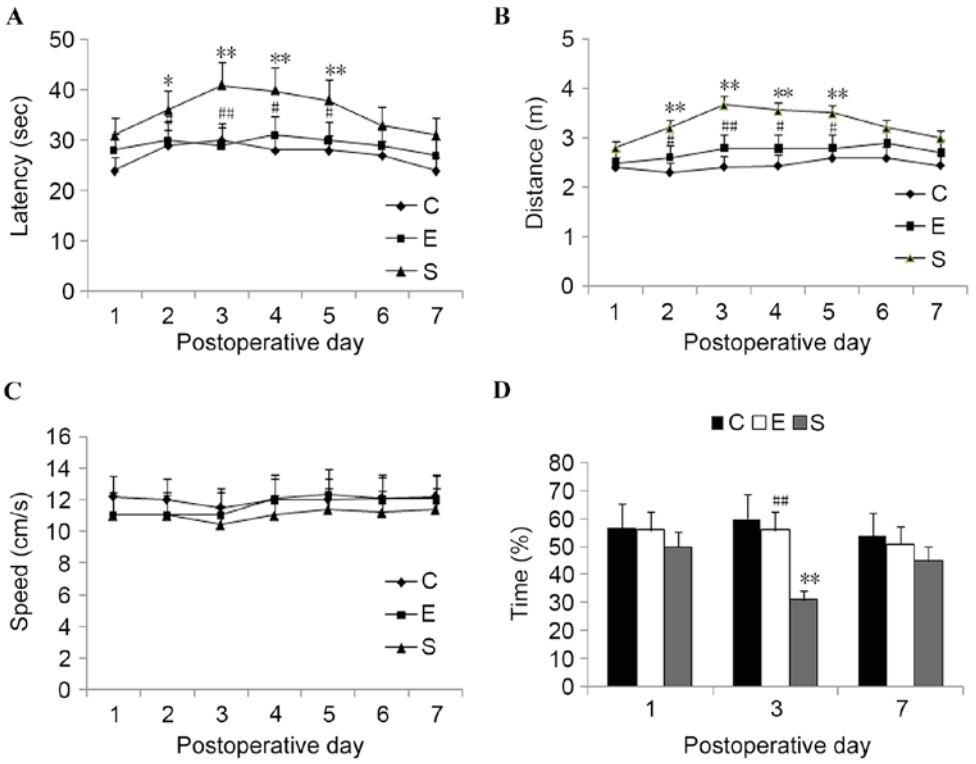


Figure 1. Morris water maze analysis of the effect of edaravone on spatial learning and memory in mice following abdominal surgery. (A) Average escape latency to find the hidden platform. (B) Average swimming distance to find the hidden platform. (C) Average swimming speed to find the hidden platform. (D) Time spent in the target quadrant following removal of the platform in the probe trial. Data are presented as the mean ± standard error of the mean. $^{*}$P<0.05 and $^{**}$P<0.01 vs. the control group at the indicated time point; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. the surgery group at the indicated time point. C, control group (n=16); E, edaravone group (n=16); S, surgery group (n=16).

antibody (1:100; KGA702; Kaiji, Nanjing, China) at 37˚C for 30 min, followed by a 10-min incubation with DAB solution at room temperature. Nuclei were counterstained with hematoxylin. Samples were examined using an Olympus BX51 optical microscope (Olympus Corporation, Tokyo, Japan; magnification, x400). Cells exhibiting brown nuclei under DAB staining were considered to be apoptotic. The mean integrated OD of TUNEL-positive staining in the hippocampal samples was determined.

*Statistical analysis*. SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Comparison between groups was made using one-way analysis of variance, followed by the Student-Newman-Keuls test. Data were presented as the mean ± standard error of the mean. P<0.05 was considered to indicate a statistically significant difference.

## Results

*Edaravone attenuates surgery-induced cognitive impairment*. Results from the MWM tests revealed that, compared with those in the surgery group, the escape latency was significantly reduced in mice treated with edaravone on postoperative day 2 (P<0.05) and postoperative days 3 to 5 (P<0.01; Fig. 1A). The swimming distance was also significantly reduced in the edaravone group from postoperative days 2 to 5 compared with the surgery group (P<0.05: Fig. 1B). No significant difference was detected in the escape latency or swimming distance between the edaravone group and the control group (P>0.05). There was no significant difference in the swimming speed among the three experimental groups (P>0.05; Fig. 1C).

In the probe trial, it was demonstrated that the mice treated with edaravone spent a significantly longer time in the target quadrant compared with mice in the surgery group on post-operative day 3 (P<0.01; Fig. 1D). However, comparison of the cumulative time spent in the target quadrant demonstrated no significant difference between the edaravone and control groups on postoperative days 1, 3 and 7.

In order to evaluate spatial working memory function, T-maze tests were conducted. On postoperative days 1, 3 and 7, a similar learning curve was detected between control mice and mice from the edaravone group (P>0.05). However, compared with mice in the surgery group, a significantly superior performance was demonstrated in the edaravone group when increasing the interval between the sample and choice runs to 90 and 180 sec on postoperative days 1 and 3 (P<0.05; Fig. 2). These results indicate that edaravone administration significantly attenuates surgery-induced cognitive impairment in mice.

*Edaravone reduces neuronal loss in mice after surgery*. Neuronal loss, particularly in the hippocampal region, commonly occurs during stress injury. In order to examine whether edaravone inhibited neuronal loss as a result of surgery, histological changes in the hippocampus on postoperative day 3 were examined. No signs of histopathological abnormalities were observed in the hippocampal samples from the control mice; however, mice in the surgery group exhibited severe

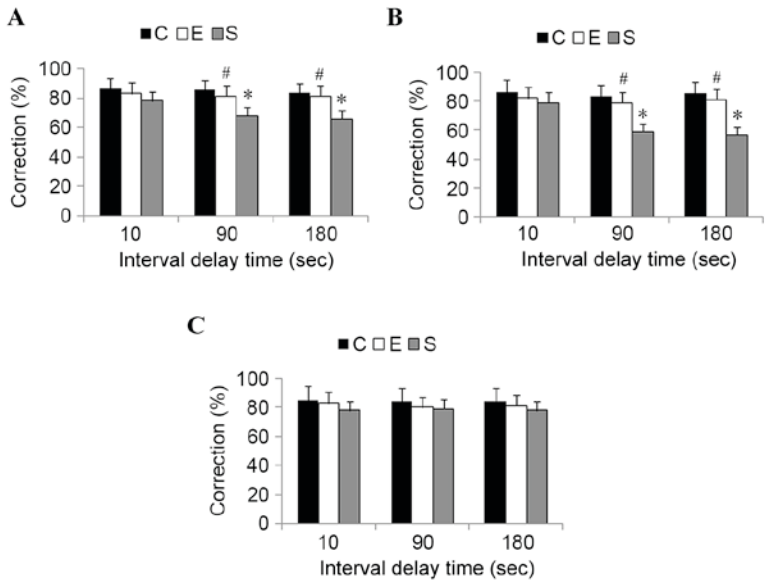


Figure 2. T-maze tests to determine the effect of edaravone on spatial working memory in mice after abdominal surgery. Mice in the different experimental groups were subjected to T-maze tests with interval delay times between the sample and choice trails of 10, 90 and 180 sec on postoperative day (A) 1, (B) 3 and (C) 7. Data are presented as the mean ± standard error of the mean. *P<0.05 vs. the control group at the indicated time point; #P<0.05 vs. the surgery group at the indicated time point. C, control group (n=16); E, edaravone group (n=16); S, surgery group (n=16).

neuronal damage in the hippocampus. Edaravone significantly reduced neuronal loss in the hippocampus, as compared with those in surgery group (P<0.01; Fig. 3). These results suggest that edaravone may inhibit hippocampal neuronal death induced by surgery.

*Edaravone downregulates GRP78 and CHOP expression levels in the hippocampus after surgery*. To determine the potential involvement of ERS in edaravone-mediated neuroprotection, the expression levels of ERS-related proteins, including GRP78 and CHOP, were measured. Results indicated that, compared with control mice, the protein expression levels of both GRP78 and CHOP were significantly upregulated in the hippocampus on postoperative day 3 in the surgery group (P<0.01; Fig. 4). Administration of edaravone significantly reduced the upregulation of GRP78 and CHOP expression levels in the hippocampus after surgery (P<0.01; Fig. 4). Compared with the control mice, no significant difference was demonstrated in the GRP78 and CHOP expression levels of mice treated with edaravone. These results indicate that the protective role of edaravone in mice after surgery may be associated with its capability to inhibit ERS.

*Edaravone inhibits hippocampal neuron apoptosis after surgery*. To evaluate cell apoptosis, TUNEL staining was performed. On postoperative day 3, mice that only received abdominal surgery exhibited significant cell apoptosis in the hippocampus, with an increased number of TUNEL-positive cells, when compared with control mice (P<0.01; Fig. 5). Edaravone application significantly prevented cell apoptosis in the hippocampus induced by surgery (P<0.01; Fig. 5). No significant difference was detected in the number of TUNEL-positive cells between the edaravone and control groups. These results suggest that edaravone may exert neuroprotection in mice after surgery through inhibiting hippocampal neuron apoptosis.

## Discussion

General anesthesia has been demonstrated to have an important role in the pathogenesis of POCD (20,21); however, other studies have indicated that there is no causative relationship between general anesthesia and POCD (3,22). To rule out the potential influences of general anesthesia on cognitive impairment, the present study established a murine model of abdominal surgery under local anesthesia. Results demonstrated that, under local anesthesia, the abdominal surgical intervention resulted in cognitive impairment in mice; however, treatment with edaravone was able to attenuate cognitive decline induced by surgery. The present study indicated that the therapeutic effect of edaravone on POCD may be related to its efficacy in inhibiting ERS-induced apoptosis in mice after surgery.

By using MWM tests, the present study compared the spatial memory in the three experimental groups. The results demonstrated that the escape latency, as well as the swimming distance, of mice that underwent surgery were longer, and the time spent in the target quadrant was significantly shorter than those demonstrated by mice in the control and edaravone groups. These results suggest that abdominal surgery resulted in spatial memory defects in mice, which were able to be effectively prevented by the administration of edaravone. Furthermore, the present study evaluated the working memory in the three experimental groups of mice using T-maze tests, the difficulty of which was manipulated by systematically varying the interval between the sample and choice run trials. Consistent with the MWM test results, edaravone was able to prevent the impairments of the working memory induced by surgery in mice. These findings suggest that edaravone prevented the cognitive decline in mice following surgery.

In addition, the present study demonstrated that surgery resulted in neuronal apoptosis in the hippocampus of mice,

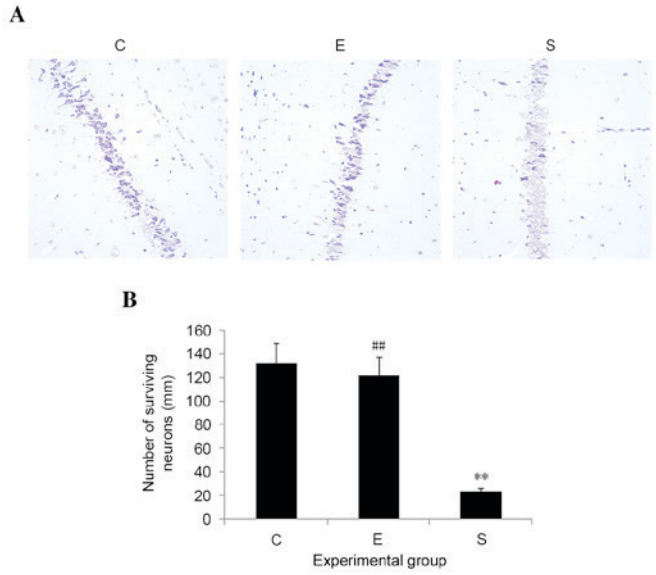


Figure 3. Effect of edaravone on neuronal loss in the hippocampus of mice following surgery. (A) Representative microphotographs of Nissl-stained hippocampal sections from the three experimental groups (magnification, x400). (B) Number of surviving neurons in the hippocampus of mice from the three experimental groups. Data are presented as the mean ± standard error of the mean. **P<0.01 vs. the control group at the indicated time point; ##P<0.01 vs. the surgery group at the indicated time point. C, control group (n=16); E, edaravone group (n=16); S, surgery group (n=16).

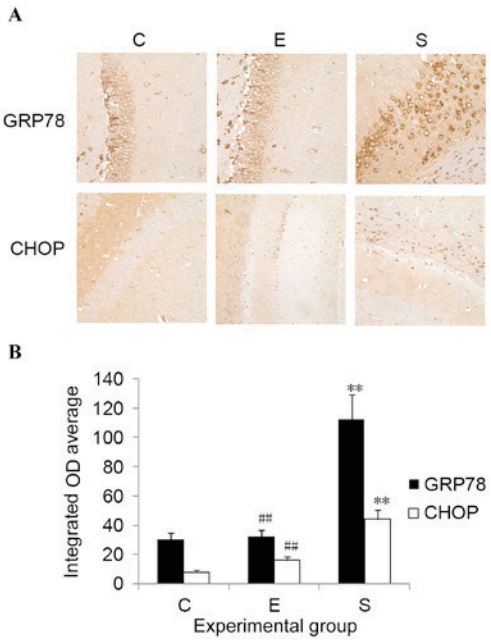


Figure 4. Immunohistochemistry of GRP78 and CHOP in the hippocampal region of mice. (A) Hippocampal sections derived from mice in the three experimental groups immunostained with anti-GRP78 or anti-CHOP antibodies (magnification, x400). (B) ODs of GRP78- and CHOP-positive cells. Data are presented as the mean ± standard error of the mean. **P<0.01 vs. the control group at the indicated time point; ##P<0.01 vs. the surgery group at the indicated time point. CRP78, glucose-regulated protein 78; CHOP, CCAAT-enhancer-binding homologous protein; OD, optical density; C, control group (n=16); E, edaravone group (n=16); S, surgery group (n=16).

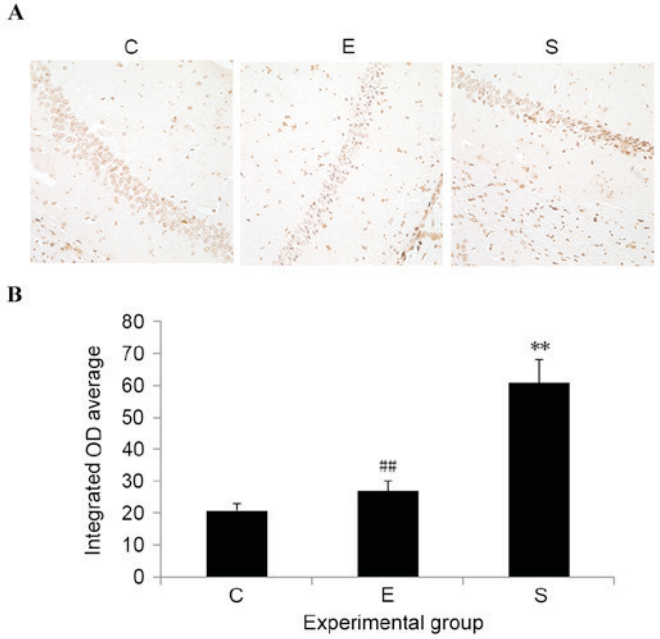


Figure 5. Representative micrographs of TUNEL staining in the hippocampus of mice from the three experimental groups. (A) Hippocampal sections derived from mice from the three experimental groups were stained with TUNEL solution and representative micrographs were captured (magnification, x400). (B) ODs of TUNEL-positive cells. Data are presented as the mean ± standard error of the mean. **P<0.01 vs. the control group at the indicated time point; ##P<0.01 vs. the surgery group at the indicated time point. OD, optical density; C, control group (n=16); E, edaravone group (n=16); S, surgery group (n=16); TUNEL, terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling.

with the presence of an increased number of TUNEL-positive cells in the hippocampal region of the brain. Apoptosis is a complex intracellular cascade, with multiple signals and pathways involved in the initiation of cellular apoptosis. However, the upstream mechanism of surgery-induced neuronal apoptosis remains unknown.

It is understood that stress may result in cognitive dysfunction (23). In the present study, mice were given peripheral surgery under local anesthesia. Therefore, it is not possible to completely exclude the potential influences of stress on postoperative cognitive decline. A previous study demonstrated that cellular stress results in ERS by enhancing the phosphorylation of eukaryotic translation initiation factor 2A (24).

Emerging evidence suggests that neuronal cell apoptosis induced by ERS is a predominant pathological issue in several neurological disorders (25,26). ERS-driven apoptosis is accompanied by the increased expression of ERS indicators, such as CHOP and GRP78 (27). CHOP is a transcription factor, the physiological level of which is relatively low; however, the protein expression of CHOP may be strongly induced in response to ERS under diverse pathological conditions (28). Similarly, upregulated expression of GRP78 is often regarded as an indicator of ERS (29).

In the present study, significant upregulation of CHOP and GRP78 expression levels were found in the hippocampus of mice following surgery. Furthermore, the number of TUNEL-positive cells was significantly increased in the hippocampal region of mice that received abdominal surgery. It is likely that surgery results in hippocampal cell apoptosis by inducing ERS, ultimately contributing to neuronal loss and cognitive impairment in mice. Notably, treatment with

edaravone significantly suppressed the protein expression levels of CHOP and GRP78 and reduced the number of TUNEL-positive cells in the hippocampus of mice following surgery. Thus, it was hypothesized that edaravone yielded a neuroprotective effect against surgery-induced ERS and hippocampal apoptosis, and therefore improved spatial learning and working memory in mice following surgery.

In conclusion, the findings of the present study demonstrated that ERS-mediated neuronal apoptosis in the hippocampus has an important role in POCD. The edaravone-induced amelioration of cognition may be partly attributed to its potency of inhibiting ERS-induced neuronal apoptosis in the hippocampus after surgery.

## Acknowledgements

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