

Original Article

Resveratrol alleviates learning and memory impairment in aged rats after general anesthesia with sevoflurane and nitrous oxide via SIRT1-p53 signaling pathway

Wan-Xia Xiong¹, Zong-Tao Chai², Bei Wang¹, Guo-Xia Zhou³, Jing Cang¹, Zhang-Gang Xue¹, Sheng-Jin Ge¹

¹Department of Anesthesia, Zhongshan Hospital and Department of Anesthesiology, Shanghai Medical College, Fudan University, Shanghai 200032, China; ²Liver Cancer Institute and Zhongshan Hospital, Fudan University, Shanghai 200032, China; ³Department of Anesthesia, Shanghai Fifth People's Hospital, Fudan University, Shanghai 200240, China

Received June 11, 2016; Accepted August 2, 2016; Epub November 15, 2016; Published November 30, 2016

Abstract: The aim of this study was to evaluate the effects of resveratrol on learning and memory impairment in aged rats after general anesthesia with sevoflurane and nitrous oxide (N₂O), and explore the potential mechanism. 18-month-old Sprague-Dawley rats were randomly divided into anesthesia group (receiving 1.3% sevoflurane and 50% N₂O for 4 hours) and control group. 48 hours after anesthesia, spatial learning and memory were tested by the Morris Water Maze experiment. The neurotoxicity and expression of silent information regulator 1 (SIRT1) in the hippocampus were measured. Moreover, the effect of resveratrol on spatial learning and memory after anesthesia in aged rats was also investigated. The results showed that general anesthesia triggered neuronal apoptosis and, subsequently, induced impairment of learning and memory ability. Neuronal apoptosis induced by anesthetics triggered endogenous SIRT1 expression. Moreover, the SIRT1 expression and p53 deacetylation was increased in rats pretreated with resveratrol, which subsequently inhibited neuronal apoptosis and improved behavioral performance. In conclusion, our results demonstrated that general anesthesia with sevoflurane plus 50% N₂O impaired learning and memory ability in aged rats, induced neuronal apoptosis and increased SIRT1 expression. Pretreatment with resveratrol improved the ability of learning and memory, inhibited neuronal apoptosis by increasing the expression of SIRT1 in aged rats after general anesthesia.

Keywords: SIRT1, p53 deacetylation, learning and memory, resveratrol, general anesthesia, aged rat

Introduction

Many elderly patients may experience difficulties in learning, memory, concentration, and attention after surgery and anesthesia [1]. The mechanisms are not well-understood, and general anesthesia has been implicated as a possible cause [2]. Several studies suggest that exposure to isoflurane, nitrous oxide, or combination of isoflurane-nitrous oxide can cause persistent memory impairment in aged rodents [3-5].

Silent information regulator 1 (SIRT1), a member of the sirtuin family of class III histone deacetylases, is expressed in the brain and mediates intracellular responses that promote cell survival, enhance the repair of DNA damage, and reduce cell division, which plays im-

portant protective roles in nervous system diseases [6, 7]. In animal models of Alzheimer disease and amyotrophic lateral sclerosis, studies have shown that SIRT1 is upregulated, which is thought to be a neuroprotective adaptation response [8]. SIRT1 can deacetylate numerous proteins such as forkhead box class O family members, p53 tumor suppressor, and nuclear factor-κB. p53 plays an essential role in response to a multitude of cellular stress, whereas its acetylation correlates well with p53 stabilization and activates transcription of downstream targets [6]. In this regard, SIRT1 overexpression is shown to inhibit p53 transcriptional activity and p53-dependent apoptosis in response to DNA damage [9, 10].

Resveratrol, a polyphenolic component in grapes and red wine, has been reported to pos-

sess a wide range of biological and pharmacological activities, including anti-oxidant, anti-inflammatory, anti-mutagenic, and anti-carcinogenic effects [11, 12]. In a model of Alzheimer disease, resveratrol reduced neurodegeneration in the hippocampus and prevented learning impairment [8]. Recent studies have focused on its neuroprotective effects through the promotion of SIRT1 expression in several neurodegenerative disease models in vivo and in cultured cells in vitro. However, little is known about the role of SIRT1 in neurotoxicity induced by inhalation anesthetics so far.

In the present study, the effect of resveratrol on general anesthesia-induced learning deficits, memory loss, and neuronal apoptosis was evaluated and the related mechanisms were also elucidated in aged rats in vivo.

Materials and methods

Animals

18-month-old Sprague-Dawley rats (male, 500-600 g) were purchased from the Shanghai Medical Laboratory Animal Center (Shanghai, China). Rats were housed under constant temperature ($23\pm1^{\circ}\text{C}$) in a light-controlled vivarium (12-hour light/12-hour dark cycle) with free access to food and water. All the experimental protocols used in the present study were approved and monitored by the Ethical Committee of Animal Experiments, Zhongshan Hospital, Fudan University (Shanghai, China).

Anesthesia in rats

Rats were randomized to receive 1.3% sevoflurane (Baxter, Deerfield, IL) in 50% N_2O or 50% oxygen for 4 hours in an anesthetizing chamber at a flow rate of 3 L/min to maintain the end-tidal CO_2 at approximately 32 mmHg. The rats spontaneously breathed sevoflurane and N_2O . The inhaled and exhaled gas concentrations were monitored (Datex; Capnomac, Helsinki, Finland) and pulse oximeter oxygen saturation (SpO_2) was measured continuously during anesthesia. The temperature was maintained at $37\pm0.5^{\circ}\text{C}$ by applying a warming blanket to the rats. To discontinue the anesthetics, 100% oxygen was administered for 10 minutes, and the rats were subsequently removed from the anesthetizing chamber to terminate the treatment. The rats were recovered for 48 hours to avoid the confounding influence of residual

anesthetics before Morris water maze (MWM) testing. The rats in control group received 50% oxygen in their home cage at flow rates identical to those of anesthetized animals for 4 hours, but arterial oxygen saturation was not measured to prevent the introduction of stress as a confounding variable. The blood pressure was not measured during anesthesia, since several studies have shown that hypotension during surgery is not associated with increased risk for the development of POCD in elderly patients [1, 13]. SaO_2 remained within the physiological acceptable range in the exposed rats ($97\%\pm2\%$).

Morris water maze

Rats were consecutively released into the water facing the wall of the pool from four randomly assigned points (northeast (EN), northwest (NW), southwest (WS), and southeast (SE)). The platform was located in the southeast quadrant. In all trials, rats were allowed to search for the platform for 60 seconds. Once the rats located the platform, they were allowed to remain on it for 15 seconds. If a rat failed to find the platform within 60 seconds, it was gently guided to the platform and allowed to stay on the platform for 15 seconds; the escape latency was accepted as 60 seconds. The release point differed in each trial, and different sequences of release points were used from day to day. Rats were trained with the platform in a fixed location four trials per day for 6 consecutive days. The probe test was performed on day 7. The platform was removed from the tank and the rats were allowed to swim in the maze for 60 seconds. Swimming distance, speed, and latency were recorded by video tracking mounted on the ceiling and digital images were analyzed by water maze software (HVS image, United Kingdom).

Experimental procedures

In experiment 1, aged rats were randomly divided into two groups: control group ($n = 40$) and anesthesia group ($n = 40$). 48 hours after anesthesia, total 11 rats in the two groups were killed and the hippocampi from six rats in the anesthesia group and five rats in the control group was quickly dissected and stored at -80°C for subsequent testing. The other rats were deeply anesthetized and perfused transcardially with saline followed by 300 ml ice-cold

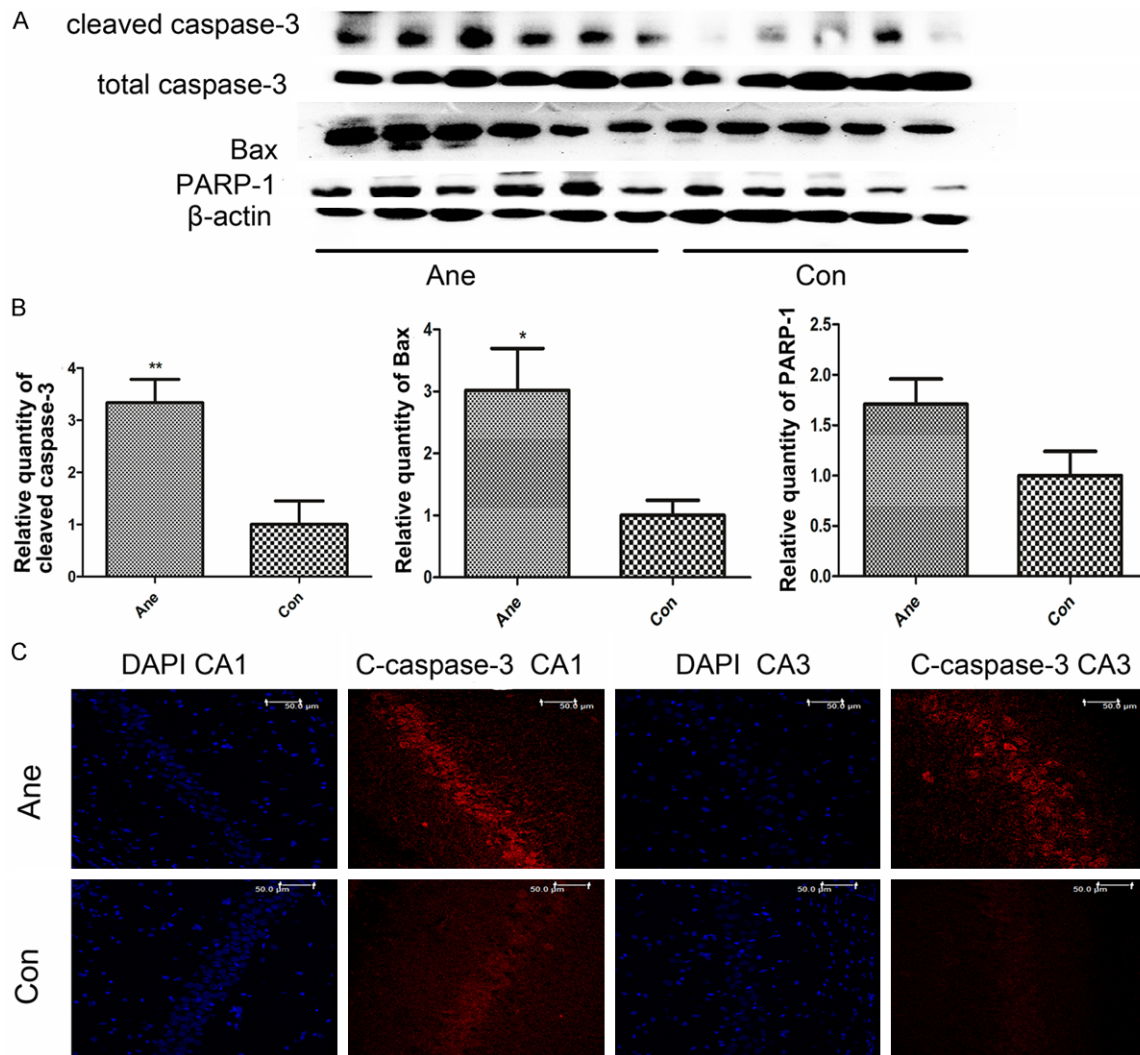


Figure 1. Neuronal apoptosis increased in the hippocampus at 48 hours after general anesthesia. $\bar{x} \pm SD$. A: The levels of cleaved caspase-3 and Bax in Ane group increased significantly compared to those in Con group. $n = 6$ for Ane group, $n = 5$ for Con group. B: Relative levels of cleaved caspase-3, Bax and PARP-1 were quantified. C: The expression of cleaved caspase-3 in CA1 and CA3 areas was detected by immunofluorescence staining. Scale bar: 50 μm , $n = 5$ per group. $**P < 0.01$; $*P < 0.05$.

4% paraformaldehyde solution. The brains were dissected and fixed in 4% paraformaldehyde overnight. The remaining rats in each group were subjected to MWM testing. The rats were killed after probe test on day 7.

In experiment 2, aged rats were randomly divided into two groups: anesthesia group ($n = 15$) and anesthesia with resveratrol group ($n = 12$). Resveratrol (Sigma, MO) was prepared in a stock solution in DMSO and further diluted in PBS. Rats were intraperitoneally injected with 100 mg/kg/day resveratrol or vehicle for 7 days. At the end of the 7th day, general anesthesia was administered to all the rats. 48 hours after general anesthesia, all the rats

were subjected to MWM testing, and killed on day 7.

Western blotting

Hippocampal tissues were subjected to SDS-PAGE (10% gels) and then transferred to the polyvinylidene difluoride membrane. The membranes were incubated in blocking buffer for 2 hours at RT and were incubated with the primary antibodies overnight. The primary antibodies used in this study were as follows: SIRT1 (1:500, Cell Signaling Technology, Danvers, MA); PARP-1 (Poly(ADP-ribose) polymerases-1; 1:1000, Santa Cruz); cleaved caspase-3 (1:500, Cell Signaling Technology); Bax (1:500,

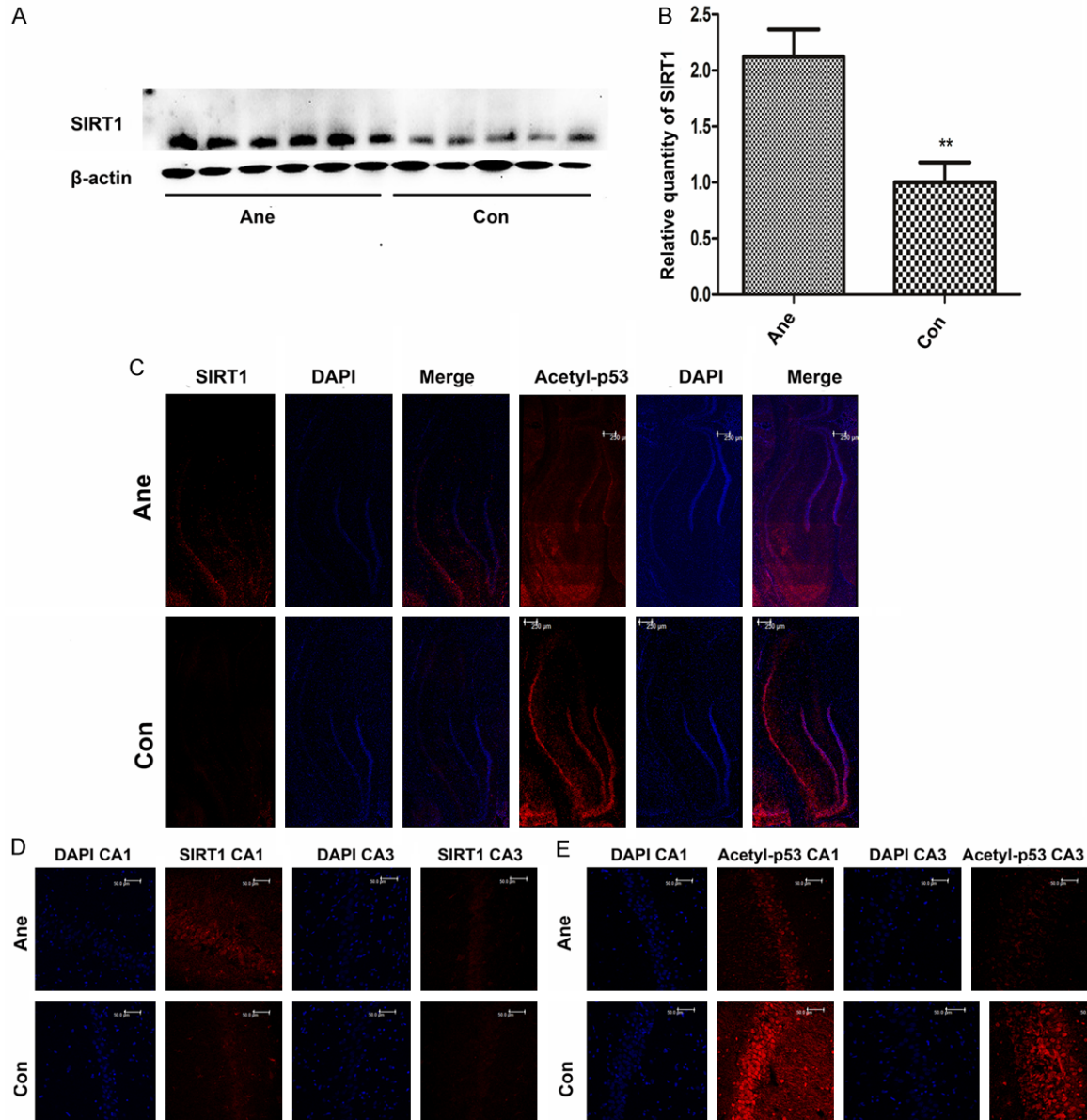


Figure 2. SIRT1 expression and p53 deacetylation at 48 hours after general anesthesia. $\bar{x} \pm \text{SD}$. **A:** SIRT1 expression was detected by Western Blot analysis. $n = 6$ for Ane group, $n = 5$ for Con group. **B:** The relative level of SIRT1 was quantified. **C:** The expressions of SIRT1 and acetyl-p53 in all the subfields of the hippocampus were detected by immunofluorescence staining. Scale bar: 250 μm . **D, E:** The expressions of SIRT1 and acetyl-p53 in CA1 and CA3 areas was detected by immunofluorescence staining. Scale bar: 50 μm , $n = 5$. $^{**}P < 0.01$.

Santa Cruz); and β -actin (Santa Cruz). Then the membranes were incubated with the secondary antibody (Santa Cruz). Blots were visualized with an ECL detection kit (Pierce, Rockford, IL, USA) and analyzed using Quantity One 1-D Analysis Software (Bio-Rad, San Francisco, CA).

Immunofluorescence

The brains were placed in 10% sucrose and then in 30% sucrose (in 0.1 M phosphate buf-

fer) at 4°C. Coronal sections were cut on a microtome. After blocking of nonspecific epitopes with 10% goat serum in PBS with 0.5% Triton-100 for 2 hours at room temperature, five sections (thickness, 20 μm ; space, 100 μm) of each rat (six rats per group) were incubated with primary antibodies for 48 hours at 4°C, respectively. Then, the sections were incubated for 4 hours at room temperature with secondary antibodies (Alexa Fluor-488 and Alexa Fluor-555; Invitrogen). The primary anti-

bodies were SIRT1 (1:100), acetylated p53 (acetylated p53 at lysine 381; 1:250; Abcam, Cambridge, USA), cleaved caspase-3 (1:100). Fluorescence analyses were performed using a confocal laser (Leica QWin, Germany).

Statistical analysis

All analyses were performed using SPSS 18.0 software (SPSS, Chicago, IL). The escape latencies and the swimming speed were analyzed using repeated-measures ANOVA. Unpaired two-tailed Student *t* test was used to evaluate the significance of differences of other results, and $P < 0.05$ was considered significant.

Results

Experiment 1

Neurotoxicity induced by general anesthesia in aged rats: We first determined whether general anesthesia with sevoflurane plus N_2O induced neurotoxicity in the hippocampus. We observed a significant increase in the levels of cleaved caspase-3 (3.3-fold; $P = 0.006$) and Bax (3-fold; $P = 0.03$). We also found an increase tendency in the expression of PARP-1, a DNA damage sensor, at 48 hours after general anesthesia, although there was no significant difference ($P = 0.07$) (**Figure 1A, 1B**). Moreover, the expression of cleaved caspase-3 was significantly increased in the anesthesia group as assayed by immunofluorescence staining (**Figure 1C**).

SIRT1 protein expression and p53 deacetylation

Previous study found that SIRT1 could mediate the deacetylation of p53 and attenuate stress-induced apoptosis by reducing the ability of p53 to induce the transcription of the proapoptotic factor Bax [15]. Based on this, we hypothesized that SIRT1 was involved in the prevention of anesthetics-mediated neurotoxicity through the deacetylation of p53. Interestingly, the results of western blot analysis demonstrated a 112.2% increase in SIRT1 expression in rats from anesthesia group compared to those from control group ($P = 0.0062$, **Figure 2A, 2B**). We performed immunofluorescence staining assay and observed a marked reduction in acetyl-p53 expression compared to control group (**Figure 2C-E**).

Behavioral results

The performance of rats in the MWM was showed in **Figure 3**. The rats in anesthesia group had significant longer latency as compared with the control group ($P = 0.041$ on day 2, $P = 0.033$ on day 5, and $P = 0.003$ on day 3) (**Figure 3A**). Moreover, a significant day effect was found but no day * group interaction was noted ($P = 0.558$). The latencies decreased significantly across sessions during the 6 days of training ($P < 0.001$), indicating that the rats both in control group and anesthesia group had successfully learned to navigate to the platform. Additionally, no significant differences were found in the swimming speeds ($P = 0.11$) (**Figure 3B**), suggesting that the impairment of cognitive functions did not effect motor ability.

To investigate the effect on spatial memory, the probe trial was conducted on day 7 (9 days after general anesthesia), and the percentage of time spent and distance traveled in the target quadrant (southeast) where the platform had been located in the navigation phase was analyzed. The rats in control group displayed a significant preference for the target quadrant in comparison with the other three control quadrants ($P < 0.05$), while the rats in anesthesia group did not spend significant more time in the southeast quadrant than in southwest quadrant (**Figure 3C**). However, the anesthesia group showed a significant reduced percentage of the time crossing over the target quadrant compared to the control group ($P = 0.022$) (**Figure 3D**), and similar results were obtained for the distance traveled in the target quadrant ($P = 0.009$) (**Figure 3D**).

The changes in SIRT1 expression and neurotoxicity after probe test

Rats were killed and the brains were dissected immediately after the probe trials on day 7 to further evaluate the neurotoxicity induced by anesthesia with sevoflurane plus N_2O . Western blot analysis showed a 2.6-fold increase in cleaved caspase-3 ($P = 0.033$) and a 46% increase in PARP-1 ($P = 0.034$) levels, whereas the level of Bax was slightly increased in the hippocampus of rats in anesthesia group compared to control group with no statistical difference (**Figure 4A, 4B**). Meanwhile, for immunofluorescence staining, we found that the cleaved caspase-3 expression was significantly

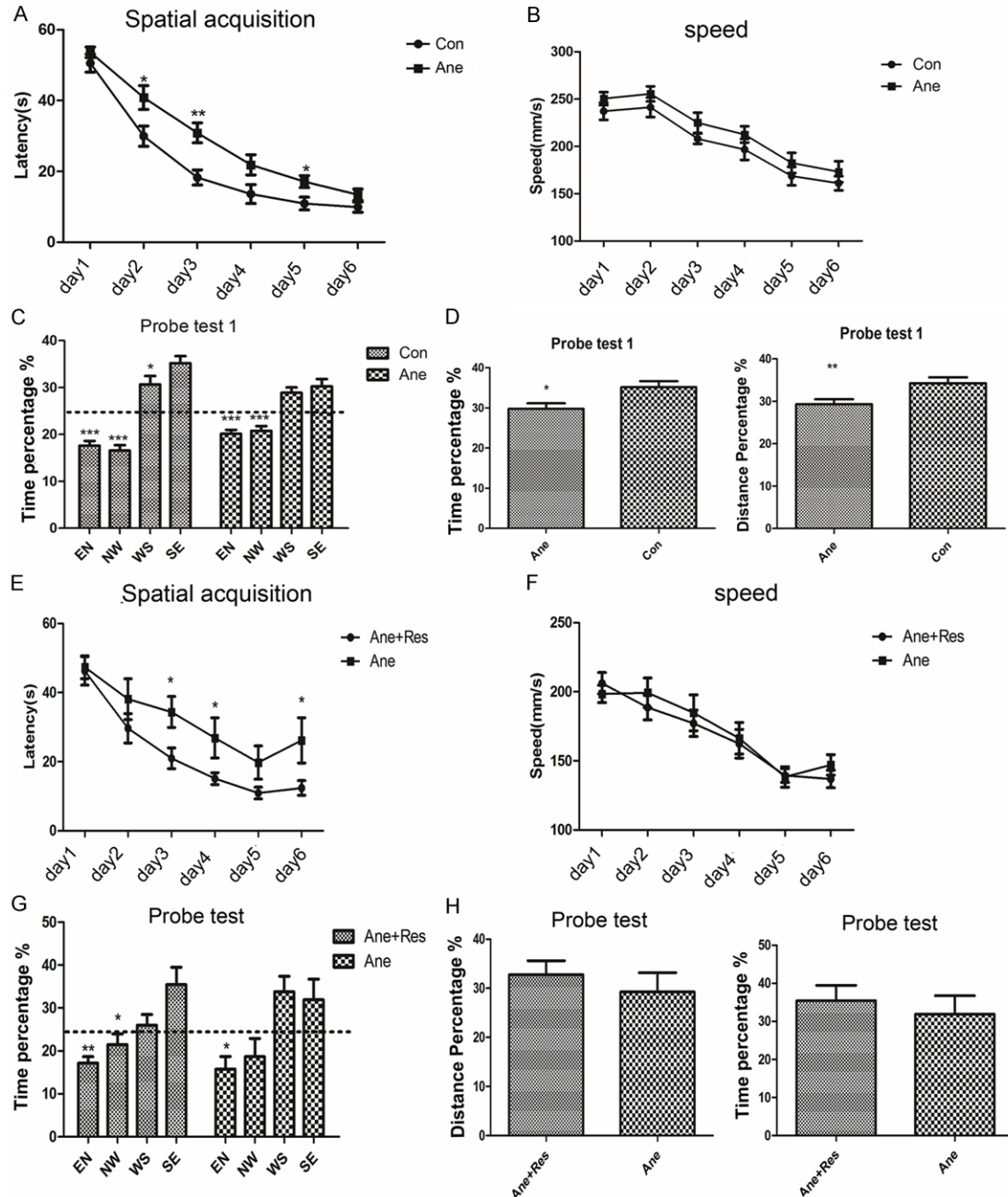


Figure 3. Behavioral results of the Morris Water Maze assay. $\bar{x} \pm \text{SEM}$. A: The mean escape latency (in seconds) in the phase of spatial acquisition. The significant differences were found on day 2 ($P = 0.041$), day 3 ($P = 0.033$) and day 5 ($P = 0.003$). B: There was no significant difference in the swimming speed between the two groups during the training days ($P = 0.11$). C: The percentage of time spent in each quadrant during the probe test 1 conducted on day 7. D: The percentage of time spent and distance traveled in the target quadrant in the probe test 1. E: The escape latency in the phase of spatial acquisition. The significant difference was found on day 3 ($P = 0.02$), day 4 ($P = 0.049$) and day 6 ($P = 0.044$). F: No significant difference was observed on the swimming speed between the two groups. G: The percentage of time spent in each quadrant during the probe test. H: The percentages of time spent and distance traveled in the target quadrant. * $P < 0.05$; ** $P < 0.01$.

increased in rats exposed to anesthetics (Figure 4C).

Next, we evaluated the changes in SIRT1 expression. On day 7, western blot analysis

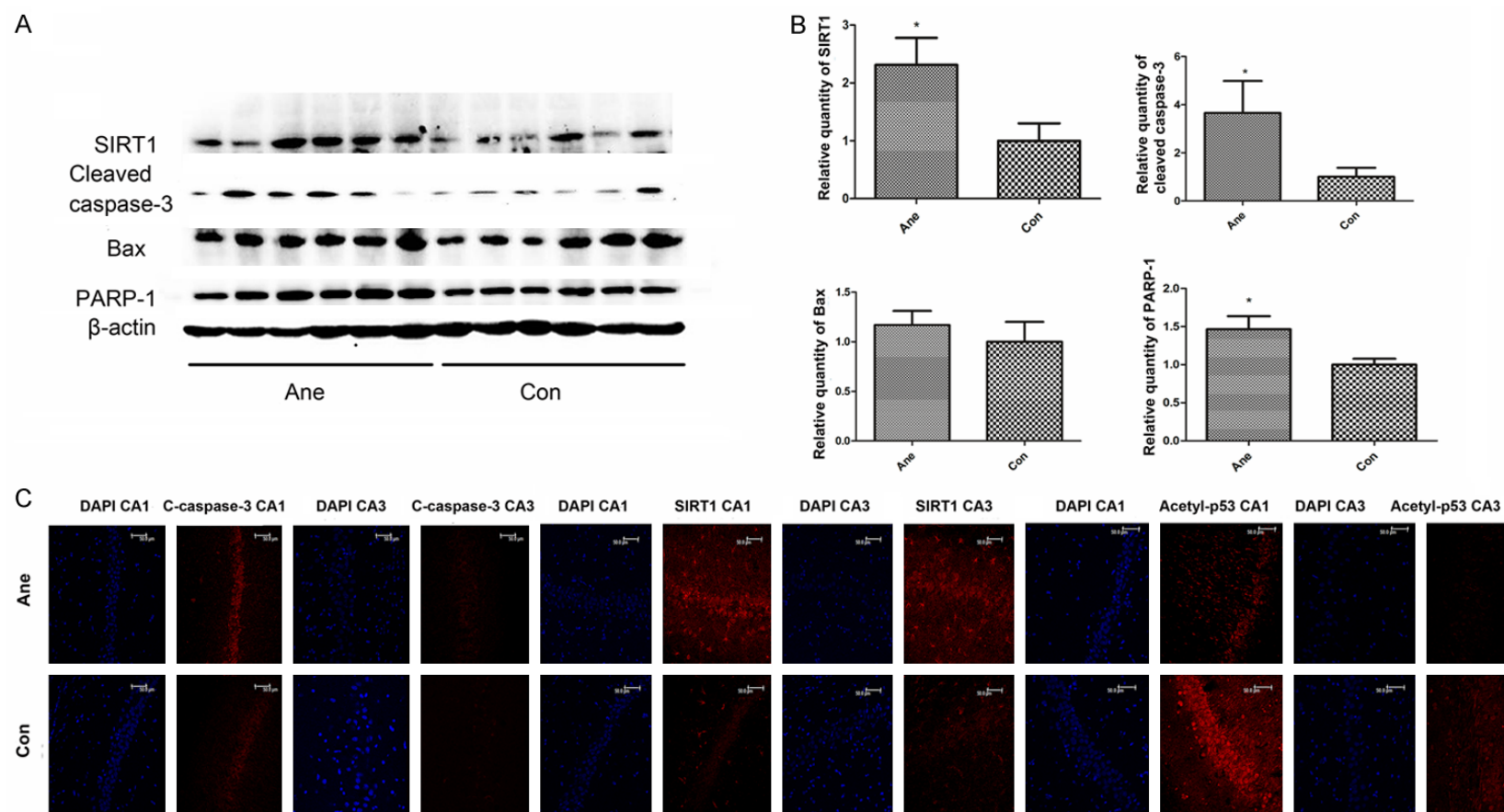


Figure 4. SIRT1 expression and neurotoxicity after probe test. $\bar{x} \pm \text{SD}$. A: The Western Blot results showed a significant increase in the expressions of SIRT1, cleaved caspase-3 and PARP-1 in the hippocampus of exposed rats compared to the control rats, while the expression of Bax was only slightly increased, $n = 6$. B: The relative levels of SIRT1, cleaved caspase-3, Bax, PARP-1 were quantified. C: The expressions of cleaved caspase-3, SIRT1 and acetyl-p53 in the CA1 and CA3 areas of the hippocampus were detected by immunofluorescence staining. Scale bar: 50 μm , $n = 6$. * $P < 0.05$; ** $P < 0.01$.

showed a 131% increase in SIRT1 expression in the anesthesia group ($P = 0.03$) (**Figure 4A, 4B**). In consistent with western blot assay, as assayed by immunofluorescence staining and shown in **Figure 4C**, the anesthetized rats exhibited increased SIRT1 expression and decreased acetyl-p53 expression.

Experiment 2

Neuroprotective effect of resveratrol in aged rats anesthetized with sevoflurane plus N₂O: To examine whether resveratrol can protect neurons against general anesthesia-induced damage in vivo, rats were treated daily with resveratrol 100 mg/kg or vehicle for 7 days before exposure to sevoflurane plus N₂O. Immediately after probe trial the rats were killed. The expressions of SIRT1 and acetyl-p53 were measured in the hippocampus. We observed that the expression of SIRT1 was increased 2.6-fold ($P = 0.004$) (**Figure 5A, 5B**). Immunofluorescence staining showed that SIRT1 expression was enhanced, and acetyl-p53 expression was significantly decreased (**Figure 5C, 5D**). Next, neuronal apoptosis was assessed. We found that resveratrol pretreatment decreased the staining of cleaved caspase-3 compared to the anesthesia group (**Figure 5D**). Furthermore, western blot analysis showed that the levels of cleaved caspase-3, Bax and PARP-1 were significantly decreased in the resveratrol pretreatment group ($P = 0.01$ and $P = 0.008$, respectively) (**Figure 5A, 5B**), whereas the level of bcl-2 was increased ($P = 0.02$) (**Figure 5A, 5B**). These data provided evidence that pretreatment with resveratrol increased SIRT1 expression and enhanced its deacetylated activity, which played some role in alleviating neurotoxicity in the hippocampus induced by sevoflurane plus N₂O.

Resveratrol improved cognitive function

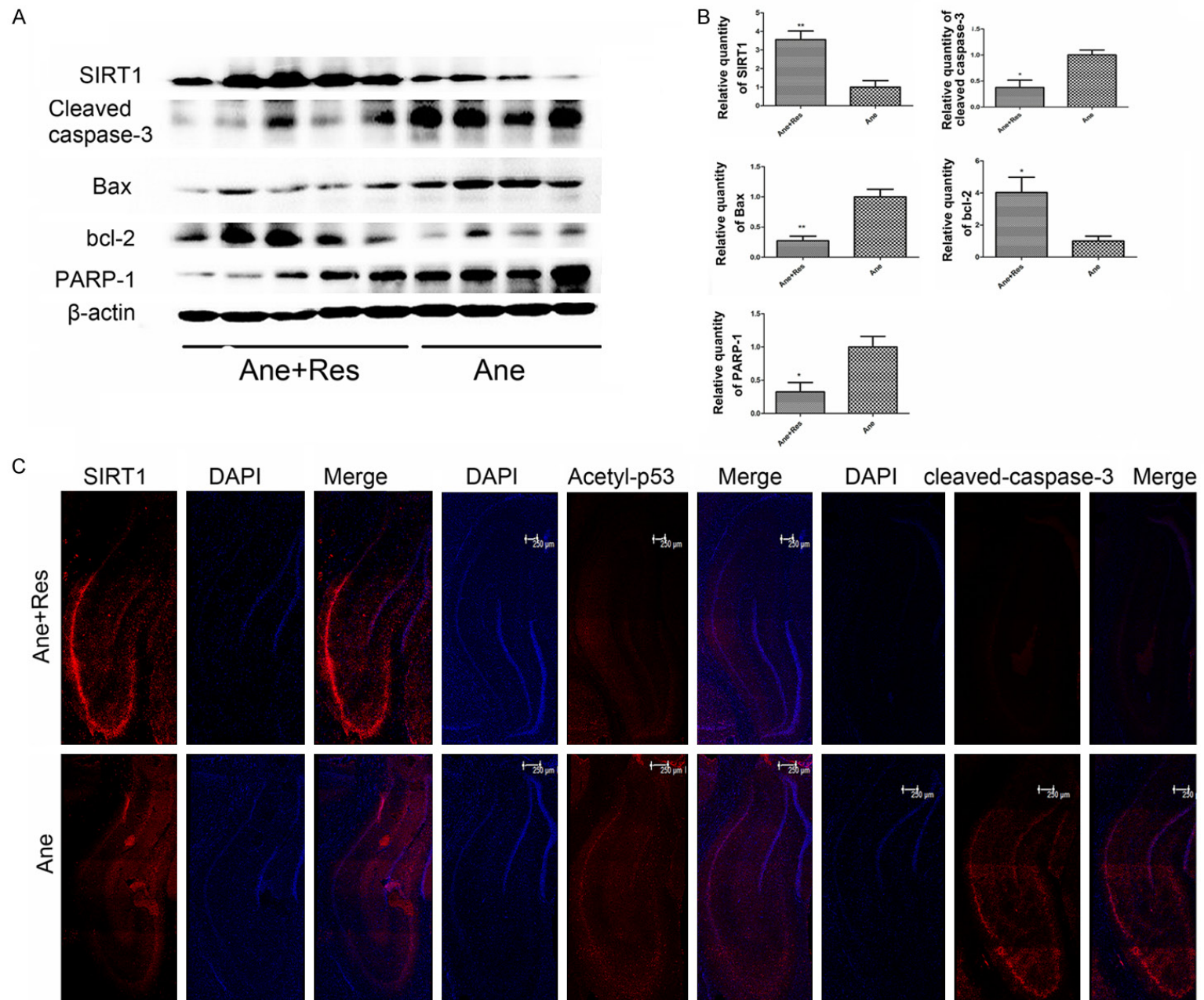
To determine whether the neuroprotection of resveratrol could protect against cognitive dysfunction, MWM testing was performed. The results indicated that the rats in resveratrol pretreatment group had significant shorter latency on several individual days compared to anesthesia group ($P = 0.020$ on day 3, $P = 0.049$ on day 4, and $P = 0.044$ on day 6) (**Figure 3E**). However, no statistical difference was observed regarding the swimming speed between the experiment groups ($P = 0.912$) (**Figure 3F**).

For the probe test, the rats in the resveratrol pretreatment group spent more time in the target quadrant than in the other three quadrants (significant differences in the northeast and the northwest, $P = 0.01$ and $P = 0.02$, respectively), while the rats in the anesthesia group spent no more time in the target quadrant than in the southwest and northwest quadrants ($P = 0.87$ and $P = 0.08$, respectively) (**Figure 3G**). There was no significant difference in the time spent in the target zone and the distance traveled (**Figure 3H**). Thus, resveratrol pretreatment selectively protected against the spatial memory deficit induced by general anesthesia.

Discussion

Learning and memory impairment after operation may occur in all age groups, but elderly patients seem to be at increased risk [14]. The aged brain differs from the younger brain in several respects, such as size, distribution, type of neurotransmitters, metabolic function, and capacity for plasticity, suggesting that the aged brain might be more susceptible to anesthetic-mediated neurotoxicity [15]. A growing body of laboratory evidence suggests that general anesthesia with isoflurane, N₂O, isoflurane plus N₂O, or sevoflurane may be neurotoxic to aging brains and may lead to cognitive dysfunction. In the present study, we demonstrated impairment in the hippocampus-dependent learning and memory performance in 18-month-old anesthetized rats, which is consistent with another report [16]. In the recall phase, which was performed 24 hours after the last acquisition session, we found the impairment of memory recall in the probe trial.

The underlying mechanisms remain unclear. Several pathomechanisms have been postulated, including age-related neuroinflammation [17], amyloid β peptide accumulation and cell apoptosis [18]. Studies have proposed that inhalational anesthetics induced apoptosis by a disruption of intracellular calcium homeostasis [19, 20]. Zhang et al [21] demonstrated that treatment with 2% isoflurane for 6 hours increased proapoptotic factors and decreased anti-apoptotic factors in cultured cells and in mice. The current study found that 48 hours after general anesthesia, the levels of proapoptotic markers cleaved caspase-3, Bax, and DNA damage sensor PARP-1 significantly increased,



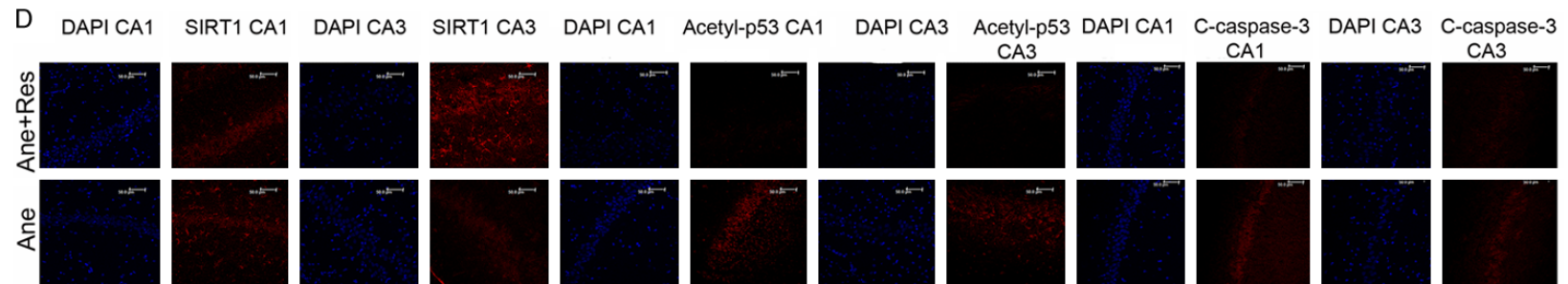


Figure 5. Effect of resveratrol on the neurotoxicity and SIRT1 expression after general anesthesia. $\bar{x} \pm SD$. A: The expressions of SIRT1, cleaved caspase-3, Bax and PARP-1 were assessed by Western Blot. $n = 5$ for Res+Ane group, $n = 4$ for Ane group. B: The relative levels were quantified. C: The expressions of SIRT1 and acetyl-p53 in all the subfields of hippocampus was assessed by immunofluorescence staining. Scale bar: 250 μm . D: The expressions of SIRT1, acetyl-p53 and cleaved caspase-3 in the CA1 and CA3 areas of the hippocampus were detected by immunofluorescence staining. Scale bar: 50 μm , $n = 6$. * $P < 0.05$; ** $P < 0.01$.

whereas less difference was noted in Bax and cleaved caspase-3 on day 7 compared with that noted after 48 hours. However, when inhibiting neuronal apoptosis by pretreatment with resveratrol, behavioral performance was improved in the navigation phase. Thus, the results suggest that cleaved caspase-3 and Bax, the down targets of p53, are major contributors to neurotoxicity, indicating that p53-dependent apoptosis is likely responsible for the impaired behavioral performance of the anesthetized rats.

SIRT1 came to the attention of the medical profession after finding it associated lifespan-prolonging activity with caloric restriction [22]. SIRT1 is highly expressed in the developing central nervous system, and numerous studies using pharmacological or genetic manipulation have shown neuroprotection with SIRT1 activation in central nervous system injuries [23]. In fact, part of the lifespan extension of SIRT1 may be attributed to its protective effect against neuronal degeneration. SIRT1 has been demonstrated to have beneficial effects in several neurodegenerative disease models, including amyotrophic lateral sclerosis [8] and Alzheimer disease [24]. As one of the deacetylated targets of SIRT1, p53 is the first nonhistone protein and shown to be regulated functionally by acetylation and deacetylation, and its acetylation plays a key role in controlling promoter-specific activation of p53 targets during stress responses. Deacetylation by SIRT1 decreases binding of p53 to its targets and represses transcriptional activity, thereby suppressing p53-mediated apoptosis [25]. In the current study, we found that neurotoxicity induced by anesthetics triggered the overexpression of SIRT1 and its deacetylation of p53 shifted the function of p53 to cell protection rather than neuronal death, thus providing neuroprotection against the neuronal insult.

To further confirm whether the exogenous stimulation of SIRT1 prevents neuron injury, resveratrol, a potential SIRT1 activator, was administered. Resveratrol directly induced SIRT1 expression at transcriptional or translational levels [26] and induced the deacetylation of endogenous p53 in cells [27]. Studies have shown that knockdown or knockout of SIRT1 inhibited the resveratrol-mediated changes in gene expression and protein posttranslational

modifications, including p53 deacetylation [28], providing a direct link between resveratrol and SIRT1. In our current study, rats pretreated with resveratrol exhibited better performance during the navigation phase of the MWM than the rats in the vehicle plus anesthesia group. Consistent with the behavioral results, the significant decreases in the markers of cell apoptosis, including Bax and cleaved caspase-3, could explain some of the behavioral improvement in the resveratrol plus anesthesia treatment group.

Conclusively, the major original findings of the present study are summarized as follows: First, we demonstrated that aged rats exposed to 1.3% sevoflurane plus 50% N₂O had impaired learning and memory for at least 1 week. Second, results showed that exposure to sevoflurane plus N₂O induced hippocampal neuronal apoptosis accompanied by endogenous increase in SIRT1 expression. Third, we showed that pretreatment with SIRT1 activator resveratrol promoted neuronal survival and protected against the neurotoxicity induced by general anesthesia in aged rats. Fourth, our behavioral studies demonstrated that resveratrol also enhanced acquisition of spatial memory and protected against the learning and memory impairment induced by general anesthesia *in vivo*. Thus, resveratrol, by activating the SIRT1 pathway, enhanced neuronal survival against neurotoxicity and improved behavioral performance, thereby providing a possible pathway for prevention and treatment of learning and memory impairment induced by general anesthesia.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Sheng-Jin Ge, Department of Anesthesia, Zhongshan Hospital, Fudan University, No. 180 Fenglin Road, Shanghai 200032, China. Tel: +86-21-64041990-2331; Fax: +86-21-64038472; E-mail: ge.shengjin@fudan.edu.cn

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