

# Nobiletin ameliorates isoflurane-induced cognitive impairment via antioxidant, anti-inflammatory and anti-apoptotic effects in aging rats

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**Abstract.** A recent study reported that nobiletin is an active ingredient in *Fructus Aurantii immaturus* and *Pericarpium Citri Reticulatae*, which may be capable of preventing ischemic stroke. Therefore, the present study aimed to determine the neuroprotective effects of nobiletin, and to evaluate whether it could ameliorate isoflurane-induced cognitive impairment via antioxidant, anti-inflammatory and anti-apoptotic effects in aging rats. Male Sprague-Dawley rats (age, 18 months) were used to analyze the neuroprotective effects of nobiletin. Morris water maze test was used to determine cognitive competence. Enzyme-linked immunosorbent assay and western blot analysis were also used to quantify nuclear factor- $\kappa$ B, tumor necrosis factor (TNF)- $\alpha$ , IL-1 $\beta$ , IL-6, glutathione, (GSH), GSH-peroxidase, superoxide dismutase and malondialdehyde concentration and relevant protein expression levels. Cognitive competence was increased in isoflurane-treated rats following treatment with nobiletin. In addition, as expected, nobiletin exerted antioxidant, anti-inflammatory and anti-apoptotic effects on isoflurane-induced cognitive impairment in aging rats. Treatment with nobiletin induced the activation of phosphorylated (p)-Akt, p-cAMP response element binding protein (CREB) and brain-derived neurotrophic factor (BDNF) protein expression and reduced the levels of B-cell lymphoma 2-associated X protein (Bax) in isoflurane-induced rats. In conclusion, the present study demonstrated that nobiletin may ameliorate isoflurane-induced cognitive impairment through antioxidant, anti-inflammatory and anti-apoptotic effects via modulation of Akt, Bax, p-CREB and BDNF in aging rats. These findings provide support for the molecular

mechanisms underlying the effects of nobiletin treatment on isoflurane-induced damage.

## Introduction

Inhalational anesthetics have been used in clinical practice for >170 years; they have been extensively applied due to their numerous advantages, including anesthetic efficacy and safety, and the ease at which they can be used to regulate anesthetic depth (1). In the United States, the annual number of cases where general anesthesia is used is ~40,000,000, and anesthesia has an important role in the safety and success of surgery. In recent years, inhalational anesthetics have been reported to protect against ischemic injury in the cardiovascular system, brain, kidney and other important organs (2); and the potential clinical application of these drugs may be wider than at present. However, further studies are required on how inhalational anesthetics induce unconsciousness, immobilization and analgesia, and other general anesthetic effects. In addition, it is necessary to determine how inhaled anesthesia induces intraoperative awareness and postoperative agitation, nausea and vomiting, and postoperative cognitive decline (POCD), and how it impacts the intelligence and central nervous system development of young children (3). Such issues have attracted extensive clinical attention. Clarification of the aforementioned pathogenetic mechanisms not only has far-reaching significance on revealing the underlying mechanism of general anesthesia, but may also be used to guide clinical medication, improve the safety of clinical anesthesia, and for the development of novel general anesthetics and antagonists (4). Understanding the underlying mechanisms of anesthesia is conducive to understanding the neuroscience of consciousness, memory, perception, movement and awakening (5).

The pathogenetic mechanism of POCD is complex, and research regarding the mechanism is diverse. It has previously been indicated that halothane can induce cognitive impairment (6). In addition, it has been reported that increased hippocampal inflammatory cytokine expression may induce transitory cognitive impairment (7). In mice with an interleukin (IL)-1 receptor knockout, peripheral operation-induced hippocampal neuronal inflammation and IL-1 $\beta$ -induced autoimmune response are associated with memory impairment.

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In addition, continuous excessive expression of hippocampal IL-1 $\beta$  may lead to contextual memory and spatial memory impairment of mice (8).

Nobiletin (Fig. 1) is a type of polymethoxyflavonoid, which is predominantly found in *Pericarpium Citri Reticulatae*, a traditional Chinese medicine (9). Nobiletin exists in the pericarp of citrus fruits, and exerts extensive physiological effects that are beneficial to health. Therefore, nobiletin has attracted clinical attention (10). As well as its anti-inflammatory, antioxidant and anticancer activity, nobiletin has roles in anti-atherosclerosis, reducing blood glucose levels, liver protection and nerve nutrition (11). Previous studies have demonstrated that rat hippocampal neurons cultivated in nobiletin may enhance the signal channel of protein kinase A/extracellular signal-regulated kinase/cAMP response element binding protein (CREB) to alleviate memory deterioration caused by P-amyloid protein in mice with Alzheimer's disease model and amyloid precursor protein genetically modified, and to improve learning and memory disorder caused by cerebral ischemia (12,13). The aim of the present study was to determine the neuroprotective effects of nobiletin, and to evaluate whether it could ameliorate cognitive impairment via antioxidant, anti-inflammatory and anti-apoptotic effects in isoflurane-treated aging rats.

## Materials and methods

**Ethical approval and experimental animals.** The animal protocol was approved by the Standing Committee on Animals at Shandong University (Jinan, China). Male Sprague-Dawley rats (age, 18 months) were acquired from the Institute of Experimental Animals, Shandong University. All rats were given *ad libitum* access to food and water, and were maintained under controlled laboratory conditions: 12/12 h light/dark cycle, 55 $\pm$ 5% humidity and 23 $\pm$ 2 $^{\circ}$ C.

**Rat model of isoflurane-induced cognitive impairment.** All aging rats were allowed to acclimate to the environment for 1 week, and were then randomly separated into four groups: i) Isoflurane group (n=20); ii) nobiletin (10) group (n=20); iii) nobiletin (25) group (n=20); and iv) sham group (n=10). Rats in the isoflurane and nobiletin (10) and (25) groups were administered 1.4% isoflurane in a 100% oxygen environment for 2 h in an anesthetization chamber. Rats were maintained at 37.5 $\pm$ 0.5 $^{\circ}$ C using a heating pad. In the nobiletin (10) and (25) groups, the rats were intraperitoneally injected with nobiletin (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) at 10 or 25 mg/kg/day for 3 days, respectively (1).

**Behavioral testing.** A circular pool (150x50 cm; depth, 31 cm; 22 $\pm$ 1 $^{\circ}$ C) was used to conduct the Morris water maze test. The pool area was divided into SW, NW, SE and NE quadrants. A transparent platform (diameter, 9 cm) 2 cm below water level was placed in the center of the SW quadrant. All rats were trained to find the escape platform in order to test reference memory. The rats underwent eight trials for 30 min every day. The maximum swimming time was set at 120 sec and ended with the animal finding the platform. Each rat was gently guided to the platform, which remained for 30 sec, if the time limit was exceeded. Escape latency, path length (length taken

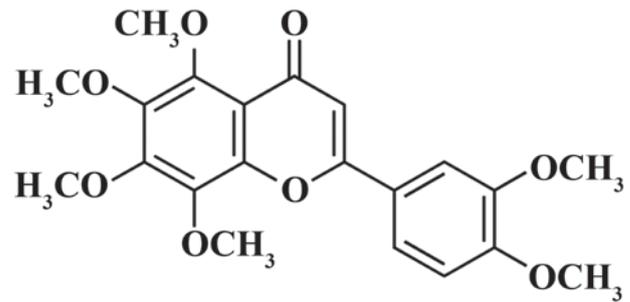


Figure 1. Chemical structure of nobiletin.

to reach the platform) and swimming speed were all analyzed. After the last reference memory test, the spatial probe test was performed. Briefly, the rats were placed into the water and were allowed to swim for 120 sec. Path length and time spent in the target quadrant were recorded. The number of platform crossings were analyzed using Actimetrics motion detection software for the Morris Water Maze (Actimetrics Software, Evanston, IL, USA).

**Enzyme-linked immunosorbent assay (ELISA).** Whole blood samples were immediately collected by centrifugation at 4,000 x g for 10 mins at 4 $^{\circ}$ C to measure serum nuclear factor (NF)- $\kappa$ B, tumor necrosis factor (TNF)- $\alpha$ , IL-1 $\beta$  and IL-6 concentration using ELISA kits obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Glutathione (GSH; Nanjing Jiancheng Bioengineering Institute), GSH peroxidase (GSH-PX; Nanjing Jiancheng Bioengineering Institute), superoxide dismutase (SOD; Elabscience Biotechnology Co. Ltd., Wuhan, China) and malondialdehyde (MDA; Elabscience Biotechnology Co. Ltd.) concentrations were also determined using ELISA kits, according to the manufacturers' protocols.

**Western blot analysis.** The rats were anesthetized with 30 mg/kg pentobarbital sodium and then sacrificed by decollation. Hippocampi were then immediately removed and maintained in liquid nitrogen. Frozen hippocampi were subsequently weighed, and ~50 mg tissue was homogenized in RIPA buffer with protease inhibitors (Beyotime Institute of Biotechnology, Haimen, China). The homogenates were centrifuged at 12,000 x g for 10 min at 4 $^{\circ}$ C, and the supernatant was collected in order to measure protein content using bicinchoninic acid assay kit according to the manufacturer's protocol (Beyotime Institute of Biotechnology). Equal amounts of protein samples (50  $\mu$ g) were separated by 10-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were transferred to polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). After blocking with 5% skimmed milk in Tris-buffered saline 1% Tween-20 for 1 h, the membranes were probed with the following primary antibodies: Anti-B-cell lymphoma 2-associated X protein (Bax; cat. no. sc-6236; 1:500; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-phosphorylated (p)-Akt (cat. no. sc-135650; 1:500; Santa Cruz Biotechnology, Inc.), anti-p-CREB (cat. no. sc-81486; 1:500; Santa Cruz Biotechnology, Inc.), anti-brain-derived neurotrophic factor

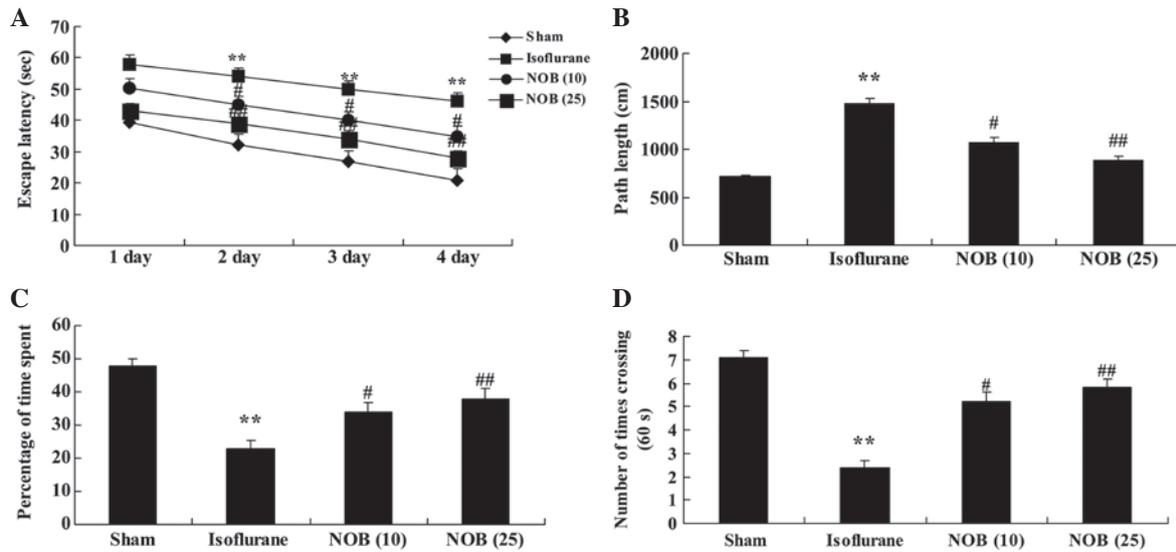


Figure 2. Memory testing of isoflurane-treated rats. (A) Escape latency, (B) path length, (C) time spent in target quadrant and (D) number of times the rat crossed the platform. \*\*P<0.01 compared with the sham group; #P<0.05, ##P<0.01 compared with the isoflurane group. NOB (10), 10 mg/kg nobiletin; NOB (25), 25 mg/kg nobiletin.

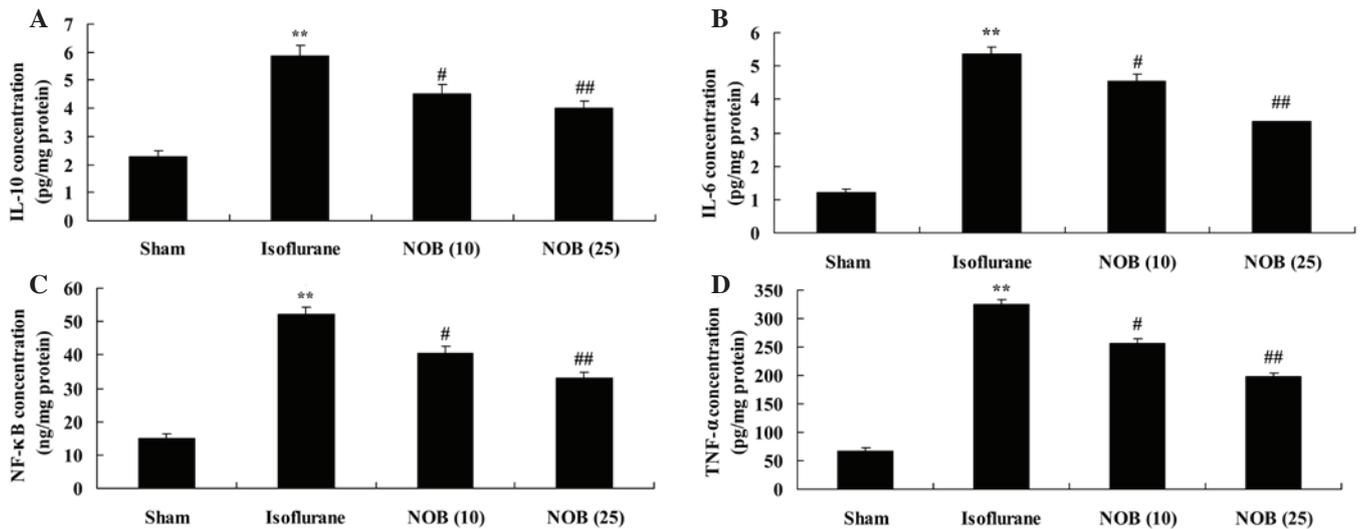


Figure 3. Antioxidant effects of nobiletin in isoflurane-treated rats. (A) Glutathione peroxidase (GSH-PX), (B) GSH, (C) superoxide dismutase (SOD) and (D) malondialdehyde (MDA) concentrations. \*\*P<0.01 compared with the sham group; #P<0.05, ##P<0.01 compared with the isoflurane group. NOB (10), 10 mg/kg nobiletin; NOB (25), 25 mg/kg nobiletin.

(BDNF; cat. no. sc-20981; 1:500; Santa Cruz Biotechnology, Inc.) and anti-β-actin (cat. no. sc-130656; 1:500; Santa Cruz Biotechnology, Inc.) overnight at 4°C. Proteins were detected using horseradish peroxidase-conjugated anti-rabbit secondary antibodies (cat. no. sc-2054; 1:1,000; Santa Cruz Biotechnology, Inc.) at room temperature for 1 h. Blots were visualized using an enhanced chemiluminescence kit (Santa Cruz Biotechnology, Inc.). The membranes were analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

**Statistical analysis.** Results are presented as the mean ± standard deviation and were analyzed using one-way analysis of variance, followed by Tukey post-hoc multiple comparisons test using GraphPad Prism version 4.0 (GraphPad Software,

Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

## Results

**Memory testing.** To examine whether nobiletin exerted neuroprotective effects on isoflurane-induced cognitive impairment, memory testing was conducted. As shown in Fig. 2A and B, treatment with isoflurane induced an increase in escape latency and path length compared with in the sham group. Treatment with nobiletin effectively decreased the escape latency and path length in a dose-dependent manner. In addition, there was a significant reduction in time spent in the target quadrant and the number of times the rat crossed the platform in the isoflurane model group compared with in the sham group (Fig. 2C

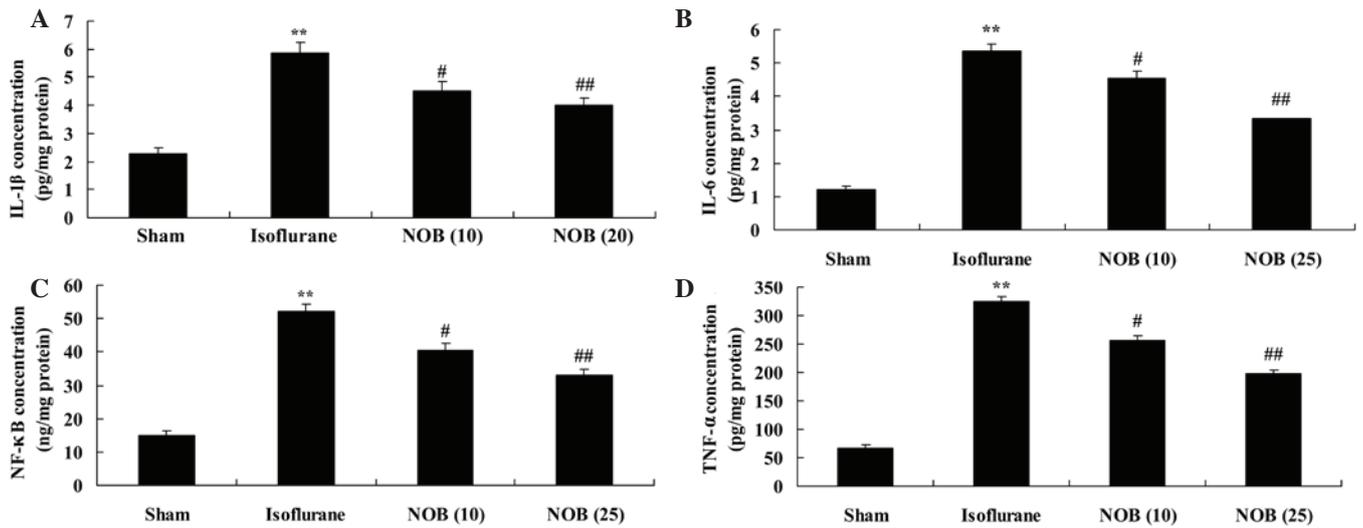


Figure 4. Anti-inflammatory effects of nobiletin in isoflurane-treated rats. (A) Interleukin (IL)-1 $\beta$ , (B) IL-6, (C) nuclear factor (NF)- $\kappa$ B and (D) tumor necrosis factor (TNF)- $\alpha$  concentrations. \*\* $P < 0.01$  compared with the sham group; # $P < 0.05$ , ## $P < 0.01$  compared with the isoflurane group. NOB (10), 10 mg/kg nobiletin; NOB (25), 25 mg/kg nobiletin.

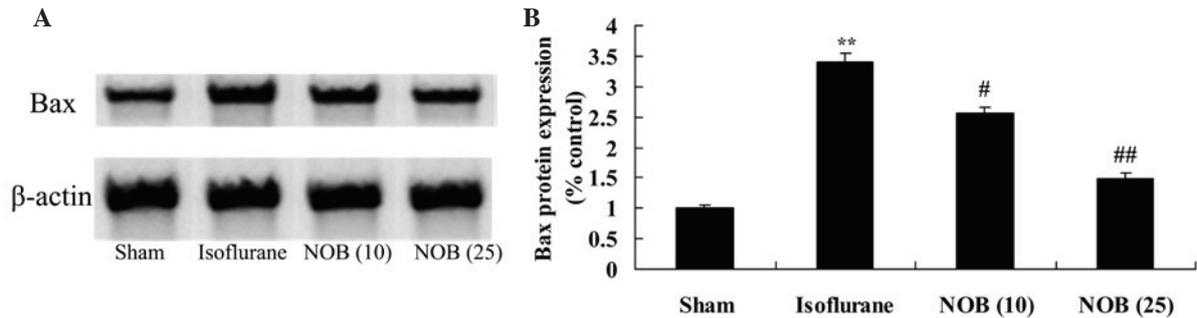


Figure 5. (A) Protein expression levels of B-cell lymphoma 2-associated X protein (Bax) were detected using western blot analysis. (B) Semi-quantitative analysis of Bax protein expression in isoflurane-treated rats. \*\* $P < 0.01$  compared with the sham group; # $P < 0.05$ , ## $P < 0.01$  compared with the isoflurane group. NOB (10), 10 mg/kg nobiletin; NOB (25), 25 mg/kg nobiletin.

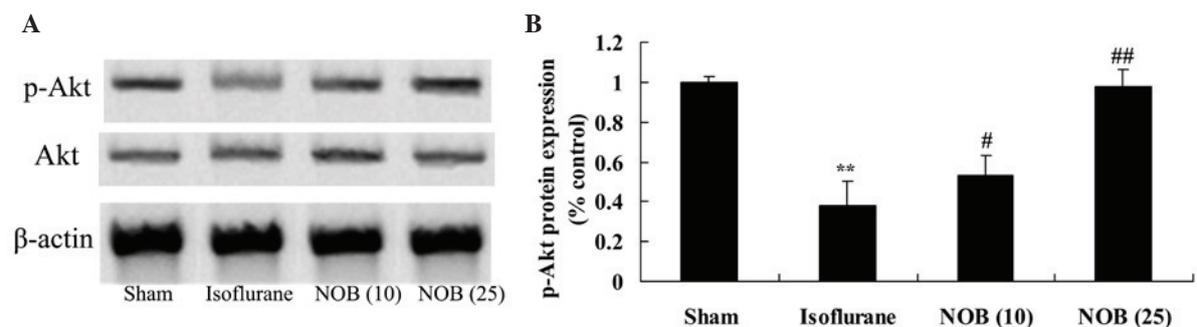


Figure 6. (A) Protein expression levels of phosphorylated (p)-Akt were detected by western blot analysis. (B) Semi-quantitative analysis of p-Akt protein expression in isoflurane-treated rats. \*\* $P < 0.01$  compared with the sham group; # $P < 0.05$ , ## $P < 0.01$  compared with the isoflurane group. NOB (10), 10 mg/kg nobiletin; NOB (25), 25 mg/kg nobiletin.

and D). However, treatment with nobiletin significantly increased time spent in the target quadrant and the number of times the rat crossed the platform (Fig. 2C and D).

**Antioxidant effects.** To examine whether nobiletin exerted neuroprotective effects on isoflurane-induced oxidative damage, GSH-PX, GSH, SOD and MDA concentrations

were analyzed using ELISA kits. As shown in Fig. 3A-D, isoflurane inhibited GSH-PX, GSH and SOD concentrations, whereas MDA concentration was increased in the isoflurane group compared with in the sham group. Conversely, treatment with nobiletin significantly increased GSH-PX, GSH and SOD concentrations, and reduced MDA concentration in isoflurane-treated rats (Fig. 3A-D).

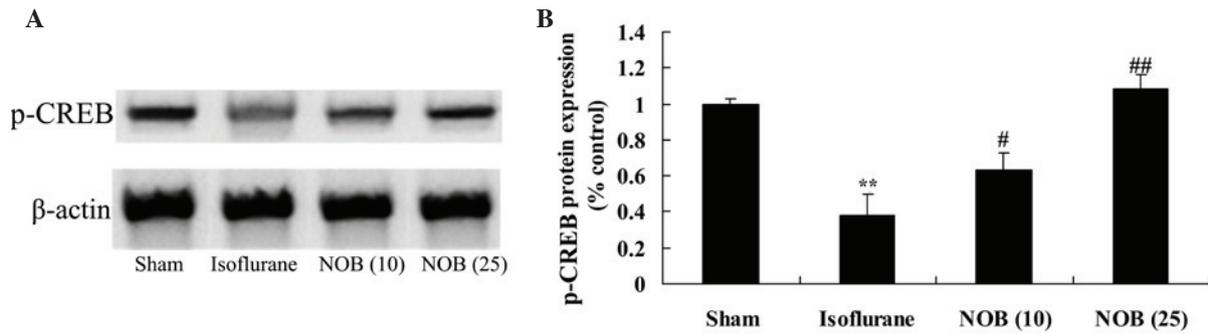


Figure 7. (A) Protein expression levels of phosphorylated-cAMP response element binding protein (p-CREB) were detected by western blot analysis. (B) Semi-quantitative analysis of p-CREB protein expression in isoflurane-treated rats. \*\*P<0.01 compared with the sham group; #P<0.05, ##P<0.01 compared with the isoflurane group. NOB (10), 10 mg/kg nobiletin; NOB (25), 25 mg/kg nobiletin.

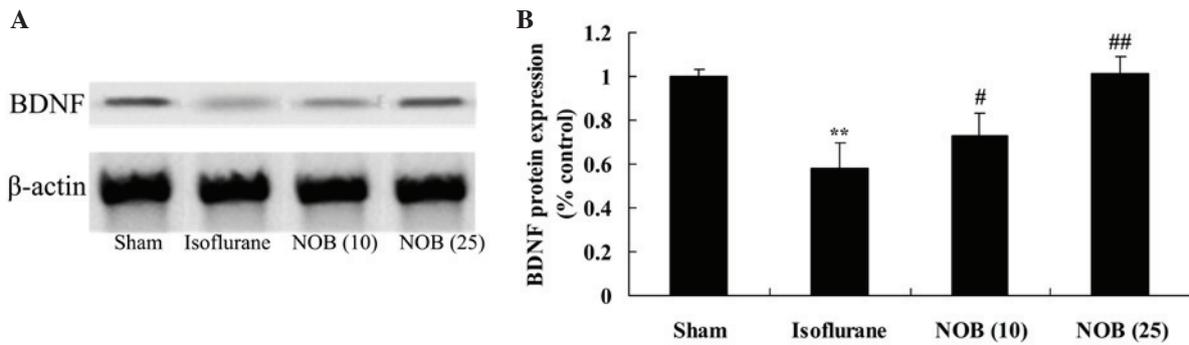


Figure 8. (A) Protein expression levels of brain-derived neurotrophic factor (BDNF) were detected by western blot analysis. (B) Semi-quantitative analysis of BDNF protein expression in isoflurane-treated rats. \*\*P<0.01 compared with the sham group; #P<0.05, ##P<0.01 compared with the isoflurane group. NOB (10), 10 mg/kg nobiletin; NOB (25), 25 mg/kg nobiletin.

**Anti-inflammatory effects.** To examine whether nobiletin exerted neuroprotective effects on isoflurane-induced inflammation, NF- $\kappa$ B, TNF- $\alpha$ , IL-1 $\beta$  and IL-6 concentrations were analyzed using ELISA kits. As shown in Fig. 4A-D, isoflurane enhanced NF- $\kappa$ B, TNF- $\alpha$ , IL-1 $\beta$  and IL-6 concentrations compared with in the sham group. However, treatment with nobiletin significantly reduced NF- $\kappa$ B, TNF- $\alpha$ , IL-1 $\beta$  and IL-6 concentrations compared with in the isoflurane-treated rats (Fig. 4A-D).

**Protein expression of Bax.** The present study assessed the effects of nobiletin on the protein expression levels of Bax by western blot analysis. Bax protein expression was activated by isoflurane compared with in the sham group (Fig. 5). Conversely, treatment with nobiletin significantly suppressed Bax protein expression in isoflurane-treated rats (Fig. 5).

**Protein expression of p-Akt.** The protein expression levels of p-Akt were detected in every group; p-Akt protein expression was decreased following treatment with isoflurane compared with in the sham group (Fig. 6). However, treatment with nobiletin markedly increased p-Akt protein expression compared with in the isoflurane-treated group (Fig. 6).

**Protein expression of p-CREB.** As shown in Fig. 7, exposure to isoflurane markedly decreased the protein expression levels of p-CREB compared with in the sham group. Treatment with nobiletin increased p-CREB protein expression compared with in the isoflurane-treated group (Fig. 7).

**Protein expression of BDNF.** To investigate the mechanism underlying the effects of nobiletin on isoflurane-induced cognitive impairment, the protein expression levels of BDNF were detected using western blot analysis. Compared with the sham group, BDNF protein expression was decreased following exposure to isoflurane anesthesia (Fig. 8). Conversely, the protein expression levels of BDNF were increased following treatment with nobiletin (Fig. 8).

## Discussion

The primary cause of POCD is the use of anesthesia, including frequently-used isoflurane, sevoflurane and other inhalational anesthetics, as well as intravenous anesthetics, such as ketamine and propofol (4). Surgical and anesthetic complications can accelerate POCD. POCD induced by isoflurane and other inhalational anesthetics is a complex issue, which has attracted attention from surgeons and anesthesiologists (14). At present, several studies have focused on cognitive impairment induced by isoflurane; however, whether the concentration of frequently-used isoflurane (1-3%) is able to induce cognitive impairment in patients undergoing surgery, as well as neuronal apoptosis associated with cognitive function, requires further study (15-17). The present study demonstrated that the neuroprotective effects of nobiletin ameliorated isoflurane-induced cognitive impairment in rats.

Proinflammatory and anti-inflammatory cytokines exist in the body; the balance between them is regulated by

the neuroendocrine and immune systems. An imbalance between proinflammatory and anti-inflammatory cytokines is a significant cause leading to inflammatory injury (18). Among the types of inflammatory damage that affect visceral organs, IL-1 $\beta$  is one of the earliest proinflammatory cytokines. The synergistic effects of IL-1 $\beta$  and TNF- $\alpha$  activate the inflammatory reaction-associated transcription factor, NF- $\kappa$ B, in immune cells and non-immune cells, which induces the inflammatory cascade reaction, promotes aggregation of granulocytes, and lead to tissue damage (19). The present study demonstrated that nobiletin significantly reduced isoflurane-induced NF- $\kappa$ B, TNF- $\alpha$ , IL-1 $\beta$  and IL-6 concentrations in rats. Guan *et al* (20) reported that nobiletin attenuated reactive oxygen species (ROS) production and the expression of nuclear NF- $\kappa$ B p65 in a rat model of carotid artery injury (20). Jang *et al* (12) revealed that nobiletin ameliorated scratching behavior via inhibiting the activation of NF- $\kappa$ B, activator protein-1 and p38 in mice.

Dysfunctional energy metabolism and oxidative damage have a role in cognitive impairment, since brain tissue is particularly sensitive to tissue and free radical damage (21). In addition, the brain tissue exerts strict demands on oxygen, and accounts for 20% of human body oxygen consumption. However, GSH has obviously decreased in brain tissues of isoflurane-induced aged rats (22). Sufficient evidence has revealed the relationship between oxidative stress and cognitive function. Antioxidants are able to reverse the memory impairment of aged rats (23,24). Through lipid metabolite detection and behavioral testing, it has been demonstrated that oxidative damage can aggravate transitory cognitive impairment (25). The learning capacity of mice is higher compared to that of aged rats. SOD is an essential antioxidant *in vivo*; however, a large amount of SOD may enhance oxidative stress (25). Therefore, decreased SOD levels may reflect increasing of oxidative stress levels. MDA is a lipid peroxidation product associated with increased ROS levels. MDA levels can reflect the degree of body lipid peroxidation, or indirectly reflect the degree of tissue and cell damage (22). The present study demonstrated that nobiletin significantly increased GSH-PX, GSH and SOD concentrations, and reduced MDA concentration in isoflurane-treated rats. Lo *et al* (11) reported that nobiletin inhibits low-density lipoprotein oxidation in THP-1 cells.

The Akt signal transduction pathway is associated with growth, proliferation and regulation of differentiation (26). The Akt pathway promotes survival, and its activation has an important role in nerve cell protection, particularly in hypoxic ischemic neuronal injury. It has recently attracted extensive attention (27). The activation of Akt can promote endothelial cell survival, decrease nerve damage, reduce inflammatory cell death and obstruct damage to thermoregulatory neurons (27). The present study demonstrated that nobiletin markedly increased isoflurane-induced p-Akt protein expression. Zhang *et al* (28) reported that nobiletin was able to activate p-Akt, p-CREB, BDNF and B-cell lymphoma 2 (Bcl-2) pathways in order to protect against cerebral ischemia in rats.

Apoptosis is a complex type of programmed cell death, which is associated with the regulation of several genes. The Bcl-2 gene family has an important role during the regulatory process. The Bcl-2 family can be divided into two categories,

according to effects on the apoptotic process: Proapoptotic genes, including Bax, Bcl-2 antagonist/killer 1 and Bcl-2 associated agonist of cell death; and anti-apoptotic genes, including Bcl-2, Bcl-extra large and Bcl-w. Interactions between Bcl-2 family proteins may affect cell survival and apoptosis. The interaction between Bax and Bcl-2 is of particular importance. Increased levels of Bax promote cell apoptosis; however, increased levels of Bcl-2 inhibit cell apoptosis. In the present study, nobiletin significantly suppressed Bax protein expression in isoflurane-treated rats. Malik *et al* (29) suggested that nobiletin may ameliorate cisplatin-induced acute kidney injury by increasing the expression of Bax, and exerting antioxidant, anti-inflammatory and anti-apoptotic effects.

Previous studies regarding learning memory behaviors have indicated that CREB, as 'the third messenger', is important in the long-term memory process (30-32). It is believed, as the optimal nuclear transcription factor, to adapt to external stimulus as well as long term memory. Both the cortical neuronal at plasticity forming process and hippocampal neuron under long range increase stimulation and memory training task, CREB phosphorylation and CRE reporter gene expression can be detected. However, in mice with decreased spatial memory ability, the phosphorylation level of CREB in the hippocampus has been reported to be markedly decreased (33). In the present study, the protein expression levels of CREB were enhanced following treatment with nobiletin, as compared with in the isoflurane-induced model group. Zhang *et al* (28) demonstrated that nobiletin activated p-Akt, CREB, BDNF and Bcl-2 pathways for protection against cerebral ischemia in rats.

BDNF is an important member of the glial cell line-derived neurotrophic factor family, which is predominantly distributed throughout the central nervous system, particularly the hippocampal area. BDNF exerts differentiative, proliferative and nutritive effects on several types of neuron (34). In addition, it exerts promoting effects on the synthesis of neurotransmitter and neurotrophic factors, and is closely associated with learning, memory and cognitive processes (35). At present, it is believed that the occurrence of POCD is caused by neuronal necrosis and apoptosis via multiple pathways. Previous animal experiments have indicated that at the onset of POCD, the expression of BDNF is markedly enhanced, which may significantly alleviate ischemic brain injury, inhibit neuronal death, and exert neuroprotective effects (35,36). In the present study, the protein expression levels of BDNF were increased following treatment of isoflurane-treated rats with nobiletin. Zhang *et al* (28) reported that nobiletin activated p-Akt, p-CREB, BDNF and Bcl-2 pathways in order to protect against cerebral ischemia in rats. In addition, Li *et al* (37) suggested that nobiletin may ameliorate hippocampal deficits via BDNF.

In conclusion, the present study demonstrated that nobiletin exerts neuroprotective effects and ameliorates isoflurane-induced cognitive impairment in aging rats. Nobiletin exerted antioxidant, anti-inflammatory and anti-apoptotic effects via the p-Akt, p-CREB, BDNF and Bcl-2 signaling pathways.

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