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The autophagy inhibitor 3-methyladenine restores sevoflurane anesthesia-induced cognitive dysfunction and neurons apoptosis

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This study was designed to explore the effect of 3-methyladenine (3-MA) on sevoflurane anesthesia-induced cognitive dysfunction. A total of 60 C57BL/6 (5-8 months old) mice were randomly arranged into 3 groups: Control, sevoflurane (Sev) and Sev+3-MA group with 3-MA administration was performed during Sev administration. Morris water maze and Y-maze test were performed to examine the behavioral disorders. Moreover, hippocampal neuronal cell apoptosis and expression of autophagy related genes were detected. Sevoflurane induced cognitive dysfunction in mice showing significant longer escape latency, lower number of correct response, higher apoptotic neurons, and higher expression of autophagy related genes. However, additional 3-MA administration inhibited the effect of sevoflurane on cognitive dysfunction by shorting escape latency, reducing correct response number, inhibiting neurons apoptosis and autophagy genes expression. 3-MA additional administration inhibited sevoflurane anesthesia-induced cognitive dysfunction on mice. 3-MA might be usefull as an inhibitor for sevoflurane anesthesia-induced cognitive dysfunction in clinical trials.

1. Introduction

Anesthesia and surgery induced cognitive impairment is a recognized clinical phenomenon and probably the most frequent type of postoperative cognitive impairment (Grape et al. 2012; Rundshagen 2014). Cognitive dysfunction had been reported prevalent in older adults with diabetes (Rawlings et al. 2015), multiple sclerosis (Rocca et al. 2015), cardiovascular disease (Böhm et al. 2015; Char et al. 2016; Cireddu et al. 2015), as well as Alzheimer's disease (Ng et al. 2015; Parrott et al. 2015). Thus, cognitive dysfunctions are a severe problem in aged population, especially those with nerve-related diseases. As reported, cognitive dysfunction was prevalent in patients with cardiovascular disease (Cireddu et al. 2015).

Cognitive dysfunction could be induced by a variety of factors including operative anesthesia and surgery (Grape et al. 2012; Rundshagen 2014). Among the clinical anesthesia medication, sevoflurane is a commonly used for the induction and maintenance of general anesthesia (Kondo et al. 2016). However, sevoflurane could cause health damage in patients after occupational exposure (Kunze et al. 2015). Reports have been focusing on the effect of sevoflurane on cognitive dysfunction, and animal experiments have shown that sevoflurane inhalation induced nerve cell apoptosis via autophagy (Kashiwagi et al. 2015; Zhang et al. 2016). However, sevoflurane-induced neurotoxicity in H4 human neuroglioma cells was reported to be prevented by activation of autophagy (Zhou et al. 2016). Thus, an autophagy inhibitor might remit cognitive dysfunction, such as 3-methyladenine (3-MA) (Jin et al. 2016).

3-MA has been reported to inhibit autophagy and to enhance the sensitivity of cancer cells to chemoradiotherapy (Song et al. 2016; Tan et al. 2015). Ni et al. (2000) reported that 3-MA was involved in modulating the autophagy/lysosomal pathway via downregulating the expression of autophagy-related genes light chain LC3 and beclin-1. Moreover, several reports suggested that 3-MA increases apoptosis induced by cisplatin, oxaliplatin as well as tumor suppression of antineoplastic agents (Tan et al. 2015; Zhang et al. 2015). However, there is no direct evidence revealing the effect of 3-MA on sevoflurane-induced cognitive dysfunction and the mechanism behind it.

In order to investigate the effect of 3-MA on sevoflurane-induced cognitive dysfunction and the related mechanism, we studied the behavioral disorders of mice, apoptosis of neurons, and autophagy-related genes LC3 and beclin-1. This study should provide information about the modulation and mechanisms related to cognitive impairment.

2. Investigations and results

2.1. 3-MA restores Sev-induced cognitive disorder

The Morris water maze (MWM) test was used to examine spatial learning and memory of mice to examine the effect of 3-MA on Sev-induced cognitive disorder. The results showed that there was no difference in swim speed among the the mice in Control, Sev, and Sev + 3-MA groups (Fig. 1A, $p > 0.05$). However, significant differences were found in latency and and time spend in origin quadrant among the three groups (Fig. 1B and 1C, $p < 0.05$). Mice with administration of sevoflurane showed significant longer escape latency, and shorter time in original quadrant compared with those of mice in Control group. Moreover, we found that the administration of the autophagy inhibitor 3-MA in addition to sevoflurane (Sev + 3-MA group) significantly restored the behavioral deficits caused by sevoflurane (Sev group), by reducing escape latency and increasing time spent in the origin quadrant compared with mice in the Sev group ($p < 0.05$, Fig. 1B and 1C).

A similar result was found in spatial memory among the three groups by Y-maze test. Mice successfully escaped from electric shock within 5 s considered as a correct response. The numbers of correct responses of three groups of 40 trials were counted. We found that sevoflurane administration significantly reduced the correct response number of mice in the Sev group, vs Control (Fig. 1D, $p < 0.01$), and an additional administration of the autophagy inhibitor 3-MA obviously increased the number of correct responses ($p < 0.05$). Taken together, these results revealed that the autophagy inhibitor 3-MA could restore sevoflurane-induced behavioral disorders and memory deterioration to some extent.

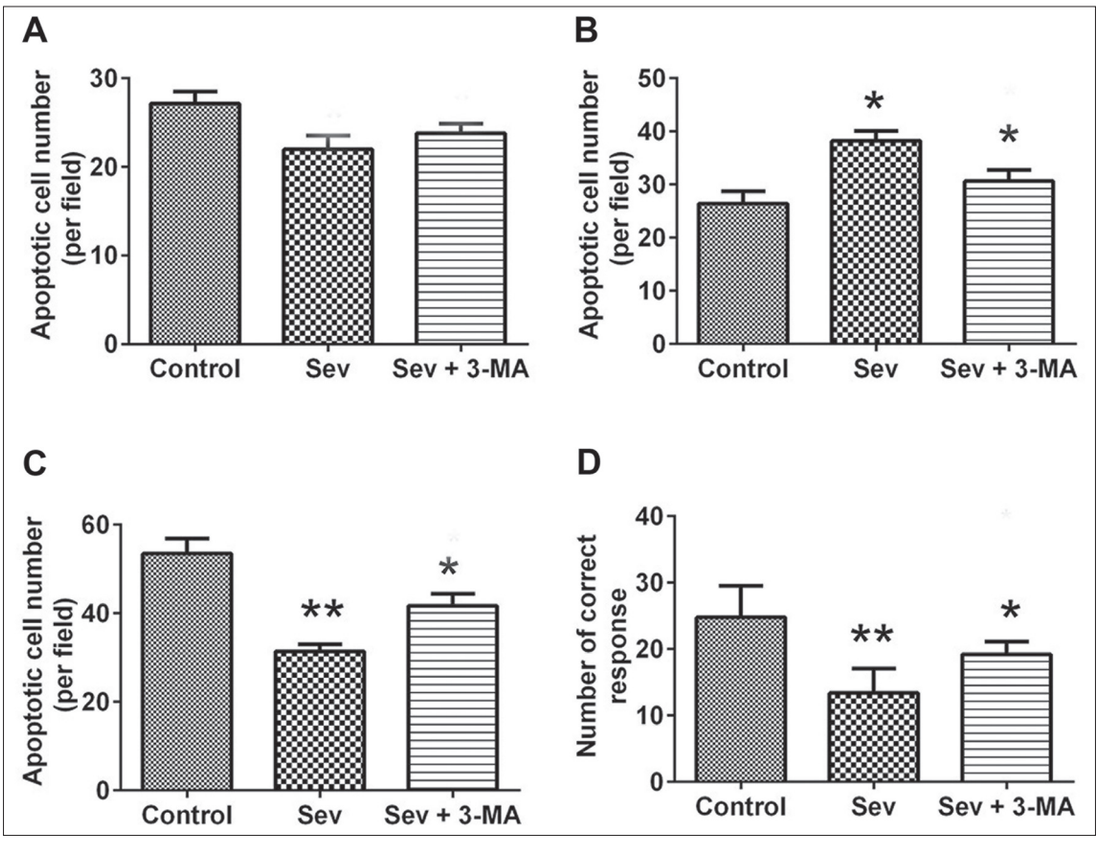

Fig. 1: Behavioral test of mice in difference treatment. A, B, and C indicates the swimming speed ($cm \bullet s^{-1}$), time taken to find the hidden platform (Latency) and time spend in the original quadrant detected using morris water maze, respectively. D, indicates the Y-maze learning task. * and ** represents the significant level at $p < 0.05$ and $p < 0.01$ vs Control or Sev, respectively.

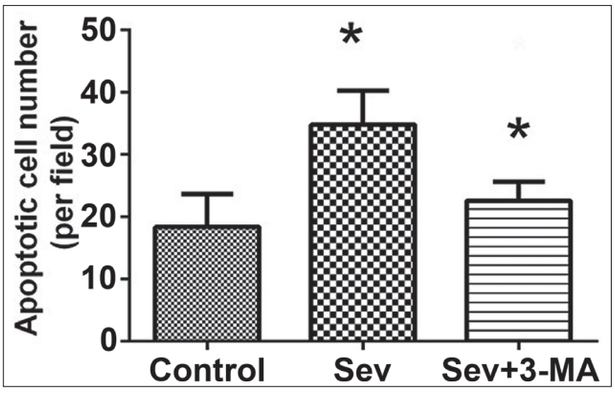

Fig. 2: Apoptosis in hippocampal neuronal cells. Apoptosis of hippocampal neuronal cells was detected using TUNEL assay, * represents the significant level at $p < 0.05$ vs Control or Sev (sevoflurane), respectively.

2.2. 3-MA inhibits Sev-induced neuronal cell apoptosis

To investigate the effect of 3-MA on neuronal cells apoptosis, we detected the apoptosis of hippocampal neuronal cells in treatment groups using TUNEL assay. The results showed that sevoflurane administration significantly induced neuron apoptosis in comparison with control (Fig. 2, $p < 0.05$). After additional administration of 3-MA, however, the number of apoptotic neurons per field in the Sev + 3-MA group significantly decreased compared with the Sev group (Fig. 2, $p < 0.05$). These data suggested that the autophagy inhibitor 3-MA could inhibit sevoflurane induced neurons apoptosis in hippocampus. This might account for the 3-MA effect on the memory deterioration.

2.3. 3-MA suppresses the expression of LC-3 and Beclin-1

To explore the 3-MA effect on mechanism of behavioral disorders, the expression of the apoptosis-related genes LC-3 and Beclin-1 mRNA and protein levels were detected using qRT-PCR and western blotting analysis, respectively. We confirmed that 3-MA administration affected the expression of LC-3 and Beclin-1 mRNA and protein. The results showed that sevoflurane administration upregulated the expression of LC-3 and Beclin-1 mRNA and protein ($p < 0.01$ or $p < 0.05$, Fig. 3). The administration of 3-MA to mice of the Sev + 3-MA group suppressed the sevoflurane-induced LC-3 and Beclin-1 expression (Fig. 2, $p < 0.05$).

3. Discussion

3-MA is known to inhibit autophagy through modulating the autophagy/ lysosomal pathway (Song et al. 2016; Tan et al. 2015). In this present study, we explored the influence of sevoflurane on cognitive impairment and behavioral disorders in a mice model, and the effect of 3-MA on cognitive impairment. The results showed that sevoflurane inhalation successfully resulted in cognitive impairment by reducing spatial learning and memory ability, and promoting neuron cell apoptosis as well as the expression of LC3 and Beclin-1. However, the administration of 3-MA could reduce sevoflurane-induced cognitive reduce and expression of LC3 and Beclin-1 genes. Sevoflurane is commonly used for general anesthesia, while anesthesia induced cognitive impairment is probably the most frequent type of postoperative cognitive impairment, especially in aged population (Grape et al. 2012; Rundshagen 2014). In this study, mice treated with sevoflurane showed a significant damage in learning ability, showing a longer escape latency of morris water maze and a lower number of correct response in the Y-maze test. These showed that sevoflurane inhalation resulted in behavioral disorders in mice.

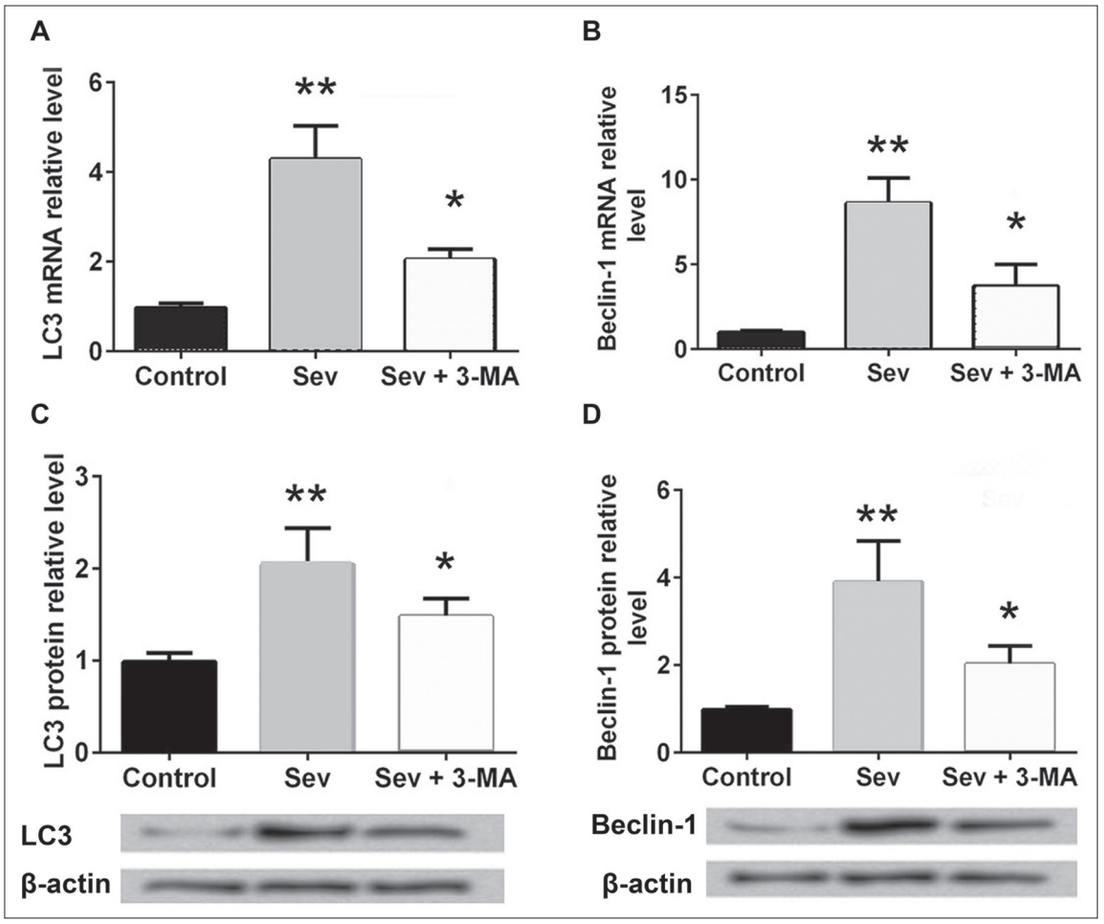

Fig. 3: Expression of autophagy related genes expression in hippocampal neuronal cells. A and B, mRNA expression levels of LC-3 and Beclin-1 in hippocampus by qRT-PCR; C and D, protein expression levels of LC-3 and Beclin-1 in hippocampus by Western blotting analysis. * and ** represents the significant level at $p < 0.05$ and $p < 0.01$ vs Control or Sev (sevoflurane), respectively.

As reported, sevoflurane inhalation induced nerve cell apoptosis via autophagy in aged rats (Kashiwagi et al. 2015; Zhang et al. 2016), and sevoflurane-induced neurotoxicity in cancer cells could be prevented by activation of autophagy (Zhou et al. 2016). This demonstrated that sevoflurane contributes to cell autophagy. However, the roles of autophagy in diseases progression were different and depended on stimulations' difference. As reported by Zhang et al. (2016), sevoflurane inhalation could promote hippocampal nerve cell apoptosis and induce cognitive dysfunction in aged rats. Sun et al. (2015a) revealed that upregulation of neuronic autophagy *via* activation of P2X7R and other factors in the hippocampus is associated with cognitive dysfunction. On the contrary, reports have shown that lipopolysaccharides could cause persistent cognitive dysfunction accompanied by inhibition of autophagy in mice (Huang et al. 2016), and that autophagy inhibitor 3-MA addition aggravates cognitive impairment of diabetic mice (Li et al. 2016). Moreover, there were some studies revealing that neuronal apoptosis by exposure to 2% sevoflurane may not contribute to long-term cognitive dysfunction (Lu et al. 2016). This demonstrated that autophagy had a dual character in cognitive impairment mechanism, and neurodegenerative diseases (Mohammadi et al. 2016). In this study, we confirmed that sevoflurane inhalation induced cell apoptosis as well as cognitive impairment suggesting that sevoflurane induced cognitive impairment was positively associated with neurons apoptosis temporarily or permanently. Moreover, 3-MA administration inhibited sevoflurane induced neurons apoptosis, which to a certain degree contributed to alleviated cognitive impairment.

Autophagy in neuron cells plays a great role in neurodegenerative diseases via modulation of autophagy-related genes, such LC3 and Beclin-1 (Li et al. 2015). LC3 is an indispensible autophagy initiator which travels between the nucleus and cytoplasm and plays an important role in cellular recycling process of autophagy, autophagosome biogenesis and completion (Huang et al. 2015; Wilkinson et al. 2015). Beclin 1 is an indispensible regulator of multiple trafficking pathways including autophagy, and plays a role in the degradative process of macroautophagy (O'Brien et al. 2015; Rohatgi et al. 2015). Moreover, Beclin 1 might act as a tumor suppressor in many human tumors (Rohatgi et al. 2015), and the acetylation of Beclin 1 could inhibit maturation of autophagosome and thus promote tumor growth (Sun et al. 2015b). Additionally, 3-MA has been reported to inhibit autophagy through modulating the autophagy/lysosomal pathway, as well as autophagy-related genes LC3 and Beclin-1 (Song et al. 2016; Tan et al. 2015). Ni et al. (2010) showed that LC3 and Beclin-1 expression levels in recurrent seizures were significantly elevated and 3-MA treatment could suppress the emerged expression of LC3 and Beclin-1. In this study, we demonstrated that the expression of LC3 and Beclin-1 as well as autophagy could be upregulated by sevoflurane inhalation, and 3-MA treatment could reduce the elevated LC3 and Beclin-1 levels and degradative autophagy. This demonstrated that 3-MA might serve as an inhibitor of sevoflurane-induced neuronal autophagy.

Taken together, this study showed that sevoflurane inhalation induced cognitive dysfunction in mice, showing significant longer escape latency, lower number of correct responses, higher apoptotic neurons, and higher expression of autophagy related genes. On the contrary, 3-MA addition to sevoflurane inhalation inhibited the sevoflurane-induced cognitive impairment. Sevoflurane-induced hippocampal nerve cells autophagy, expression of autophagy-related genes LC3 and Beclin-1 were all significantly inhibited by 3-MA addition. This suggested that 3-MA could remit

sevoflurane-induced cognitive impairment and might be explored as a therapeutic strategy against sevoflurane-induced cognitive dysfunction during surgery or anesthesia.

4. Experimental

4.1. Animal and group treatment

A total of 60 C57BL/6 (5-8 months old) mice were purchased from the Charles River Breeding Laboratory, Inc. (North Wilmington, MA, USA). Mice were randomly arranged into 3 groups: Control group (n = 20) received air inhalation in an anesthetizing chamber at 37 ± 0.5 °C for 2 h, sevoflurane (Sev) group (n = 20) received 2.5% Sev in 100% O_2 at the identical conditions, and Sev+3-MA group (n = 20) received 2.5% Sev at the identical conditions. 3-MA at 100 nmol•μl^{-1} was given during Sev administration for 20 min every day for two days. Twelve hours after Sev administration, half of the animals in each group were used to perform the morris water maze (MWM) and Y-maze test. Next, half of the animals in each group were decapitated and brain tissues were rapidly removed and prepared for TUNEL assay, quantitative reverse transcription-polymerase chain reaction (RT-PCR) and western blotting analysis. All experimental procedures were approved by the Fifth Affiliated Hospital of Sun Yat-Sen University.

4.2. Morris water maze task

Morris water maze (MWM) was used to examine spatial learning and memory of mice as described. Maze was placed in a room with dimmed lights, and mice were immersed in water and forced to find the submerged platform in water. MWM composed of a circular water pool (100 cm in diameter, 50 cm in height) with 30 cm depth of water and a platform (12 cm in diameter, 29 cm in height) below the water surface (1 cm). Water temperature was kept at 25 ±2 °C to prevent hypothermia. Maze was divided into 4 quadrants and mice were forced to finish swim in the four quadrants of MWM (six trials per day), for five consecutive days. Mice were immersed in the center of one of the three quadrants without the hidden platform. At the end of each swim, mice were towel dried and rested for 30 min. Each mouse was allowed for a maximum of 60 s to find the platform. Performances of mice related to swim ability were videotaped by a video camera on the ceiling, and time taken to find the hidden platform (latency, s), path length (cm) and swimming speeds (cm•s^{-1}) were analyzed using image tracking software (2020 Plus Tracking System; HVS Image). The spatial learning and memory of mice were evaluated by the latency.

4.3. Y-maze learning task

Mice spontaneous alternation performances in the Y-maze were tested as previously described (He et al. 2014). The maze was composed of 3 arms with equal length of 40 cm, equal height of 13 cm, and width of 8 cm. At the beginning of each test, mice were placed in one arm and allowed to move freely in the Y-maze during a 3-min session. Then, the illuminated light located at the end of the arm indicated a 50 V electric current would pass through the floor of that arm (non-safe regions) for 5 s, and the other two arms were safe during this period (safe regions). The correct response was regarded by the successful escape to a safe region from the non-safe region within the 5 s. Mice got 9 consecutive correct responses were considered to have learned the task. The test was repeated 24 h later and the number of correct responses was measured to assess the spatial memory of mice.

4.4. TUNEL assay

The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was employed to detect the apoptosis of hippocampal neuronal cells in treatment groups, by quantifying cells with DNA strand breaks (Tulsulkar and Shah 2013). In brief, paraffin-embedded hippocampal tissue sections of 4 μm were de-paraffinized with xylene and graded series of ethanol. *In situ* apoptosis detection of apoptotic hippocampal neuronal cells was done by TUNEL assay using colorimetric TUNEL kit (EMD Millipore, Billerica, MA, USA) as per manufacturer's instructions. Cells in five arbitrarily selected fields were counted under × 40 magnification using a Zeiss Axioskop-2 microscope (Carl Zeiss, Inc. Jena, Germany) and the percentage of TUNEL positive cell numbers was calculated as positive cells × 100/total number of cells.

4.5. RNA isolation and quantitative RT-PCR

Total RNA was extracted and prepared using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. For detection of LC-3 and Beclin-1, the first-strand cDNA was synthesized. The mRNA expression levels of LC-3 and Beclin-1was determined by using Ssofast EvaGreen Supermix Kit (BIO-RAD) and ABI 7500 system (Applied Biosystems, Carlsbad, Calif., USA). The primers of apoptosis-related genes LC-3, Beclin-1 and internal control gene β-actin were synthesized by Shanghai Sangon Biologic Engineering Technology and Services Co., Ltd. (Shanghai, China). The reaction conditions were: denaturing 95 °C for 5 min, denaturing at 94 °C, annealing and elongation at 60 °C for 40 cycles. All reactions were run in triplicate. The relative mRNA expression level was calculated by the $2^{-\Delta\Delta Ct}$ method.

4.6. Western blotting

The mice were killed and the hippocampus tissues were separated and proteins were prepared for Western blotting analysis. SDS-PAGE was performed on 10 % polyacrylamide SDS-PAGE, and separated proteins were then electrophoretically transferred to PVDF membrane. After blocking nonspecific binding sites with bovine serum albumin (BSA), membranes were incubated with primary antibodies against LC-3 (1:1000, Millipore, Billerica, MA, USA), Beclin-1 (1:1000, Cell Signal Technology Inc., Danvers, MA, USA), and β-actin (1:1000, BD Transduction Laboratories, Breda, the Netherlands) at 4 °C overnight. The membrane was washed, and then incubated with HRP-conjugated secondary antibodies for 1 h. The western blot was visualized using the ECL Plus detection system (Amersham Pharmacia Biotech, Inc.), followed by imaging and quantification of protein bands.

4.7. Statistical analysis

All data are expressed as the mean±standard deviation of three independent experiments. Differences between groups were assessed by student t-test or analysis of variance ANOVA. $p < 0.05$ was considered statistically significant.

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Conflicts of interest: Authors declare no conflict of interest.

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