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Mechanism of gastrodin in cell apoptosis in rat hippocampus tissue induced by desflurane

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Abstract. This study investigated the protective effect ofgastrodin on cell apoptosis in rats hippocampus tissues induced by desflurane to explore its mechanism. A total of 36 rats were randomly divided into three groups: Blank control group (C group, n=12), desflurane anesthesia group (DF group, n=12) and gastrodin treatment group (GT group, n=12). Rats in DF group were treated with anesthesia using desflurane. Rats in GT group were treated with gavage using gastrodin and the same treatment as DF group. After the experiment, novel object recognition test and water maze test were performed. The hippocampus tissues were taken from the rat after the behavioral experiment; then the number of apoptotic cells was detected using the terminal deoxynucleotidyltransferase-mediated dUTP nick end labelling (TUNEL) kit, and the mRNA and protein expression levels of p38 and interleukin-1 (IL-1) were detected via semi-quantitative polymerase chain reaction (PCR) and western blot analysis. After the desflurane anesthesia, novel object recognition showed that compared with that in DF group, the exploration capacity of novel objects in GT group was increased (P<0.01). The water maze test showed that the escape latencies in DF group, T1 in GT group was significantly shortened, but T2 was significantly prolonged (P<0.01). TUNEL assay showed that the number of apoptotic cells in hippocampus tissues in GT group was significantly fewer than that in group DF (P<0.01). Semi-quantitative PCR and western blot analysis showed that the expression levels of p38 and IL-1β in GT group were lower than those in DF group (P<0.01). The results show that gastrodin has a protective effect on the apoptosis of hippocampus cells of rats induced by desflurane. Its protection mechanism may be realized through decreasing the increased p38 and IL-1β expression levels induced by desflurane, thus blocking the p38 mitogen-activated protein kinase (p38 MAPK) pathway.

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Introduction

Postoperative cognitive dysfunction often leads to mental disorders, personality changes, memory loss, anxiety and other mental symptoms, which is caused by central nervous system damage due to surgery or anesthesia, seriously affecting the life quality of patients (1,2). It has been reported that the commonly-used anesthetics, such as isoflurane and ketamine, can cause postoperative cognitive dysfunction. Studies have shown that the application of isoflurane during surgery can lead to a significant decline in cognitive and memory abilities of patients (3,4). Li *et al* (5) found that the hippocampus cell apoptosis is the reason for the decline in cognitive and memory abilities, and the overexpression of inflammatory factors [p38 and interleukin-1β (IL-1β)] is the leading cause of apoptosis. Gastrodin is the extract of *Gastrodia elata* Bl., which has liver-calming, wind-extinguishing and spasm-stopping effects, mainly used for the treatment of headache, dizziness, epilepsy and convulsion. Recent studies show that it also has the intelligence-enhancing, brain-strengthening and senescence-delaying effects (6-9). In this study, the protective effect of gastrodin on apoptosis of hippocampus cells induced by desflurane was investigated and its possible mechanism was studied to provide a theoretical basis for the clinically reasonable and standardized use of anesthetics.

Materials and methods

Instruments and materials. Desflurane (Shenzhen RWD Life Science Co., Ltd., Shenzhen China); dimethylsulfoxide (DMSO) (Sigma, St. Louis, MO, USA); TRIzol kit; reverse transcription kit (both from Invitrogen, Carlsbad, CA, USA); rabbit anti-rat p38, IL-1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) polyclonalantibodies; anti-rabbit horseradish peroxidase-labeled rabbit secondary polyclonal antibody (cat. nos. 8690, 12703, 2118 and 7074; both from Cell Signaling Technology, Beverly, MA, USA); electrochemiluminescence (ECL) liquid; developing powder (both from Invitrogen); skim milk powder (Guangzhou Sijia Biotechnology Co., Ltd., Guangzhou, China); polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA); pipettor (Eppendorf, Hamburg, Germany); polymerase chain reaction (PCR) instrument (ABI, Foster City, CA, USA); ultraviolet imaging system (Biometra, Goettingen, Germany); electronic

balance (BP121S; Sartorious, Göttingen, Germany); -80˚C refrigerator (Thermo Fisher Scientific, Darmstadt Germany); instruments of novel object recognition test and Morris water maze (Guangzhou Jiebeisi Animal Instrument Co., Ltd., Guangzhou, China); low-temperature centrifuge (Thermo Fisher Scientific); other related equipment and reagents are described in relevant parts.

Experimental animals and grouping. Male Sprague-Dawley (SD) rats weighing 220-250 g were purchased from Guangdong Animal Experimental Center [experimental animal certificate no. SCXK (Guangdong) 2013-0015]. Animals adapted to the feeding environment for 1 week before the experiment, following the circadian rhythms, in a quiet environment at 25˚C, and animals were fed with food and water freely. The above 36 SD rats were randomly divided into three groups: blank control group (C group, n=12), desflurane anesthesia group (DF group, n=12) and gastrodin treatment group (GT group, n=12). Rats in DF group were treated with anesthesia using desflurane (1.2%) for 4 h for a total of 7 days. Rats in GT group were treated with gavage using gastrodin (0.5 g/kg) at 1 h before desflurane anesthesia, as well as the same treatment as DF group. The study was approved by the Ethics Committee of Hospital of Stomatology, Jilin University (Jilin, China).

Novel object recognition test. Rats in each group were placed in the open box before the test to adapt to the environment for 300 sec. After that, each rat was disinfected using alcohol to remove the odor. Then rats were placed in an open box with two identical black objects for object recognition training. When the distance of smelling object was <2 cm or they touched the object with the limbs, it was defined as the recognition time. The time was recorded for a total of 300 sec. The novel object recognition test was performed at 24 h after the training: One of the black objects was replaced with one white object, and the novel object recognition time and the recognition index were recorded; recognition index = novel object recognition time/total object recognition time.

Morris water maze test. One platform was placed in a swimming pool with a diameter of 120 cm and a depth of 50 cm, and the water in the pool was kept 1 cm above the platform. Ink was added into the pool to make the platform invisible. The pool was divided into four quadrants, and one fixed point in each quadrant was determined as the entry point, from which the animals were put into the pool to let them swim freely and find the platform. When they stayed on the platform for 20 sec, it was deemed as finding the platform. The escape latency (T1) was the time from coming into the pool to finding the platform, and the target quadrant exploration time (T2) was the residence time of rats in the quadrant where the platform was located. All rats were trained for 5 days, and all data were recorded by the supporting image tracking and recording software of the instrument. T1 and T2 were recorded accurately and the rats were wiped dry after that. After the training, the platform was removed and exploratory experiment was performed. T2 of each group was recorded and analyzed statistically.

Detection of apoptosis in rat hippocampus tissues via terminal deoxynucleotidyltransferase-mediated dUTP nick end labelling (TUNEL). Hippocampus tissue sections (20 μm) of rats were made according to immunohistochemical methods. After sealing, proteinase K solution was dropped onto the section, and the section was digested at 37˚C for 10 min in the wet box for permeable treatment. After that, the section was washed with phosphate-buffered saline (PBS) 3 times, and soaked in 4% paraformaldehyde for fixation for 5 min. Then 40 μl TdT and DIG-d-UTP mixed solution was dropped onto the section, and the section was placed in the wet box for labeling at 4˚C for 2 h. After that, the liquid was dried and the section was soaked in PBS for 10 min. Then 40 μl blocking solution was dropped onto the section, and the section was sealed at room temperature for 30 min. The biotinylated antibody (1:100) was added and the section was placed in the wet box, followed by incubation at 37˚C for 40 min and washing with PBS 3 times. Then the secondary antibody (1:100) was applied and the section was placed in the wet box for incubation at 37˚C for 40 min, followed by washing with PBS 3 times. The anti-fluorescence quenching sealing fluid was used, followed by observation and photography under a fluorescence microscope (Olympus, Tokyo, Japan). Those with yellow-green fluorescence were the positive cells, namely the apoptotic cells.

Detection of mRNA expression levels via semi-quantitative PCR. The total RNA in rat hippocampus sample in each group was extracted, and the reverse transcription was performed according to the instructions of PrimeScript RT reagent kit to obtain the cDNA, and the expression levels of p38 and IL-1β were detected via semi-quantitative PCR using GAPDH as the internal reference. Primer sequence is shown in Table I, and the primers were synthesized by Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China). Two microliters cDNA sample and RT-PCR reaction solutions were added to prepare the 25 μl reaction system. The mixture was shaken and mixed evenly, centrifuged at 100 x g for 30 sec and placed in the RT-PCR instrument for amplification. The reaction conditions were as follows: pre-degeneration at 95˚C for 5 min, 95˚C for 30 sec, 64˚C for 25 sec, 72˚C for 30 sec, a total of 35 cycles, and then extension at 72˚C for 7 min. After the reaction, 2% agarose gel electrophoresis was performed to separate the band under 120 V until the electrophoresis exceeded more than half of the gel. The ultraviolet imaging system was used to photograph after electrophoresis, and the results were analyzed and compared with p38/GAPDH and IL-1β/GAPDH as the indexes.

Detection of the expression levels of related proteins via western blotting. The hippocampus tissues of rats in each group were taken and the total protein was extracted from the hippocampus tissues using the protein extraction kit (Beyotime Biological Medicine Technology Co., Ltd., Shanghai, China). Then the protein was quantified using the BCA protein quantitative kit (Invitrogen). Loading sample in the equal concentration was prepared according to the protein content. Twelve percent separation gel and 5% spacer gel were prepared and 20 μl sample was added into each well for sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresed under 80 V. After the sample was separated till the

Table I. PCR primers.

Gene	Primer sequences
p38	F: 5'-GTACCACGATCCTGATGATG-3'
	R: 5'-CAGTAGTGGGATCAACAG-3'
IL-1β	F: 5'-GACCTGAGCACCTTCTTTCC-3'
	R: 5'-CTGGAGGTGGAGAGCTTTCA-3'
GAPDH	F: 5'-CTCCTCCACCTTTGACGCTG-3'

F, forward; R, reverse; PCR, polymerase chain reaction; IL-1β, interleukin-1β; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

bottom of the gel, the gel was electrophoresis. Besides, after the gel and PVDF membrane were prepared into membrane transfer 'sandwich', the protein was transferred onto the PVDF membrane for membrane transfer under 100 V. Then 5% skim milk was prepared for sealing for 2 h. After sealing, the target band was cut, and p38 and IL-1β primary antibodies (1:1,000) were used for incubation overnight at 4˚C, followed by comparative analysis with GAPDH as the internal reference. After the membrane was washed three times with TBST (5 min/time), the secondary antibody (1:5,000) was incubated for 2 h at room temperature; then after the membrane was washed three times with TBST, appropriate amount of ECL fluid (solution A and B were mixed at 1:1) was added onto each band in the dark. The plastic wrap covered bubbles were removed and the black box was firmly closed. According to the protein band fluorescence intensity, the time of tableting was determined, followed by color development and fixation in the dark, and then the band was scanned and the gray value was analyzed using ImageJ software.

Statistical analysis. Data in this study are presented as mean ± standard deviation. SPSS 19.0 software (SPSS Inc., Chicago, IL, USA) was used for data processing, and the t-test for intergroup comparison, and analysis of variance was used for comparison among groups. Homogeneity test of variance was performed. If the variance is homogeneous, Bonferroni method was used for pairwise comparison; otherwise, Welch's method was used. Dunnett's T3 method was used for multiple comparisons. P<0.05 indicated that the difference was statistically significant.

Results

Effects of gastrodin and desflurane on novel object recognition of rats. The effects of gastrodin and desflurane on the cognitive ability of rats were studied via the novel object recognition test. The results are shown in Fig. 1. It can be seen from the recognition index that compared with that in C group, the exploration capacities of novel objects in DF group and GT group were significantly decreased, and the differences were statistically significant (P<0.01); the exploration capacity of novel objects in GT group was higher than that in DF group (P<0.01).

Effects of gastrodin and desflurane on spatial learning-memory ability of rats. The spatial learning-memory ability

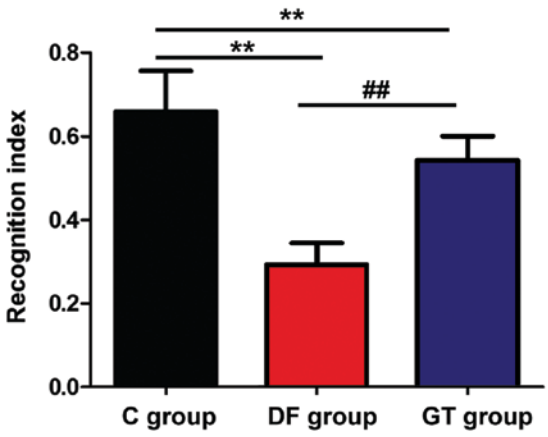

Figure 1. Novel object recognition capability of rats in each group. The recognition capacities of novel objects in desflurane anesthesia (DF) group and gastrodin treatment (GT) group are significantly higher than that in C group, and the differences are statistically significant (**P<0.01); the recognition capacity of novel objects in GT group is higher than that in DF group (##P<0.01).

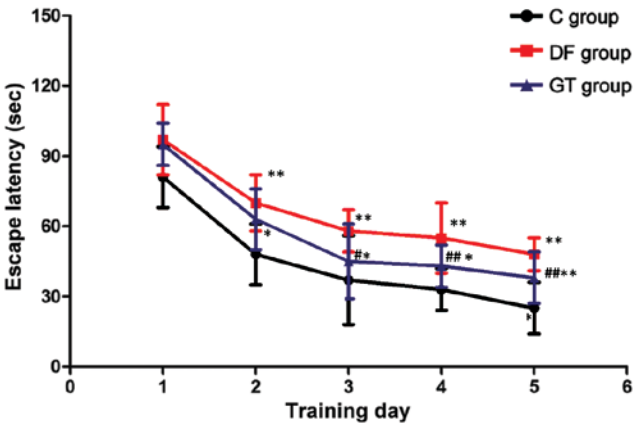

Figure 2. Exploration of learning-memory ability of rats via water maze test. T1 in desflurane anesthesia (DF) group and gastrodin treatment (GT) group from the 2nd day to the 5th day of training is significantly longer than that in C group (*P<0.05 and **P<0.01). T1 in GT group is significantly shorter than that in DF group from the 3rd day to the 5th day of training (##P<0.01).

of rats was studied via water maze test. The results are shown in Fig. 2. During the 5-day training, T1 in DF group and GT group from the 2nd day to the 5th day of training was significantly longer than that in C group (P<0.05 and P<0.01). T1 in GT group was significantly shorter than that in DF group from the 3rd day to the 5th day of training, and the difference was statistically significant (P<0.01). After the training, the exploratory experiment was performed and the results are shown in Fig. 3. The residence time in the target quadrant of rats in DF group and GT group was significantly shorter than that in C group (P<0.01), and the residence time in the target quadrant of rats in DF group was shorter than that in GT group, and the differences were statistically significant (P<0.01).

Effects of gastrodin and desflurane on hippocampus cell apoptosis of rats. The cell apoptosis in rat hippocampus tissues in each group was detected using the TUNEL kit, the number of positive cells was recorded and the apoptotic index was calculated. The results are shown in Fig. 4. Compared with

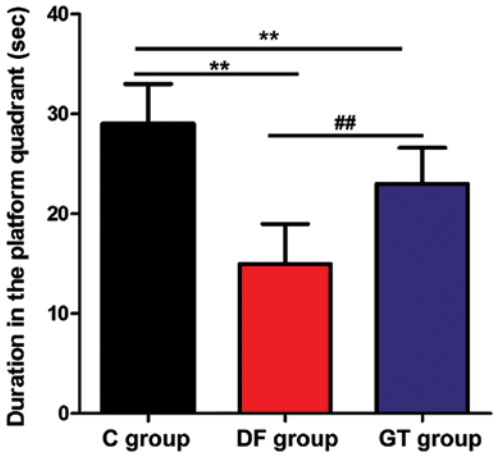

Figure 3. Exploration of learning-memory ability of rats via water maze test. The residence time in the target quadrant of rats in desflurane anesthesia (DF) group and gastrodin treatment (GT) group is significantly shorter than that in C group (**P<0.01), and the residence time in the target quadrant of rats in DF group is shorter than that in GT group ($^{\#\#}$P<0.01).

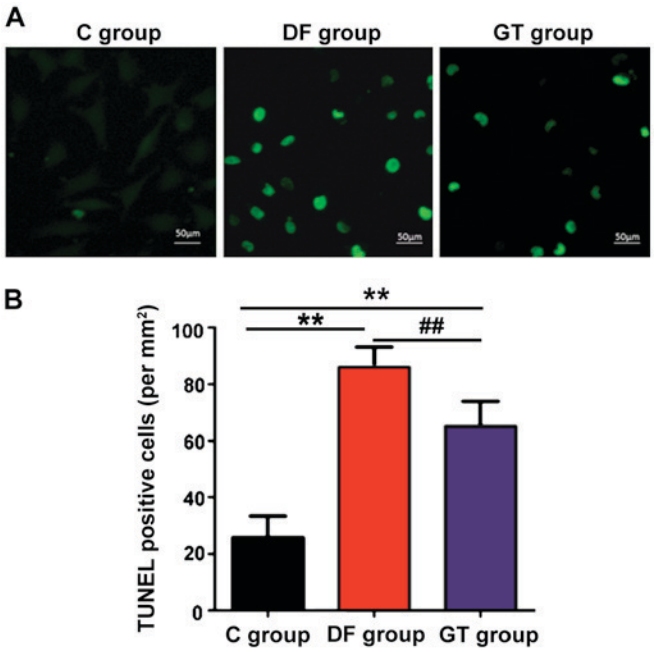

Figure 4. Detection of hippocampus cell apoptosis via TUNEL. The hippocampus cell apoptosis in desflurane anesthesia (DF) group and gastrodin treatment (GT) group is significantly higher than that in C group (**P<0.01), and the number of apoptotic cells in DF group is more than that in GT group ($^{\#\#}$P<0.01).

that in C group, the hippocampus cell apoptosis in DF group and GT group was increased significantly, and the differences were statistically significant (P<0.01); the number of apoptotic cells in DF group was more than that in GT group (P<0.01).

Detection of mRNA expression via semi-quantitative PCR. The mRNA expression of p38 and IL-1β was detected via semi-quantitative PCR. The results are showed in Fig. 5. The mRNA expression levels of p38 and IL-1β in hippocampus tissues in DF group and GT group were significantly increased compared with those in C group, and the differences were statistically significant (P<0.05 and P<0.01). Compared with those in DF group, the mRNA expression levels of p38 and IL-1β in hippocampus tissues in GT group were decreased (P<0.01).

Detection of protein expression via western blotting. The protein expression of p38 and IL-1β was detected via western blotting and the results are shown in Fig. 6. The protein expression levels of p38 and IL-1β in hippocampus tissues in DF group and GT group were significantly increased compared with that in C group (P<0.05 and P<0.01). Compared with DF group, the protein expression levels of p38 and IL-1β in hippocampus tissues in GT group were decreased (P<0.01).

Discussion

Postoperative cognitive dysfunction is the result of a variety of factors, and hippocampus cell injury is considered to be the main factor of causing the decreased cognitive and memory ability (10). The activation of a large number of inflammatory factors in hippocampus activates the relevant signal pathways to damage the hippocampus tissues, thereby affecting the learning and memory ability (11). Ramírez-Jirano *et al* (12) found that the learning and memory abilities of rats can be significantly damaged at 30 days after anesthesia with low-dose isoflurane.

In this study, rats received gastrodin 5 days before the desflurane anesthesia to explore the protective effect of gastrodin on neurological damage caused by desflurane. The novel object recognition test found that after the desflurane anesthesia, the exploration time of novel objects of rats was significantly decreased, and the water maze test showed that after the desflurane anesthesia, T1 was significantly increased, while T2 was significantly decreased compared with those in C group. The above results showed that the learning and memory ability of rats was damaged; in other words, desflurane can damage the learning and memory ability of rats. Before desflurane anesthesia, the intraperitoneal injection of gastrodin could increase the time of exploration, decrease T1 and increase T2. The above results showed that gastrodin could effectively resist the damage of rats caused by desflurane and protect the hippocampal nerve. TUNEL is the most commonly used method of studying apoptosis, which can visually label the apoptotic cells and calculate the apoptotic index to measure the apoptosis of tissue or cells. Wu *et al* (13) found that isoflurane has a direct toxic effect on the rat neurons and PC12 cells, then inducing the apoptosis, and it is thought that the toxicity of isoflurane may be realized through the destruction of endoplasmic reticulum calcium homeostasis. In this study, desflurane anesthesia significantly increased apoptosis of hippocampus cells, and early administration of gastrodin effectively protected the hippocampus tissues and reduced apoptosis. Mani *et al* (14) found that the p38 activity was increased in brain tissues of AD patients, and the same is true in rats with overexpression of amyloid protein, indicating that p38 and p38 MAPK signaling pathways are closely related to the nerve injury. IL-1β is one of the earliest pro-inflammatory cytokines, and the overexpression of IL-1β can induce the aggregation of neutrophils and injury; the activation of IL-1β will further activate p38 activity, and then activate p38 MAPK signaling pathway (15,16). Studies have shown that isoflurane anesthesia can significantly increase the IL-1β expression level

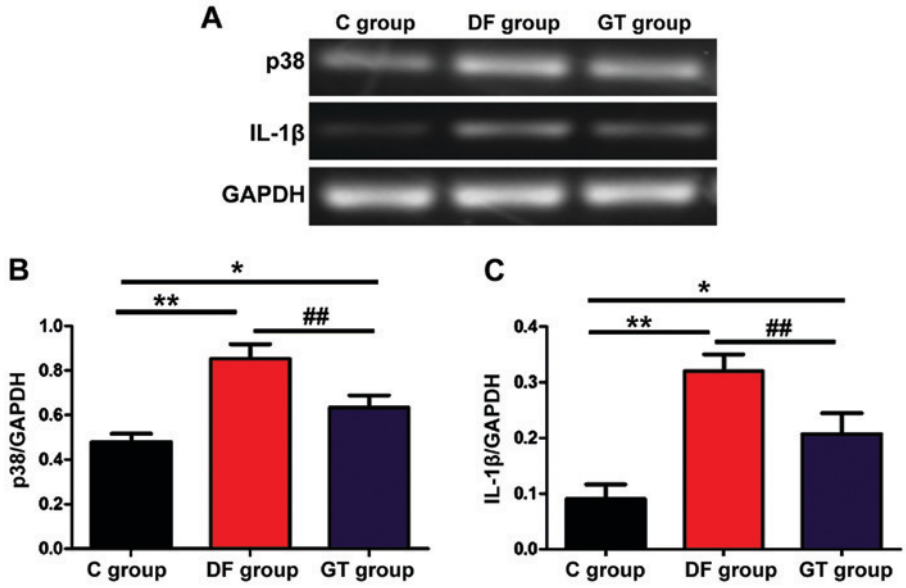

Figure 5. Detection of mRNA expression of p38 and interleukin-1β (IL-1β) via semi-quantitative PCR. The mRNA expression levels of p38 and IL-1β in hippocampus tissues in desflurane anesthesia (DF) group and gastrodin treatment (GT) group are significantly increased compared with those in C group (*P<0.05 and **P<0.01). Compared with those in DF group, the mRNA expression level of p38 and IL-1β in hippocampus tissues in GT group are decreased ($^{\#\#}$P<0.01).

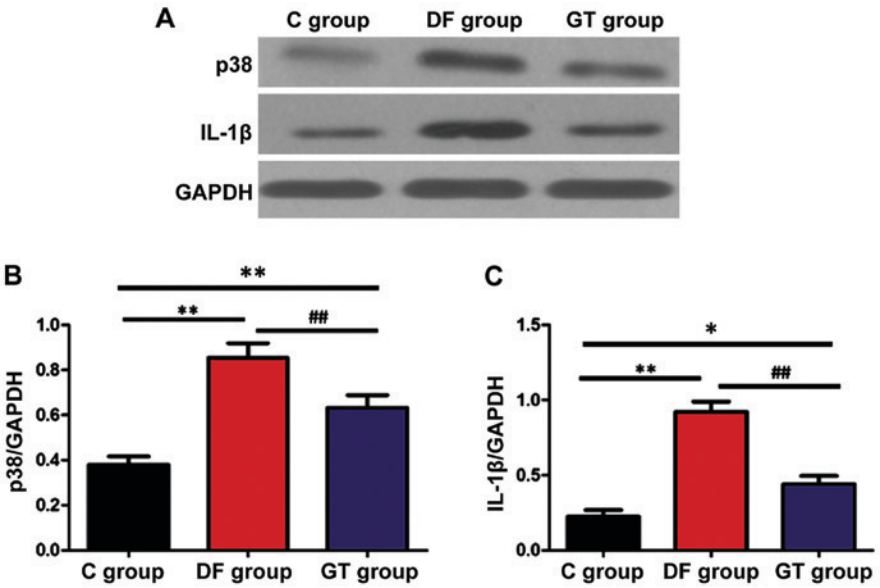

Figure 6. Detection of protein expression of p38 and interleukin-1β (IL-1β) via western blotting. The protein expression levels of p38 and IL-1β in hippocampus tissues in desflurane anesthesia (DF) group and gastrodin treatment (GT) group are significantly higher than those in C group (*P<0.05 and **P<0.01). Compared with those in DF group, the protein expression levels of p38 and IL-1β in hippocampus tissues in GT group are decreased ($^{\#\#}$P<0.01).

in hippocampus tissues, thus causing neural inflammation, and a series of central nervous system injury symptoms (17-20). In this study, the expression of p38 and IL-1β were detected via semi-quantitative PCR and western blotting. It was found that compared with those in C group, the mRNA and protein expression levels of p38 and IL-1β in DF group and GT group were significantly increased, and the expression levels of p38 and IL-1β in GT group were lower than those in DF group. The above results suggest that the hippocampus tissue apoptosis caused by desflurane may be realized by increasing the level of IL-1β, thus activating the p38 and p38 MAPK pathways.

In conclusion, desflurane anesthesia can cause hippocampus tissue apoptosis in rats, and its mechanism may be the activation of IL-1β and p38, thus activating the p38 MAPK signaling pathway. Gastrodin can reduce the levels of IL-1β and p38, and effectively protect the hippocampus tissues of rats from desflurane, thus playing a protective role in the learning, memory and cognitive abilities of rats.

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Availability of data and materials

Not applicable.

Authors' contributions

Not applicable.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Hospital of Stomatology, Jilin University (Jilin, China).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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