Abstract. Elderly patients often need repeated surgical intervention, so it is important to determine the impact of repeated exposure to anesthetics on learning and memory. Docosahexaenoic acid (DHA) is considered to be an essential nutrient for maintaining brain health. The aim of the present study was to explore the potential effects of DHA on memory impairment induced by repeated sevoflurane anesthesia in aged rats. A total of 54 Sprague Dawley aged rats (18 months) were randomly divided into the following six groups: i) Control group; ii) sevoflurane group (Sev, 2.5% for 5 min); iii) DHA group (3 g/kg); iv) Sev + DHA (0.3 g/kg) group; v) Sev + DHA (1 g/kg) group; and vi) Sev + DHA (3 g/kg) group. Morris water maze experiment was performed to evaluate the learning and memory ability of the rats following treatment. H&E staining was used to observe any histological changes. Superoxide dismutase, malondialdehyde and glutathione peroxidase levels were detected using ELISA. Immunohistochemistry and western blotting were used to determine nuclear factor erythroid-2-related factor 2 (Nrf2) and heme oxygenase 1 (HO-1) protein expression levels. Following repeated sevoflurane anesthesia, rats exhibited a prolonged escape latency. The number of times rats crossed the platform and the time spent in the target quadrant were also significantly reduced by repeated sevoflurane anesthesia. However, rats treated with Sev + DHA exhibited a reduced escape latency, whilst the number of times they crossed the platform and the time spent in the target quadrant increased compared with Sev treatment alone. Histopathological examination revealed that DHA treatment ameliorated the disordered neuron arrangement, deep staining of the neuronal nucleus pyknosis and cell edema observed in the brain tissue induced by repeated sevoflurane anesthesia. Furthermore, the protein expression levels of Nrf2 and HO-1 were demonstrated to be significantly increased in rats treated with DHA and exposed to repeated sevoflurane anesthesia compared with those in untreated rats that underwent repeated sevoflurane anesthesia. In conclusion, the present study revealed that DHA exerted protective effects against impairments in learning and memory induced by repeated sevoflurane anesthesia in aged rats, which may be associated with the Nrf2/HO-1 signaling pathway.

Introduction

Elderly patients with multiple comorbidities or diseases often need repeated anesthesia for multiple treatment. Sevoflurane is a volatile anesthetic that is widely applied in medicine, due to its efficiency, reduced risk of airway irritation compared with other inhaled anesthetics (for example, halothane and desflurane) and quick induction (~2-5 min) and recovery periods (~10-30 min); however, sevoflurane is potentially neurotoxic and has been previously reported to be associated with postoperative cognitive impairments in elderly individuals (1). A study previously performed on mice at postnatal day 6 reported that sevoflurane can mediate neurological damage and brain dysplasia by promoting oxidative stress (2).

Docosahexaenoic acid (DHA) is an unsaturated fatty acid and an important component of the neuronal cell membrane (3). DHA serves numerous key roles in signal transduction in neurons, preventing cytoskeletal protein degradation and inhibiting oxidative stress and lipid peroxidation (4). Aging is associated with changes in the DHA content in the membranes of neurons in the brain, which can contribute to memory impairment (5). Age-related decrease associated with the DHA content in the hippocampus has previously been observed in rat models of 3- and 13-month-old rats (6) in addition to 3-4-month and 24-25-month-old rats (7). The present study aimed to investigate the effects of different DHA doses on behavioral memory impairment induced by the repeated administration of sevoflurane in aged rats. In addition,
any potential side effects as a result of repeated sevoflurane anesthesia were also explored.

A previous study has demonstrated that DHA can induce activation of nuclear factor erythroid-2-related factor 2 (Nrf2) and subsequently heme oxygenase 1 (HO-1) (8). The Nrf2/HO-1 signaling pathway is a protection system that exists in a number of organs and is activated in response to a number of different stressors, such as radiation, UV light, air pollution and toxins (9). The Nrf2/HO-1 signaling pathway serves numerous functions, including anti-oxidation and anti-inflammatory responses, reduction of mitochondrial damage and regulation of cell death (10‑13). A previous study has reported that Nrf2/HO-1 signaling can mediate a neuroprotective role by delaying the occurrence of Alzheimer's disease in a mouse model (14). However, to the best of our knowledge, there is no evidence of the role of this pathway after repeated anesthesia.

In the present study, an aged rat model of repeated sevoflurane anesthesia was established to explore the effects of DHA treatment on sevoflurane-induced behavioral memory impairments. The aim of the present study was to provide a theoretical basis for DHA treatment in improving behavioral memory impairment and its underlying molecular mechanisms.

Materials and methods

Experimental animals. A total of 54 aged Sprague Dawley (SD) male rats (Jinan Peng Yue Experimental Animal Breeding Co., Ltd.; production license no. SCXK (LU) 20140007; age, 18 months; weight, 540±50 g) were used in the present study. The housing conditions for the animals were as follows: Room temperature, 20-26°C with daily temperature difference ≤4°C; relative humidity 40-70%; and 12-h light/dark cycles. During the experimental periods, all rats had free access to food and water. Animal health was monitored twice a day. No adverse effects of the treatment were observed during the experiment. All experimental protocols were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (15) and were approved by the Animal Protection and Use Committee of The Affiliated Yantai Yuhuangding Hospital of Qingdao University (Yantai, China).

Animal grouping, anesthesia and DHA administration. The SD rats were randomly divided into the following six groups (n=9): i) Blank control group (control); ii) sevoflurane group (Sev; 2.5%; duration, 5 min); iii) DHA group (3 g/kg); iv) Sev + DHA (0.3 g/kg) group, v) Sev + DHA (1 g/kg) group; and vi) Sev + DHA (3 g/kg). Sevoflurane was purchased from Shanghai Hengrui Pharmaceutical Co., Ltd. (cat. no. NMPN-H20070172) and DHA was purchased from Rongcheng Baihe Biotechnology Co., Ltd.

Rats in the sevoflurane-induced groups were placed in a custom-made transparent anesthesia box (clear tempered glass; 50x40x40 cm). A hole on one side of the box was made to allow for an anesthesia machine (Drägerwerk AG) to be connected. The rats were treated with 2.5% sevoflurane for 5 min and the heart rate (HR), respiratory frequency (RF) and blood oxygen saturation (BOS) of the animals were continuously examined using an electrocardiogram monitor (Nordep, Ltd.) to detect brain damage caused by hypoxia. When the rats became fully anesthetized, they were exposed to air for awakening. The animals were anesthetized once a day for 10 consecutive days. The control group received no sevoflurane treatment.

Rats in the DHA treatment groups received daily feed supplemented with DHA on days 1‑20 of the experiment. On days 11‑20, rats in the Sev groups received daily inhalation of 2.5% sevoflurane for 5 min for repeated anesthesia. Rats in the DHA group received only DHA and were not anesthetized. The rats were trained for the Morris water maze (MWM) experiment on days 16‑20, as described below. No drugs were administered on day 21 before this test. The body weight of the rats in each group was recorded on days 1, 7, 14 and 21. The experimental design of the present study is presented in Fig. 1.

MWM experiment. The MWM experiment was used to test the learning capability of the rats by assessing their navigation abilities. MWM (Shanghai Institute of Materia Medica, Chinese Academy of Sciences) consisted of two parts: One round pool (diameter, 120 cm; height, 50 cm; stainless steel) and one movable platform. The platform was located 2 cm below the water. The water temperature of the pool was ~25°C. The pool surface was divided into four quadrants (named quadrants 1, 2, 3 and 4). The time required for the rats to locate the platform after being placed in the pool was then calculated and the rats’ swimming trajectories were automatically tracked using the EthoVision XT 14 software (Noldus Information Technology BV). This positioning navigation experiment recorded the time required for the rats to locate the platform hidden under the water's surface and examined the spatial orientation learning ability of the rats.

All rats started the MWM test at the center of any quadrant facing the pool wall. If the animal failed to locate the platform in the pool within 120 sec, the time was recorded as 120 sec and the rat was then guided to the platform for 30 sec. The rats were subsequently removed from the platform and wiped dry. After the rats rested for 60 sec, they were trained again. Rats were trained four times a day from days 16-20 for 5 consecutive days. An average of the four training incubation periods was used as the daily learning achievement of each rat and presented as the escape latency.

On day 21, 24 h following the end of the MWM positioning navigation experiment, the platform was withdrawn, and the rats were placed in the water at the center of any quadrant facing the wall. The rats were then permitted to swim for 120 sec, where the time spent in the target quadrant and the number of attempts to cross the platform were recorded.

H&E staining. After the behavioral tests were all completed on day 21, all animals were anesthetized via an intraperitoneal injection of 1% pentobarbital sodium (40 mg/kg) and sacrificed via cervical dislocation. Brain tissues were carefully extracted and washed with cold normal saline. The left brain tissue was used for histopathological examination. Tissue was fixed in 10% formalin for 24 h at room temperature. Subsequently, samples were embedded in paraffin and cut into 4 μm-thick sections. The paraffin sections were dewaxed using xylene, dehydrated using an ethanol descending gradient and incubated with hematoxylin for 10 min and eosin for 5 min at room temperature (Beijing Solarbio Science & Technology Co., Ltd.) before being rinsed with distilled water for 30 sec.

In the present study, an aged rat model of repeated sevoflurane anesthesia was established to explore the effects of DHA treatment on sevoflurane-induced behavioral memory impairments. The aim of the present study was to provide a theoretical basis for DHA treatment in improving behavioral memory impairment and its underlying molecular mechanisms.
The sections were soaked in 95% ethanol twice for 1 min and placed in xylene three times for 5 min before Permount™ mounting medium (Thermo Fisher Scientific, Inc.) was applied. Pathological changes were imaged using an optical microscope (magnification, x100; Olympus Corporation).

**ELISA.** The right brain tissue was collected from each group of rats, 4°C pre-cooled normal saline was used to obtain brain tissue homogenate at 1:10 (volume/volume). Tissue homogenate was centrifuged at 12,000 × g for 30 min at 4°C. The supernatant was collected before superoxide dismutase (SOD; cat. no. A001-3-2), malondialdehyde (MDA; cat. no. A003-1-1) and glutathione peroxidase (GSH-Px; cat. no. A005-1-2) levels were detected using assay kits (Nanjing Jiancheng Bioengineering Institute), according to the manufacturer's protocol.

**Immunohistochemistry.** The left brain tissues were fixed in 10% formalin for 24 h at room temperature and then embedded in paraffin and sectioned (5-µm). The sections were deparaffinized twice using xylene and rehydrated in a descending ethanol gradient. Endogenous peroxidase was inhibited by incubating the sections for 30 min with 3% H2O2 at 37°C. Antigen retrieval was performed using a 0.01 M citrate buffer at 95°C for 10 min. The sections were subsequently blocked for 20 min in 5% BSA (Beijing Solarbio Science & Technology Co., Ltd.) at 37°C, and incubated using a primary anti-HO-1 antibody (1:100; cat. no. ab13243; Abcam) and an anti-Nrf2 antibody (1:100; cat. no. ab8227; Abcam) overnight at 4°C. After rewarming, sections were incubated with an HRP-conjugated goat anti-rabbit IgG secondary antibody (1:1,000; cat. no. ab2721; Abcam) at 37°C for 1 h. Sections were visualized using 3,3’ dianinobenzidine (DAB) as the chromogen (Beijing Solarbio Science & Technology Co., Ltd.), then counterstained with hematoxylin for 1 min and washed in running water two times (3 min each) at room temperature. Subsequently, sections were dehydrated with ethanol of gradient concentration, cleared in xylene and placed onto a coverslip in Permount™ mounting medium (Thermo Fisher Scientific, Inc.). The samples were imaged using an optical microscope (magnification, x200; Olympus Corporation) with five fields randomly selected view. Protein expression levels were quantified using Image-Pro Plus software (version 6.0; Media Cybernetics, Inc.). The data were expressed as the percentage of positive cells/total number of cells counted.

**Western blotting.** Total protein was extracted from the right brain tissue using RIPA lysis buffer containing proteinase inhibitor cocktail (Beyotime Institute of Biotechnology). Total protein concentration was quantified using a BCA assay and total protein (50 μg protein/lane) was separated using SDS-PAGE on a 10% gel. The separated proteins were transferred onto PVDF membranes (MilliporeSigma). The membranes were blocked using 5% skimmed milk at 4°C overnight and subsequently incubated with the primary anti-Nrf2 (1:1,000; cat. no. ab137550; Abcam), anti-HO-1 (1:2,000; cat. no. ab13243; Abcam) and anti-β-actin antibody (1:2,000; cat. no. ab8227; Abcam) at 4°C overnight. Following primary incubation, the membranes were incubated with the secondary antibody, HRP-labeled sheep anti rabbit IgG (1:5,000; cat. no. ab97095; Abcam) at 37°C for 1 h. Protein bands were visualized using an ECL Chemiluminescence System (Thermo Fisher Scientific, Inc.). Protein expression levels were normalized to β-actin and were semi-quantified using ImageJ software version 1.46 (National Institutes of Health).

**Statistical analysis.** All experimental data were analyzed using SPSS 20.0 (IBM Corp.) and are presented as the mean ± standard deviation. One-way ANOVA was used to perform statistical comparisons among ≥3 groups followed by
Table I. Changes in rat body weight (n=9/group).

| Group                  | Day 1            | Day 7            | Day 14           | Day 21           |
|-----------------------|------------------|------------------|------------------|------------------|
| Control               | 584.52±21.16     | 598.62±22.98     | 613.62±21.08     | 627.62±23.17     |
| DHA (3 g/kg)          | 585.43±20.32     | 601.17±20.34     | 617.34±21.67     | 633.19±24.58     |
| Sev                   | 583.26±19.74     | 597.13±20.69     | 603.41±22.12     | 608.41±23.36     |
| Sev + DHA (0.3 g/kg)  | 582.31±19.42     | 598.43±19.86     | 606.51±20.82     | 614.12±21.91     |
| Sev + DHA (1 g/kg)    | 584.65±20.97     | 599.49±21.06     | 608.27±21.94     | 616.39±22.63     |
| Sev + DHA (3 g/kg)    | 583.36±20.09     | 599.78±21.43     | 611.03±22.06     | 619.56±23.74     |

a,b P<0.05 vs. control; a,b P<0.05 vs. DHA. DHA, docosahexaenoic acid; Sev, sevoflurane.

Figure 2. Changes in RF, BOS and HR in rats recorded in each group. *P<0.05 vs. control; ^P<0.05 vs. DHA (3 g/kg); #P<0.05 vs. Sev. RF, respiratory frequency; BOS, blood oxygen saturation; HR, heart rate; DHA, docosahexaenoic acid; Sev, sevoflurane.

Results

Effects of sevoflurane and DHA on the body weight, HR, RF and BOS of rats. The body weight of rats in each treatment group increased gradually over 21 days. The body weight of the rats in the Sev group increased at a markedly slower rate (Table I). On day 21, the body weight of the rats in the Sev group was significantly lower compared with that in the control and DHA groups.

The RF in the Sev group was significantly reduced compared with those in the control and DHA groups (Fig. 2). The BOS in the Sev + DHA (3 g/kg) group was not significantly reduced compared with control and DHA groups. The HR in the Sev, Sev + DHA (0.3 g/kg), Sev + DHA (1 g/kg) and Sev + DHA (3 g/kg) groups were significantly increased compared with that in the control or DHA groups. The HR in Sev + DHA (1 g/kg) and Sev + DHA (3 g/kg) groups were significantly decreased compared with the Sev group. No significant differences were seen between the control and DHA groups (Fig. 2).

DHA improves spatial learning in rats following repeated sevoflurane treatment. Compared with that in the control and DHA groups, the escape latency period of rats in the Sev group was significantly increased from 16-20 days (Fig. 3). Compared with that in the Sev group, the escape latency of rats in the Sev + DHA (0.3 g/kg), Sev + DHA (1 g/kg) and Sev + DHA (3 g/kg) groups was decreased, where DHA exhibited a dose-dependent effect. Sev + DHA (1 g/kg) group was significantly decreased compared with the Sev group from 17-20 days. The effect mediated by the Sev + DHA (3 g/kg) group was demonstrated to be significantly different compared with that in the Sev group (Fig. 3).

DHA improves memory in rats following repeated sevoflurane treatment. Compared with that in the control and DHA groups, the number of times the rats crossed the platform and the time rats stayed in the target quadrant were significantly decreased in the Sev group. Compared with that in the Sev group, the number of times the rats crossed the platform and the time rats stayed in the target quadrant in the Sev + DHA (0.3 g/kg), Sev + DHA (1 g/kg) and Sev + DHA (3 g/kg) groups were decreased, where DHA exhibited a dose-dependent effect. In particular, the effects exerted by the Sev + DHA (1 g/kg) and Sev + DHA (3 g/kg) groups were statistically significant compared with those in the Sev group (Fig. 4B and C).

Histopathological examination of the rat hippocampus following repeated sevoflurane and DHA treatment. Hippocampus section images in the control and DHA
Figure 3. Escape latency of the rats in each treatment group. *P<0.05 vs. control; †P<0.05 vs. DHA (3 g/kg); ‡P<0.05 vs. Sev. DHA, docosahexaenoic acid; Sev, sevoflurane.

Figure 4. Changes in the memory capability of rats in the different treatment groups. (A) Swimming trajectory of rats in each group. (B) Number of times rats in each group crossed the platform. (C) Time of stay in target quadrant. *P<0.05 vs. control; †P<0.05 vs. DHA (3 g/kg); ‡P<0.05 vs. Sev. DHA, docosahexaenoic acid; Sev, sevoflurane.
(3 g/kg) groups were observed to be normal, whereas tissues from rats in the Sev, Sev + DHA (0.3 g/kg), Sev + DHA (1 g/kg) and Sev + DHA (3 g/kg) groups all exhibited pathological changes in the hippocampus (Fig. 5). Pathological changes observed in the Sev group included the disordered arrangement of neurons, deep staining of neuronal nucleus pyknosis, cell edema and microglia foaming, with certain areas exhibiting a small amount of cell necrosis. These aforementioned pathological changes in the Sev + DHA (0.3 g/kg) and Sev + DHA (1 g/kg) groups appeared to be slightly reduced compared with those in the Sev group. However, neurons in these two DHA groups also exhibited a degree of disordered arrangement, deep staining of the neuronal nucleus pyknosis and cell edema. The pathological changes in the Sev + DHA (3 g/kg) group appeared to have been alleviated compared with those in the Sev + DHA (0.3 g/kg) and Sev + DHA (1 g/kg) groups.

Effects of repeated sevoflurane treatment and DHA on SOD, MDA and GSH-Px levels. SOD and GSH-Px levels were significantly decreased in the Sev group compared with those in the control and DHA groups (Fig. 6). DHA treatment resulted in marked increases in SOD and GSH-Px levels in rats exposed to repeated sevoflurane anesthesia, where there was a significant difference between Sev and Sev + DHA (3 g/kg) groups (Fig. 6). No significant differences in SOD, GSH-Px or MDA levels were observed between the control and DHA groups. In the Sev group, MDA levels were significantly increased compared with those in the control and DHA groups (Fig. 6).
DHA treatment resulted in a marked decrease in MDA levels in rat brain samples following exposure to repeated sevoflurane anesthesia, where significant differences were observed between the Sev group and the Sev + DHA (1 g/kg) or Sev + DHA (3 g/kg) groups (Fig. 6). These results suggested that DHA treatment may reduce oxidative stress caused by repeated sevoflurane anesthesia.

**Effects of sevoflurane and DHA treatment on Nrf2 and HO-1 protein expression.** The background color of immunohistochemical staining is uniform and does not affect the cell count. The cell count was calculated based on the strong staining of the brown parts. The results of immunohistochemical staining revealed that compared with that in the control and DHA groups, Nrf2 and HO-1 staining in the Sev and Sev + DHA treatment groups increased in a DHA dose-dependent manner. Compared with the Sev group, Nrf2 and HO-1 staining in Sev + DHA treatment groups significantly increased. Staining in the Sev + DHA (3 g/kg) group was the highest (Fig. 7).

Compared with those in the control and DHA groups, the protein expression levels of Nrf2 and HO-1 in the Sev group were significantly increased (Fig. 8). The Nrf2 and HO-1 protein expression levels in the Sev + DHA treatment groups were increased further compared with those in the Sev group. In the Sev + DHA treatment groups, the Nrf2 and HO-1 protein expression levels were significantly increased compared with those in the Sev group (Fig. 8). These results suggested that DHA may alleviate damage caused by repeated sevoflurane anesthesia on the brain tissue of rats by increasing the Nrf2 and HO-1 protein expression levels.

**Discussion**

In the present study, following repeated exposure to sevoflurane anesthesia, rats were observed to exhibit decreased spatial learning capabilities, aimless movement, reduced memory capacity, required more time for space exploration and crossing the platform. A previous study demonstrated that long-term exposure to sevoflurane (2.5% for 30 min) can lead to neurodevelopmental disorders, impaired learning and memory in 7-day-old and 15-day-old rats (16). Furthermore, a clinical study in elderly individuals has also reported that exposure to sevoflurane is associated with a decline in postoperative...
cognitive function (17). The neurotoxicity of sevoflurane is time- and dose-dependent (18). Another study previously demonstrated that exposure of pregnant rats to sevoflurane can lead to brain damage in the neonatal offspring within 2 weeks of birth (19). In laboratory animals (rat and mouse) exposed to sevoflurane during the peak period of neurodevelopment, synaptic plasticity and long-term potentiation were indicated to be affected, as learning and memory capabilities were decreased (20,21). In addition, a previous study presented that sevoflurane repeated exposure (2 h daily for 5 consecutive days) can damage the learning and memory of 16-18 months old male rats (22). In the present study, behavioral experiments in aged rats revealed that the learning and memory abilities of those in sevoflurane-exposed groups were lower compared with those in the control group. The results of the present study are consistent with those reported by the previous studies aforementioned.

DHA is a structural plasma membrane component that is important for normal brain function and can be readily obtained from fish oil (23). DHA is therefore applied as a health product or nutritional supplement (23). A previous study reported that the incidence of neurodegenerative diseases is lower in populations that adopt a Mediterranean diet, which may be associated with the long-term intake of foods with a high DHA content (24). In previous studies, DHA and/or eicosapentaenoic acid supplements were reported to improve cognitive function and protect against neuroinflammation and oxidative stress in rodents (25,26). In the present study, following the administration of different doses of DHA in aged rats exposed to repeated sevoflurane anesthesia, spatial exploration and navigational abilities were ameliorated in a dose-dependent manner. These results suggested that DHA may effectively reverse spatial learning and memory impairments induced by repeated sevoflurane anesthesia. Histopathological examination of the rat brain tissues also revealed that DHA can prevent brain damage to alleviate cognitive impairment in rats exposed to repeated sevoflurane anesthesia. Furthermore, the effect of DHA was enhanced in a dose-dependent manner. These results suggested that DHA may exert protective effects against learning and memory impairment induced by repeated sevoflurane anesthesia in aged rats.

Activation of the Nrf2/HO-1 signaling pathway serves an important role in ameliorating brain injury and is key to the anti-oxidative stress response in the body (27). Nrf2 nuclear translocation is important for HO-1 activation (28). In the present study, SOD, GSH-Px and MDA levels were detected in rat brain tissues. The results demonstrated that DHA ameliorated oxidative stress induced by repeated sevoflurane anesthesia in aged rats. Immunohistochemistry and western blotting were used to verify whether the DHA-induced
anti-oxidative stress effects were mediated through the Nrf2/HO-1 signaling pathway. The results demonstrated that Nrf2 and HO-1 protein expression was increased in each of the DHA dose groups compared with that in the Sev group in a dose-dependent manner. Furthermore, Nrf2 and HO-1 protein expression levels in the Sev group were also increased compared with control and DHA group. A previous study indicated that activation of the Nrf2/HO-1 signaling pathway is the main mechanism of cellular defense against oxidative stress (29). It can therefore be hypothesized that the increased Nrf2 and HO-1 protein expression levels may be induced as a cellular defense mechanism. However, this was not explored further in the present study. Therefore, in future studies, experiments will be required to verify the molecular mechanism through which DHA mediates its effects in the repeated sevoflurane anesthesia model.

In conclusion, the results of the present study indicated that DHA exhibited protective effects against learning and memory impairment in aged rats, which was induced by repeated sevoflurane anesthesia. The increased Nrf2 and HO-1 protein expression levels suggested that the mechanism may associated with the Nrf2/HO-1 signaling pathway.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

MT and XLZ designed the study. MT, YXW and DGL performed the experiments. MT, YXW and DGL performed data analysis, interpreted the data and acquired samples. MT, YXW and XLZ contributed to pathological analysis. MT and XLZ confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by Animal Protection and Use Committee of The Affiliated Yantai Yuhuangding Hospital of Qingdao University (Yantai, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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