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Estrogen receptor beta treats Alzheimer's disease☆

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Abstract

In vitro studies have shown that estrogen receptor β can attenuate the cytotoxic effect of amyloid β protein on PC12 cells through the Akt pathway without estrogen stimulation. In this study, we aimed to observe the effect of estrogen receptor β in Alzheimer's disease rat models established by intraventricular injection of amyloid β protein. Estrogen receptor β lentiviral particles delivered *via* intraventricular injection increased Akt content in the hippocampus, decreased interleukin-1β mRNA, tumor necrosis factor α mRNA and amyloid β protein levels in the hippocampus, and improved the learning and memory capacities in Alzheimer's disease rats. Estrogen receptor β short hairpin RNA lentiviral particles delivered *via* intraventricular injection had none of the above impacts on Alzheimer's disease rats. These experimental findings indicate that estrogen receptor β, independent from estrogen, can reduce inflammatory reactions and amyloid β deposition in the hippocampus of Alzheimer's disease rats, and improve learning and memory capacities. This effect may be mediated through activation of the Akt pathway.

Key Words

neural regeneration; neurodegenerative diseases; estrogen; estrogen receptor β; Alzheimer's disease; amyloid β protein; inflammatory cytokines; Akt signaling pathway; cognition; neural protection; photographs-containing paper; neuroregeneration

Research Highlights

Intraventricular injection of estrogen receptor β can (1) improve the learning and memory abilities of Alzheimer's disease rats, (2) increase Akt content in the hippocampus of Alzheimer's disease rats, and (3) reduce interleukin-1β mRNA, tumor necrosis factor α mRNA and amyloid β protein content in the hippocampus of Alzheimer's disease rats.

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INTRODUCTION

Amyloid β protein deposition in cerebral cortex and hippocampus is the key pathological change in Alzheimer's disease[1-2], so how to reduce amyloid β protein content and attenuate the neurotoxicity are research hotspots.

Growing evidence suggests that estrogen binding of estrogen receptors in nerve cells can reduce amyloid β protein deposition and antagonize its cytotoxic effects in Alzheimer's disease, providing a potential therapeutic effect[3-7]. However, large amounts of estrogen have been reported to cause increased incidence of breast cancer and endometrial cancer[8-9], thus restricting its clinical application. Estrogen receptor β is a subtype of estrogen receptors and is highly expressed in the nervous system[10-11]. Estrogen receptor β plays a biological role through an estrogen-independent pathway[12-13]. A previous study by our research group also found that estrogen

receptor β, without estrogen stimulation, still attenuates the cytotoxic effect of amyloid β protein on PC12 cells through the Akt pathway[14]. However, to date there is no *in vivo* experimental evidence of such an effect of estrogen receptor β.

Therefore, amyloid β protein-induced Alzheimer's disease model rats were injected with estrogen receptor β gene or estrogen receptor β short hairpin RNA plasmid, leading to overexpression or low expression of estrogen receptor β in the brain, with the aim of observing changes in amyloid β protein and inflammatory factor expression in the rat hippocampus, and exploring the underlying mechanism.

RESULTS

Quantitative analysis of experimental animals

After Alzheimer's disease models were established, 18 rats were randomly divided into three groups: a model group, an estrogen receptor β group and a short hairpin RNA group, and were intraventricularly injected with PBS solution, lentiviral vector expressing estrogen receptor β and lentiviral vector expressing estrogen receptor β short hairpin RNA, respectivrly. All 18 rats were involved in the final analysis.

Estrogen receptor β improved the learning and memory capacities of Alzheimer's disease rats

In the Morris water maze test, all tested rats showed no significant difference in escape latency and swimming distance before modeling ($P > 0.05$). Compared with those before modeling, the escape latency and swimming distance were significantly prolonged at 14 days after modeling ($P < 0.05$). Compared with the model group and the short hairpin RNA group, the escape latency and swimming distance were significantly shortened in the estrogen receptor β group ($P < 0.05$). There were no significant differences in these measures between the model group and the short hairpin RNA group ($P > 0.05$; Figure 1).

Effect of estrogen receptor β on the Akt and amyloid β protein contents in the hippocampus of Alzheimer's disease rats

Western blot analysis revealed that the expression of estrogen receptor β in the hippocampus of rats in the estrogen receptor β group was significantly higher than that in the model group ($P < 0.01$), while it was significantly lower in the short hairpin RNA group than in the model group ($P < 0.01$). This finding suggested that intraventricular injection of estrogen receptor β short hairpin RNA lentivirus vector can greatly downregulate the expression of estrogen receptor β in rat brain. Compared with the model group, Akt protein expression in the hippocampus was increased ($P < 0.05$), and amyloid β protein expression was significantly decreased in the estrogen receptor β group ($P < 0.01$), with no significant differences between the short hairpin RNA group and the model group ($P > 0.05$; Figure 2).

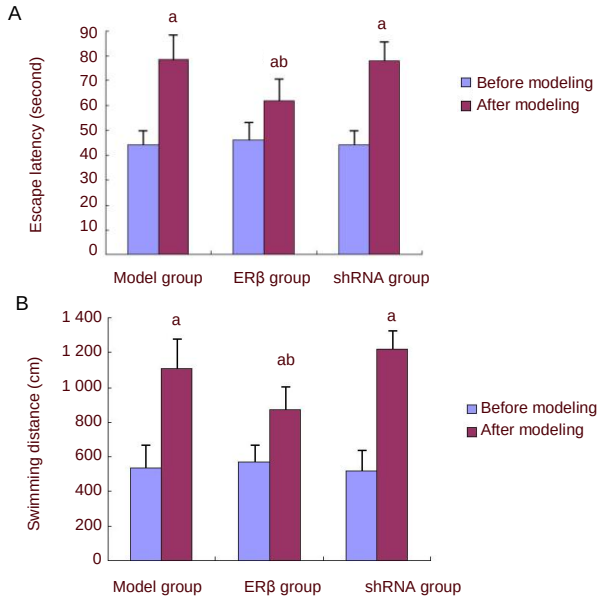

Figure 1 Effect of estrogen receptor β (ERβ) on the learning and memory capacities of Alzheimer's disease rats.

(A, B) Escape latency and swimming distance in the Morris water maze test. Data are expressed as mean ± SD, and there are six rats in each group. [a]$P < 0.05$, *vs*. before modeling; [b]$P < 0.05$, *vs*. model group and shRNA group (analysis of variance and *q* test). shRNA: Short hairpin RNA.

Estrogen receptor β attenuated inflammatory responses in the hippocampus of Alzheimer's disease rats

Real-time quantitative PCR results showed that the expression levels of interleukin-1β and tumor necrosis factor α mRNA in the hippocampus were significantly decreased in the estrogen receptor β group compared with the model group ($P < 0.05$ or $P < 0.01$), with no significant differences between the short hairpin RNA group and the model group ($P > 0.05$; Figure 3).

Estrogen receptor β reduced amyloid β protein deposition in the hippocampus of Alzheimer's disease rats

Anti-amyloid β protein immunofluorescence staining revealed varying degrees of green punctate fluorescence in the hippocampus of rats in all three groups, mainly in the extracellular matrix, with some in neural cells (Figure 4).

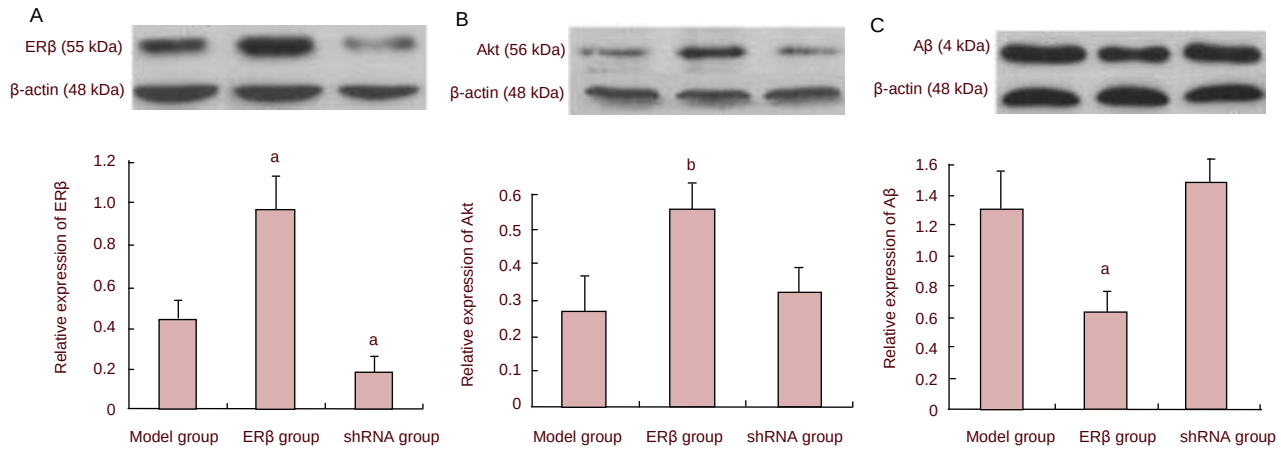

Figure 2 Effect of estrogen receptor β (ERβ) on ERβ, Akt and amyloid β protein (Aβ) expression in the hippocampus of Alzheimer's disease rats.

(A–C) ERβ, Akt and Aβ expression, respectively. Data (relative expression of target protein normalized to β-actin) are expressed as mean ± SD, and there are six rats in each group. [a]*P* < 0.01, [b]*P* < 0.05, *vs*. model group (analysis of variance followed by *q* test). shRNA: Short hairpin RNA.

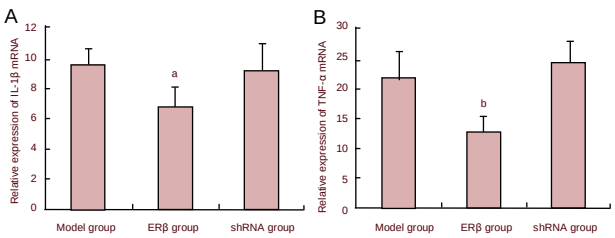

Figure 3 Expression of interleukin-1β (IL-1β) and tumor necrosis factor α (TNF-α) mRNA in the rat hippocampus of the model group, estrogen receptor β group and shRNA group.

(A, B) IL-1β and TNF-α mRNA expression. Data (relative expression of target mRNA normalized to β-actin) are expressed as mean ± SD, and there are six rats in each group. [a]*P* < 0.01, [b]*P* < 0.05, *vs*. model group (analysis of variance followed by *q* test). shRNA: Short hairpin RNA.

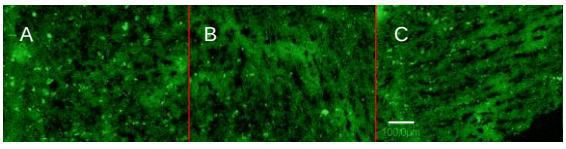

Figure 4 Effect of estrogen receptor β on amyloid β protein expression in the hippocampus of Alzheimer's disease rats (immunofluorescence, confocal laser microscopy, scale bar: 1 000 μm).

The strong positive punctate fluorescence is amyloid β protein (FITC labeled).

In the model group (A), a large amount of amyloid β protein appeared aggregated, forming sheet amyloid plaques. In the estrogen receptor β group (B), the fluorescence intensity was significantly decreased, and no significant difference in fluorescence intensity was observed between the shRNA group (C) and the model group.

A large number of amyloid β protein-aggregated sheet-shaped starch-like protein plaques were visible in the model group and the short hairpin RNA group. Compared with the model group (1.87 ± 0.22), the fluorescence intensity in the estrogen receptor β group (1.12 ± 0.17) was significantly decreased (*P* < 0.05), and there was no significant difference in fluorescence intensity between the short hairpin RNA group (1.99 ± 0.16) and the model group (*P* > 0.05).

DISCUSSION

Lentivirus vectors can be used to transfer genes into the nervous system and mediate their expression; thus, they are an important tool for the research of neurodegenerative disease pathogenesis and gene therapy, and have been widely applied in the treatment of Alzheimer's disease[15-18]. Compared with other vectors, lentiviral vector has a high transfection efficiency, stable expression and low immunogenicity, with no cellular immune responses at the site of injection[19-20]. In addition, lentiviral vectors can efficiently infect and integrate into non-dividing cells, and efficiently infect neurons, so they have great advantages for use in the nervous system[21]. Lentiviral particles carrying target gene or short hairpin RNA can be injected into the lateral ventricle on the lesion side or injected directly into the lesion site, serving as a form of gene therapy[22-23]. However, direct injection into the lesion site requires multiple points of injection, causing great injury and difficulty during the operation, while intraventricular injection is relatively simple, with little injury but a requirement for high virus titers[24]. The present experiment aimed to verify whether estrogen receptor β could attenuate the toxicity of amyloid β protein toward

hippocampal neurons, so a lentiviral vector was considered to be the most appropriate means of introducing the transgene or short hairpin RNA, with an appropriate virus titer for intraventricular injection. A western blot assay confirmed that estrogen receptor β is expressed in the brains of Alzheimer's disease rats, and that its expression was increased or decreased after intraventricular injection of lentiviral vectors carrying the estrogen receptor β gene or estrogen receptor βshort hairpin RNA.

Amyloid β protein can cause central nervous system inflammation and oxidative stress, damage cholinergic neurons and induce apoptosis. It is thought to be the leading pathogenic cause of Alzheimer's disease[25-29]. After amyloid β protein was added to the culture medium, the expression levels of tumor necrosis factor α and interleukin-1 in PC12 cells increased, and the incidence of PC12 cell apoptosis was significantly raised[14]. After PC12 cells were transfected with estrogen receptor β in the absence of estrogen stimulation, intracellular expression of inflammation factor significantly decreased and the apoptosis rate also declined, suggesting that estrogen receptor β attenuated amyloid β protein toxicity toward the cells and prevented apoptosis in a non-estrogen-binding manner[14]. In the present study, overexpression of estrogen receptor β in male rats with Alzheimer's disease still reduced tumor necrosis factor α mRNA and interleukin-1β mRNA expression. In addition to the inflammatory reaction, western blotting and immunofluorescence staining analysis revealed that overexpression of estrogen receptor β reduced amyloid β protein deposition in the hippocampus of rats with Alzheimer's disease. The results of Morris water maze testing[30] showed that compared with the model group and short hairpin RNA group, the escape latency and swimming distance of rats were significantly shortened in the estrogen receptor β group. This evidence supports the possibility that overexpression of estrogen receptor β can improve learning and memory abilities as well as spatial orientation ability in Alzheimer's disease rats.

Interestingly, although the overexpression of estrogen receptor β led to reduced inflammatory reactions and amyloid β protein deposition, short hairpin RNA silencing of estrogen receptor β mRNA downregulated the expression of estrogen receptor β without any exacerbation of cerebral inflammation and amyloid β protein deposition. Moreover, low expression of estrogen receptor β had no impact on the behaviors of Alzheimer's disease rats. One possible reason for this may rely on the low content of estrogen receptor β in the central nervous system, while only high concentrations have been shown to have a neuroprotective effect. The low expression level cannot affect the pathological process of Alzheimer's disease, so the inhibition of estrogen receptor β expression does not aggravate the pathology of Alzheimer's disease.

A previous study found that, independent from estrogen receptors, estrogen receptor β plays anti-inflammatory and antiapoptotic roles through the Akt pathway[14]. Akt is a serine/threonine protein kinase, located at an important crossing point of multiple signaling pathways; it can regulate cell growth, differentiation and apoptosis[31-33]. Activation of the Akt pathway can reduce the cytotoxicity of amyloid β protein[34-35]. Experimental results indicate that overexpression of estrogen receptor β can increase the content of Akt in the brain, but low expression of estrogen receptor β has no effect on Akt content. The interaction between estrogen receptor β and Akt may be one potential mechanism underlying the neuroprotective effects of estrogen receptor β, and may explain how low expression of estrogen receptor β has no effect on the pathological changes in Alzheimer's disease.

MATERIALS AND METHODS

Design

A randomized controlled animal experiment.

Time and setting

Experiments were performed from June 2011 to February 2012 in the Laboratory of Neurology, Medical Transformation Center, Bethune First Hospital of Jilin University, China.

Materials

Eighteen clean, healthy, male Wistar rats, aged 8–10 weeks and weighing 280–320 g, were provided by the Animal Experimental Center of Jilin University (license No. SCXK (Ji) 2003-0002). All rats were fed in separate cages at 21 ± 2°C, 30–35% humidity under a 12-hour day/night cycle, and allowed free access to food and water. All experimental disposals were performed in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology of China[36].

Methods

Establishment of a rat model of Alzheimer's disease

Alzheimer's disease models were established according to the methods described by Nabeshima *et al* [37]. In brief, amyloid β protein (1–40) polypeptide (ANASPEC Inc., San Jose, CA, USA) was dissolved in a 60 μM solution

containing 35% acetonitrile and 1% trifluoroacetate acid (Wanxingda Chemical Co. Ltd., Jinan, Shandong Province, China), and stored in the Alzet micro-injection pump (Alzet, Cupertino, CA, USA). Rats were anesthetized by subcutaneous injection of ketamine (100 mg/mL, 100 mg/kg), xylazine (20 mg/mL, 2.5 mg/kg) and acetylpromazine (2.5 mg/mL, 2.5 mg/kg; Wanxingda Chemical Co., Ltd.,), and fixed in a brain stereotactic instrument (Ruiwode Life Science Co., Ltd., Shenzhen, Guangdong Province, China). After 75% ethanol disinfection, a 2.5-cm-long sagittal incision was made along the midline, exposing the skull. The coordinates of needle entry point were defined (anterior 0.3 mm to the bregma, ventral 3.6 mm, left 1.1 mm[37]), and a hole was then drilled at the skull surface. After drilling, the dura mater was punctured, and the cannula was placed and inserted through the entry points into the left lateral ventricle. The cannula was connected *via* the catheter and syringe pump, and the pump was placed on the rat back skin. The wound was sutured, and the rats were released from the brain stereotactic instrument and housed in the controllable incubator at 37°C for 60 minutes. After that, rats were fed in separate breeding cages and received daily injections of 300 pmol amyloid β protein *via* syringe pump, for 14 consecutive days.

Estrogen receptor β intervention

Prior to connection of the syringe pump, rats in the model group were injected with PBS into the left ventricle through the catheter; rats in the estrogen receptor β group were injected with lentiviral-CMV-estrogen receptor β plasmid (25 μL, 10^8 TU/mL; Santa Cruz Biotechnology, Santa Cruz, CA, USA); and rats in the short hairpin RNA group were injected with lentiviral-CMV-estrogen receptor β short hairpin RNA plasmid (25 μL, 10^8 TU/mL; Shanghai Jikai Gene Chemical Technology Co., Ltd., Shanghai, China).

Morris water maze test for assessing behavioral changes in rats

Before modeling and 14 days after syringe pump implantation, the behaviors of Alzheimer's disease rats were detected according to Liu's method[38]. In brief, a circular water tank 150 cm in diameter and 60 cm in height was filled with water to a depth of 30 cm, at 26 ± 1°C. There were four equidistant points on the tank wall, which was divided into four quadrants, and a small platform located in the center of one quadrant, 2 cm below the water. Starting from the western point, rats were placed on the platform for 30 seconds and then entered the water tank; a video camera was fixed on the top of water tank to record the time it took rats to find the platform (escape latency) and the route of rats swimming in search of the platform. All data were automatically recorded by a computer. The maximum recording time is 200 seconds.

Real-time quantitative PCR detection of tumor necrosis factor α and interleukin-1β mRNA expression

All rats were killed under anesthesia after behavioral tests, the brains were harvested after cardiac reperfusion, and fresh left hippocampal tissues were collected. According to the Finch's method[39], tumor necrosis factor α mRNA and interleukin-1β mRNA expression was determined by PCR as follows: Trizol (Sigma, St. Louis, MO, USA) was added to extract total RNA. The extracted RNA content was assayed using a type UV-240 ultraviolet spectrophotometer (Beijing Kaiao Technology Development Co., Ltd., Beijing, China); the A_{260}/A_{280} ratio of total RNA was 1.80–2.00. Total RNA (2 μg) was reverse transcribed into cDNA using a Superscript III Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA, USA). The synthesized cDNA was subjected to real-time quantitative PCR using a SYBR Green PCR Kit (Beijing TransGen Biotech Co., Ltd., Beijing, China) as follows: 1 μL of specific primer (0.5 μL upstream primer and 0.5 μL downstream primer), 12.5 μL of 2 × SYBR Green QPCR Master Mix, 2.5 μL of diluted cDNA and 9 μL of nuclease-free PCR-grade water were mixed and denaturated at 95°C for 5 minutes, followed by 40 cycles of 95°C for 30 seconds, 60°C for 1 minute and 72°C for 30 seconds. PCR product SYBR green fluorescence was detected, with β-actin as the internal reference for quantitative product concentration.

The sequences of specific primers for tumor necrosis factor α and interleukin-1β mRNAs were obtained from GenBank, designed using Primer Premier 5.0 software (Premier, Vancouver, Canada), and synthesized in the Shanghai Institute of Biological Engineering, China. Specific sequences are shown in Table 1.

Table 1 The sequences of specific primers for tumor necrosis factor alpha (TNF-α) and interleukin-1β (IL-1β)

Gene	Sequence	Length (bp)
TNF-α	Upstream primer: 5'-TCT CAA AAC TCG AGT GAC AAG-3' Downstream primer: 5'-AGT TGG TTG TCT TTG AGA TCC-3'	426
IL-1β	Upstream primer: 5'-AAC TGT CCC TGA ACT CAA CTG-3' Downstream primer: 5'-TGG GAA CAT CAC ACA CTA GC-3'	396
β-actin	Upstream primer: 5'-CTT CTA CAA TGA GCT GCG TG-3' Downstream primer: 5'-TCA TGA GGT AGT CAG TCA GG-3'	305

Western blot assay for estrogen receptor β, Akt and amyloid β protein expression in the hippocampus

The rat hippocampus was lysed with lysis buffer containing protease inhibitors on ice for 30 minutes, homogenized for 30 seconds, and centrifuged at 12 000 r/min for 20 minutes (centrifugal radius 6 cm). After the supernatant was discarded and lysate was diluted, the total protein concentration was detected using the BCA method[39]. Total protein (15 μg) in each sample was mixed with 4 μL of 6 × loading buffer, degenerated using boiling water for 5 minutes, and subjected to 10% SDS-PAGE discontinuous gel electrophoresis at 80 V for 40 minutes and at 110 V for 90 minutes, followed by 200 mA electrical membrane transfer for 60–90 minutes. Samples were blocked with calf serum (Boster, Wuhan, Hubei Province, China) and skim milk for 2 hours, rinsed with TBST six times (10 minutes each), and incubated with rabbit anti-estrogen receptor β, Akt and amyloid β protein polyclonal antibody (1:2 000; Santa Cruz Biotechnology) at 4°C overnight. The next day, samples were rinsed with TBST six times (10 minutes each) and incubated with horseradish peroxidase-labeled goat anti-rabbit IgM (1:200; Santa Cruz Biotechnology) at room temperature for 2 hours. ECL chemiluminescence was used to visualize protein bands. The absorbance of the scanned bands was determined using Image J (National Institutes of Health, Rockville, MD, USA). The results are expressed as the ratio of the target protein band absorbance to that for β-actin.

Immunofluorescence staining for detection of amyloid β protein expression in the hippocampus

According to the methods of Liu *et al* [38], rat hippocampal slices were stained for amyloid β protein. In brief, brain tissue frozen slices were permeated with 0.5% Triton X-100 (Hufeng Biotechnology Co., Ltd., Shanghai, China) at room temperature for 20 minutes, rinsed with 0.1 M PBS for 5 minutes, three times. After being blocked with 10% calf serum at room temperature for 1 hour, samples were rinsed with 0.1 M PBS for 5 minutes, three times, incubated with rabbit anti-amyloid β protein polyclonal antibody (1:200; Santa Cruz Biotechnology) at 4°C; rinsed with 0.1 M PBS for 5 minutes, three times, and then incubated with goat anti-rabbit IgG-FITC (1:2 000; Santa Cruz Biotechnology) diluted with 3% bovine serum albumin for 1 hour at room temperature. Samples were then rinsed with 0.1 M PBS for 5 minutes, three times, and mounted with fluorescence quenching agent. Under a confocal laser scanning microscope (Olympus, Tokyo, Japan), five visual fields were selected from each slice under 100 × magnification. The fluorescent products were quantified and the intensity of fluorescence was measured using Image Pro Plus (Media Cybernetics, Silver Spring, MD, USA) software.

Statistical analysis

Measurement data are expressed as mean ± SD and grouped data are represented by relative values. Statistical processing was performed using SPSS 17.0 software (SPSS, Chicago, IL, USA). Intergroup differences were compared using analysis of variance followed by *q* test, with a = 0.05 as the standard.

Author contributions: Zhu Tian and Qun Liu designed and evaluated the study. Yang Zhao and Sheng Bi performed data analysis. Zhu Tian drafted the manuscript. All authors were involved in performing the experiments. All authors approved the final version of the paper.

Conflicts of interest: None declared.

Ethical approval: The experiment was approved by the Animal Ethics Committee of Jilin University in China.

Author statements: The manuscript is original, has not been submitted to or is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application disputations.

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