Neuropeptide Y gene transfection inhibits post-epileptic hippocampal synaptic reconstruction

Fan Zhang1,2, Wenqing Zhao1, Wenling Li3, Changzheng Dong3, Xinying Zhang3, Jiang Wu3, Na Li3, Chuandong Liang3

1 Graduate School, Hebei Medical University, Shijiazhuang 050011, Hebei Province, China
2 Hebei General Hospital, Shijiazhuang 050071, Hebei Province, China
3 Department of Neurosurgery, People’s Hospital of Hebei Province, Shijiazhuang 050071, Hebei Province, China

Abstract
Exogenous neuropeptide Y has antiepileptic effects; however, the underlying mechanism and optimal administration method for neuropeptide Y are still unresolved. Previous studies have used intracerebroventricular injection of neuropeptide Y into animal models of epilepsy. In this study, a recombinant adeno-associated virus expression vector carrying the neuropeptide Y gene was injected into the lateral ventricle of rats, while the ipsilateral hippocampus was injected with kainic acid to establish the epileptic model. After transfection of neuropeptide Y gene, mossy fiber sprouting in the hippocampal CA3 region of epileptic rats was significantly suppressed, hippocampal synaptophysin (p38) mRNA and protein expression were inhibited, and epileptic seizures were reduced. These experimental findings indicate that a recombinant adeno-associated virus expression vector carrying the neuropeptide Y gene reduces mossy fiber sprouting and inhibits abnormal synaptophysin expression, thereby suppressing post-epileptic synaptic reconstruction.

Key Words
neural regeneration; gene therapy; neural plasticity; neurodegeneration; recombinant adeno-associated virus vector; neuropeptide Y; epilepsy; kainic acid; synaptic remodeling; mossy fiber sprouting; hippocampus; synaptophysin; neuroregeneration

Research Highlights
(1) Increasing evidence indicates that exogenous neuropeptide Y can suppress epileptic seizures. However, the most effective administration route of exogenous neuropeptide Y remains unclear.
(2) Adeno-associated virus was used as a vector to explore the effect of neuropeptide Y gene transfection on hippocampal mossy fibers, which undergo post-epileptic synaptic reconstruction.
(3) Neuropeptide Y gene transfection was found to inhibit mossy fiber sprouting.
**INTRODUCTION**

Temporal lobe epilepsy is the major type of drug-refractory epilepsy, involving neuronal circuits and fibers. Synaptic remodeling is one of the most common pathological changes after epileptic seizures. Ectopic synaptic reconstruction in the hippocampus is considered to be closely related with temporal lobe epilepsy\(^1\)\(^-\)\(^3\). Mossy fiber sprouting is the most prominent manifestation in synaptic remodeling, and it is a very complex process involving interactions between nerve cells and the epileptic seizure. However, the underlying mechanisms are not yet completely clear. Mossy fiber sprouting may trigger synaptic connections or synaptic remodeling in hippocampal CA3 pyramidal cells, which could lead to the formation of excitatory synaptic circuits, thereby increasing epileptic susceptibility\(^3\)\(^-\)\(^5\). Synaptophysin (p38) is a vesicle adhesion protein that contributes to synaptic structure and function. It is selectively localized in presynaptic vesicles and is involved in Ca\(^{2+}\)-dependent neurotransmitter release and synaptic vesicle trafficking. Synaptophysin expression can be used to accurately assess dynamic changes at the synapse, including synaptogenesis and synaptic remodeling\(^6\)\(^-\)\(^7\). Moreover, synaptophysin is also a sensitive marker of hippocampal plasticity\(^8\).

An effective drug treatment for temporal lobe epilepsy is a major challenge facing neurologists. The currently available anti-epileptic drugs mainly target neurotransmitter receptors and ion channels\(^9\). After long-term anti-epileptic drug treatment, 60–70% of patients remain sensitive to treatment, while the rest develop drug-refractory epilepsy, and ultimately, epileptic foci must be surgically resected\(^10\)\(^-\)\(^11\). Neuropeptide Y is considered to be closely related to epilepsy; it reduces neuronal excitability and has antiepileptic effects\(^12\)\(^-\)\(^13\). Different administration routes and treatment dosages of neuropeptide Y may lead to variations in therapeutic results\(^14\). Recently, a new delivery method, the use of a recombinant adeno-associated virus vector carrying the neuropeptide Y gene, has attracted widespread attention\(^15\). Gene therapy is used to treat epileptic patients through target gene transfection, thus avoiding surgical resection of nerve tissue\(^16\). As a gene transfer vector, adeno-associated virus can effectively control the expression of a single or several genes through transcriptional regulatory regions, which helps to regulate target gene expression. Moreover, the transfected viral vector is non-pathogenic and harmless to normal tissues\(^17\). This study aimed to observe the effects of adeno-associated virus-mediated neuropeptide Y expression on synaptic remodeling in rats with temporal lobe epilepsy, in a broader attempt to provide experimental support for its use in gene therapy.

**RESULTS**

**Quantitative analysis of experimental animals**

One hundred and sixty Wistar rats were used in this study, including 48 in the control group and 112 in the experimental group. A rat model of epilepsy was established by intracerebroventricular injection of kainic acid; 16 animals did not undergo successful surgery or modeling, while 96 were successful. The successful model rats were randomly divided into model and neuropeptide Y groups, respectively receiving intracerebroventricular injection of recombinant adeno-associated virus without neuropeptide Y or recombinant adeno-associated virus carrying neuropeptide Y prior to epileptic seizure. Ultimately, 144 rats were involved in the final analysis, with 48 rats in each group. From each group, 24 rats were used for detection of seizure severity using hippocampal Timm staining and were stained as brownish yellow or brown particles by Timm staining. After rats were injected with kainic acid for 2 weeks, they began to exhibit epileptic seizures in both the model and neuropeptide Y groups to a similar extent. At 4 weeks after the beginning of seizures, the severity of the epileptic episodes was significantly lower in the neuropeptide Y group compared with the model group (\(P < 0.05\)): mild seizures increased, while severe seizures decreased in number. No seizures occurred in the control group (Table 1).

**Neuropeptide Y transfection reduced episodes of epileptic seizures**

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**Neuropeptide Y transfection reduced mossy fiber sprouting in the hippocampal CA3 region of epileptic rats**

The terminals of dentate gyrus granule cell mossy fibers were abundant in Zn\(^{2+}\) and were stained as brownish yellow or brown particles by Timm staining\(^18\). After 2 weeks of kainic acid injection, Timm particles were distributed from the granule cell layer to the stratum oriens in the hippocampal CA3 region in both the model and neuropeptide Y groups. Some of these particles showed a continuous distribution. At 4 weeks, Timm particles in the hippocampal CA3 region increased, and were continuously distributed in the stratum oriens in the model group. In contrast, particles were discontinuously...
distributed and reduced in the neuropeptide Y group. Very few or no Timm particles were observed in the hippocampal CA3 region in the control group (Figure 1).

At 2 weeks after kainic acid-induced epilepsy, the model and neuropeptide Y groups had similar levels of mossy fiber sprouting in the hippocampal CA3 region. At 4 weeks, neuropeptide Y-transfected rats showed a significantly lower level of mossy fiber sprouting compared with model rats ($P < 0.05$; Table 2).

Neuropeptide Y transfection reduced hippocampal synaptophysin (p38) mRNA expression in epileptic rats

Real-time PCR assay showed that, after 2 and 4 weeks of kainic acid-induced epilepsy, hippocampal synaptophysin (p38) mRNA expression increased in epileptic rats compared with the control group. After rats were transfected with neuropeptide Y, synaptophysin (p38) mRNA expression significantly diminished ($P < 0.05$), and mossy fiber sprouting simultaneously appeared (Table 3).

Neuropeptide Y transfection reduced hippocampal p38 expression in epileptic rats

Western blot assay results showed that, at 2 and 4 weeks after kainic acid-induced seizures, p38 expression in the hippocampus of control rats was maintained at a relatively stable level. Hippocampal p38 expression increased in epileptic rats compared with the control group ($P < 0.05$). Two weeks after neuropeptide Y transfection, hippocampal p38 expression was similar to that in the model group ($P > 0.05$). At 4 weeks, hippocampal p38 expression was significantly lower than in the model group ($P < 0.05$; Figure 2).

Table 1 Effect of neuropeptide Y transfection on epileptic episodes in rats

| Group          | 2 weeks after epilepsy | 4 weeks after epilepsy |
|----------------|------------------------|------------------------|
|                | Mild episode | Severe episode | Mild episode | Severe episode |
| Control        | 0           | 0           | 0           | 0           |
| Model          | 1           | 11          | 2           | 10          |
| Neuropeptide Y | 3           | 9           | 7           | 5           |

Seizures were graded as follows: 0: No episodes; level I: wet-dog shakes, hemifacial spasm such as blinking, quivering whiskers and rhythmic chewing; level II: neck muscle convolution is manifested by nodding and tail-flicking; level III: forelimb clonic spasm on one side; level IV: bilateral forelimb clonus and standing; level V: systemic clonus, loss of balance, falling down. Levels I–III: mild seizures; levels IV–V: severe seizure. The data are numbers of rats. Twelve rats in each group were examined at each time point. *$P < 0.05$, vs. model group (two-sample t-test).
DISCUSSION

Synaptophysin is currently used as an indicator in neural plasticity research, and its expression may reflect synaptic plasticity\(^\text{19}\). Synaptophysin immunoreactivity in the hippocampal CA3 region was significantly enhanced in the acute epileptic phase, which is consistent with the Timm particle distribution, while synaptophysin expression was upregulated during the chronic phase of epilepsy, which is consistent with mossy fiber sprouting\(^\text{20-21}\). Karson \textit{et al.}\(^\text{22}\) observed synaptophysin expression at the majority of axo-somatic, axo-dendritic and axoaxonic contacts under immunofluorescence confocal microscopy. An increasing amount of research has focused on whether drug treatment can inhibit synaptic remodeling after epilepsy by modulating synaptophysin expression. After 2 weeks of kainic acid-induced epilepsy in rats, mossy fiber and synaptophysin were highly expressed in the hippocampal CA3 region, and was maintained until 4 weeks (chronic phase). These findings are in agreement with previous studies\(^\text{20-22}\). Our present experimental observations indicate that neuropeptide Y gene transfection reduces abnormal expression of synaptophysin, thereby inhibiting synaptic reconstruction.

Neuropeptide Y is a 36-amino acid peptide that is widely distributed in the central and peripheral nervous systems, especially in the hippocampus\(^\text{23-25}\).

Table 2  Effect of neuropeptide Y transfection on mossy fiber sprouting in the hippocampal CA3 region in epileptic rats

| Group           | 2 weeks after epileptic seizure | 4 weeks after epileptic seizure |
|-----------------|---------------------------------|---------------------------------|
| Control         | 0.32±0.20                       | 0.33±0.20                       |
| Model           | 2.36±0.47                       | 2.38±0.47                       |
| Neuropeptide Y  | 2.27±0.47                       | 1.09±0.42                      |

The degree of mossy fiber sprouting was evaluated with Timm staining. 0: No Timm staining; 1: occasionally seen Timm stained particles, in a discontinuous distribution; 2: Timm stained particles discontinuously distributed; 3: Timm stained particles nearly continuously distributed; 4: Timm stained particles continuously distributed; 5: Timm stained particles forming a dense laminar layer. Data are expressed as mean ± SD. There are 12 rats in each group at each time point. \(^{a}P < 0.05\), vs. control group; \(^{b}P < 0.05\), vs. model group (two-sample t-test).

Table 3  Effect of neuropeptide Y transfection on hippocampal synaptophysin (p38) mRNA expression (absorbance ratio of p38 mRNA to GAPDH mRNA) in epileptic rats

| Group           | 2 weeks after epileptic seizure | 4 weeks after epileptic seizure |
|-----------------|---------------------------------|---------------------------------|
| Control         | 1.82±0.74                       | 1.88±0.79                       |
| Model           | 7.55±1.36\(^{a}\)               | 7.60±1.41\(^{a}\)               |
| Neuropeptide Y  | 7.41±1.49\(^{a}\)               | 3.33±0.32\(^{ab}\)             |

Data are expressed as mean ± SD. There are 12 rats in each group at each time point. \(^{a}P < 0.05\), vs. control group; \(^{b}P < 0.05\), vs. model group (two-sample t-test).

Figure 2  Effect of transfected neuropeptide Y on hippocampal synaptophysin (p38) expression in epileptic rats.

(A) Western blot assay for hippocampal synaptophysin (p38) expression. (B) Quantitative results of synaptophysin (p38) expression. p38 protein was expressed as the integrated absorbance ratio of p38 band to GAPDH band. Data are expressed as mean ± SD. There are 12 rats in each group at each time point. \(^{a}P < 0.05\), vs. control group; \(^{b}P < 0.05\), vs. model group (two-sample t-test).
In mossy fibers, neuropeptide Y is transported to the nerve endings, where it is released. It then binds presynaptic Y2 autoreceptors to inhibit glutamate release from the nerve endings and inhibit N-type Ca\(^{2+}\) channels. As a result, intracellular Ca\(^{2+}\) levels are reduced, and intracellular Ca\(^{2+}\) homeostasis is maintained\cite{26-27}. Exogenous neuropeptide Y has antiepileptic effects; however, the mechanism of action remains unclear. Previous studies focused on the role of neuropeptide Y in the hippocampus, and found that neuropeptide Y inhibits hippocampal excitability by increasing GABA inhibition or reducing excitation by glutamate. Neuropeptide Y provides feedback to the presynaptic terminals via the autoreceptors to suppress glutamate secretion\cite{27-28}. Baraban et al\cite{28} showed that neuropeptide Y gene knockout mice had a shorter latency of kainic acid-induced seizures and required fewer dose of kainic acid. Furthermore, mortality significantly increased after kainic acid injection compared with the control group. Intracerebroventricular administration of neuropeptide Y prior to kainic acid injection may significantly decrease mortality. Reibel et al\cite{30} demonstrated that neuropeptide Y, injected into the hippocampus for 7 consecutive days, delayed epileptic seizures induced by kindling, while anti-neuropeptide Y immunoglobulin G injection aggravated seizure severity. To the best of our knowledge, prior to the present study, there was no data on the influence of exogenous neuropeptide Y on synaptic remodeling, including mossy fiber sprouting or synaptophysin expression. In addition, no research had evaluated methods for endogenous neuropeptide Y expression. Direct intraventricular injection in animals is the only commonly used method at present.

As researchers clarify the pathogenesis of epilepsy, gene therapy for drug-refractory epilepsy has become a major concern of molecular neurobiologists. Recombinant adeno-associated virus has many advantages as a gene transfer vector, such as negligible neurological toxicity, low immunogenicity and stable expression of the target gene\cite{31-33}. Recombinant adeno-associated virus 2/1 serotype vectors have been used to significantly inhibit kainic acid-induced epileptic seizures in rats, reduce synaptic reconstruction, and modulate learning and memory functions\cite{34-35}. In addition, enhanced green fluorescent protein, serving as a reporter gene for recombinant adeno-associated virus 2/1-mediated expression, is clearly visible by fluorescence microscopy, and fluorescence-labeled neuropeptide Y can be directly observed in the hippocampal hilar area, and the CA1 and CA3 regions\cite{36-37}. In our previous studies, we used fluorescence-labeled neuropeptide Y, and we found that neuropeptide Y, transfected using adeno-associated virus, was expressed in the CA3 region, as determined by fluorescence microscopy.

In this study, rats were given an intraventricular injection of neuropeptide Y adeno-associated virus, and then treated with kainic acid to induce epileptic seizures. There was no significant difference in seizure severity compared with the control group at 2 weeks. The neuropeptide Y group only exhibited mild seizures at 4 weeks. We also observed mossy fiber sprouting and synaptophysin protein expression in the hippocampal CA3 region. After 4 weeks of neuropeptide Y injection, mossy fiber sprouting significantly diminished, and synaptophysin protein expression was significantly reduced. The mechanism underlying the effect of neuropeptide Y on synaptic remodeling remains unclear. Some researchers have suggested that neuropeptide Y gene expression inhibits the release of the excitatory neurotransmitter glutamate from presynaptic terminals and alters synapse-related events triggered by glutamate. Synaptic remodeling, mossy fiber sprouting and synaptophysin secretion also undergo a series of changes\cite{36}. Our experimental findings show that recombinant adeno-associated virus 2/ neuropeptide Y/enhanced green fluorescent protein can suppress mossy fiber sprouting and reduce synaptophysin protein expression. Further investigation of the mechanisms underlying the effects of neuropeptide Y on synaptic remodeling are warranted.

In summary, the neuropeptide Y gene, transfected using adeno-associated virus, has clear antiepileptic effects. Additional research is required to optimize this treatment strategy for refractory epilepsy. The safety and potential adverse reactions of this method also require further study.

**MATERIALS AND METHODS**

**Design**
A comparative animal experiment focusing on neural pathophysiology.

**Time and setting**
Experiments were performed from August 2010 to August 2012 in the Clinical Research Center, Hebei General Hospital, China.

**Materials**

**Animals**
One hundred and sixty healthy, clean, adult, male Wistar
rats, aged 12–20 weeks, weighing 250–279 g, were purchased from the Experimental Animal Center of Hebei Medical University, China (license No. SCXK (Ji) 2008-1-003, certificate No. 0909106). Rats were raised in individual cages in the Clinical Research Center, Hebei General Hospital, China at 20–26°C, allowing ad libitum access to food and water. Experimental procedures were performed in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals, formulated by the Ministry of Science and Technology of China[39].

**Recombinant virus**

Recombinant 2/1 type adeno-associated virus vector (VGTC Gene Technology Co., Ltd., Beijing, China) was used for endogenous neuropeptide Y therapeutic gene and enhanced green fluorescent protein reporter gene transfection, at a gene titer of 5 × 10^{11} vector genomes.

**Drug**

Kainic acid of 99% purity was purchased from Sigma, USA (Shanghai Sigma-Aldrich (Shanghai) Trading Co., Ltd., Shanghai, China). Chemical structural formula is 2-carboxy-3-carboxymethyl1-4-isopropenylpyrrolidine and molecular formula is C_{10}H_{15}NO_{4}.

**Methods**

**Establishment of the kainic acid-induced epilepsy model and viral transfection**

Rats were intraperitoneally anesthetized with 10% chloral hydrate (3.5 mg/kg) and fixed in a stereotaxic apparatus (Shenzhen RWD Life Science Co., Ltd., Shenzhen, Guangdong Province, China). After the scalp hair was cut and the skin was disinfected with povidone iodine, a midline longitudinal incision was made on the scalp. According to the rat brain stereotaxic atlas by George Paxinos and Charles Watson[40], 10 µL recombinant adeno-associated virus 2/neuropeptide Y/ enhanced green fluorescent protein was injected into the lateral ventricle (X = −1.0 mm, Y = 1.5 mm, Z = −3.8 mm) of rats in the neuropeptide Y group using a microsyringe (Shanghai Guangzheng Medical Equipment Co., Ltd., Shanghai, China). The titer was 5 × 10^{11} µg/mL, injection time was 20 minutes and injection speed was 0.5 µL/min. Model and control groups received 10 µL saline via intracerebroventricular injection. Ten minutes later, the neuropeptide Y and model groups were injected with 2 µL kainic acid (0.4 µg/µL) into the ipsilateral hippocampal CA3 region (X = 5.3 mm, Y = 4.0 mm, Z = −6.0 mm), while the control group was injected with 2 µL recombinant adeno-associated virus without neuropeptide Y. Epilepsy was assessed with the Racine 6-level evaluation criteria immediately after modeling.

Three successive episodes of epileptic seizure above level I were regarded as successful modeling[32-38, 41].

**Rat behavioral observation**

At 2 and 4 weeks after seizure, the rat’s behavior and the severity of seizures were continuously monitored using a camera. Epileptic seizure severity was assessed using a modification of the Racine 6-level evaluation criteria[42]: 0: no episodes; level I: wet-dog shakes, hemifacial spasm such as blinking, quivering whiskers and rhythmic chewing; level II: neck muscle convulsion is manifested by nodding and tail-flicking; level III: forelimb clonic spasm of one side; level IV: bilateral forelimb clonus and standing; level V: systemic clonus, loss of balance, falling down. Levels I–III: mild seizures; levels IV–V: severe seizures.

**Timm staining for semi-quantitative observation of mossy fiber sprouting in the rat hippocampal CA3 region**

At 2 and 4 weeks after seizure, rats were anesthetized with 10% chloral hydrate and killed. Brains were rapidly removed and frozen to prepare hippocampal consecutive coronal frozen sections, at 25 µm thickness. Frozen sections were incubated with Timm medium (consisting of 50% gum arabic 60 mL, citrate buffer 10 mL, 5.67% hydroquinone 30 mL, and 71% nitric acid silver solution 0.5 mL; Department of Pathology, Hebei General Hospital, China) at room temperature for 50 minutes. Subsequently, sections were washed with distilled water, dehydrated with a graded ethanol series, cleared with xylene, and mounted. Mossy fiber sprouting in the hippocampal CA3 region was semi-quantitatively evaluated with Timm staining under the light microscope[43]: 0: no Timm staining; 1: occasionally observed Timm stained particles, in a discontinuous distribution; 2: Timm stained particles discontinuously distributed; 3: Timm stained particles nearly continuously distributed; 4: Timm stained particles continuously distributed; 5: Timm stained particles forming a dense laminar layer.

**Fluorescence real-time quantitative PCR detection of synaptophysin (p38) mRNA expression in the rat hippocampus**

At 2 and 4 weeks after seizure, rats were killed under deep anesthesia and the brains were quickly harvested. Total RNA was extracted from hippocampal tissue using Trizol (CoWin Biotech Co., Ltd. (CWBIIO), Beijing, China). Total RNA content, purity and integrity were measured using a UV spectrophotometer (type 756, Certified Genetool Inc., Pleasanton, CA, USA) and agarose gel electrophoresis (DYY-6B steady-voltage, steady-flow
electrophoresis apparatus, Beijing Liuyi Instrument Factory, Beijing, China). 2 μL RNA for each sample was reverse transcribed into cDNA, and 1 μL cDNA was used for PCR amplification. The primer sequences were as follows: synaptophysin (p38) upstream primer sequence: 5'-TTT GCC TTC TCT TAG TCT G-3'; downstream primer sequence: 5'-GCC TTT TGT TGT TCT G-3'; amplification length: 79 bp; GAPDH upstream sequence: 5'-ACC ACA GTC CAT GCC ATC AC-3'; GAPDH downstream sequence: 5'-TCC ACC CTG CTG CTG-3'; amplification length: 452 bp. SyberGreen fluorescent quantitative PCR reaction parameters were as follows: 96°C pre-denaturation for 4 minutes; 40 cycles of 94°C denaturation for 30 seconds, 58°C annealing for 30 seconds, 72°C extension for 30 seconds; 72°C fluorescence signal collection for 30 seconds. After amplification, the results were analyzed on an ABI 7300 Real-Time PCR System (PE Company, Norwalk, USA), with GAPDH as an internal reference gene to permit comparison with sample No. 1 in the control group. Finally, the relative quantitative value of target gene expression was obtained.

**Western blot analysis of p38 protein expression in the rat hippocampus**

At 2 and 4 weeks after seizure, rats were killed under deep anesthesia and brains were quickly removed. The hippocampus was dissected out and mixed with protein lysing solution (Beijing Solarbio, Beijing, China) at a 1:9 ratio to prepare a 10% protein homogenate, and the supernatant was collected after centrifugation. 10 μL supernatant was extracted for protein quantification using the BCA method\[^{42}\]. Solution containing 50 μg protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The separated proteins were transferred to polyvinylidene fluoride membranes and incubated with mouse anti-rat p38 monoclonal antibody (1:300; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse anti-rat GAPDH monoclonal antibody (1:300; Santa Cruz Biotechnology) at 4°C overnight. Then, samples were incubated with horseradish peroxidase-conjugated rabbit anti-mouse IgG (1:5 000; Santa Cruz Biotechnology) at 37°C for 1 hour and developed using chemiluminescence solution\[^{44}\]. X-ray film was exposed to the blot and then analyzed using a UVP gel scanning system (Ultra-Violet Products Ltd., Cambridge, UK). p38 protein expression levels were expressed as the integrated absorbance ratio of p38 band to GAPDH band\[^{44}\].

**Statistical analysis**

Data were analyzed using SPSS 13.0 software (SPSS, Chicago, IL, USA) and expressed as mean ± SD. Completely randomized one-way analysis of variance was applied for comparisons among groups, and two groups were compared using two-sample t-test. A P value less than 0.05 was considered statistically significant.

**Acknowledgments:** We would like to thank Professor Yubin Hao, Associate Professor Chao Wang and other staff from the Clinical Research Center of Hebei Province in China for providing great guidance and selfless help.

**Author contributions:** Changzheng Dong, Jiang Wu and Chuandong Liang provided and integrated data, and had full access to study concept and design. Fan Zhang, Xinying Zhang and Na Li analyzed data, wrote the paper and performed statistical analysis. Wenling Li validated the paper and was responsible for technical information and support. Wening Zhao supervised the study. All authors approved the final version of the manuscript.

**Conflicts of interest:** None declared.

**Ethical approval:** The experimental procedures conformed with the Animal Ethical Requirements, formulated by the Animal Ethics Committee of Hebei General Hospital 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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(Reviewed by Patel B, Norman C, Chen JF, Ye M) (Edited by Yu J, Yang Y, Li CH, Song LP)