The novel amyloid-beta peptide aptamer inhibits intracellular amyloid-beta peptide toxicity

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Abstract
Amyloid β peptide binding alcohol dehydrogenase (ABAD) decoy peptide (DP) can competitively antagonize binding of amyloid β peptide to ABAD and inhibit the cytotoxic effects of amyloid β peptide. Based on peptide aptamers, the present study inserted ABAD-DP into the disulfide bond of human thioredoxin (TRX) using molecular cloning technique to construct a fusion gene that can express the TRX1-ABAD-DP-TRX2 aptamer. Moreover, adeno-associated virus was used to allow its stable expression. Immunofluorescent staining revealed the co-expression of the transduced fusion gene TRX1-ABAD-DP-TRX2 and amyloid β peptide in NIH-3T3 cells, indicating that the TRX1-ABAD-DP-TRX2 aptamer can bind amyloid β peptide within cells. In addition, cell morphology and MTT results suggested that TRX1-ABAD-DP-TRX2 attenuated amyloid β peptide-induced SH-SY5Y cell injury and improved cell viability. These findings confirmed the possibility of constructing TRX-based peptide aptamer using ABAD-DP. Moreover, TRX1-ABAD-DP-TRX2 inhibited the cytotoxic effect of amyloid β peptide.

Key Words
neural regeneration; neurodegenerative disease; gene therapy; Alzheimer’s disease; aptamer; amyloid β peptide; amyloid β peptide binding alcohol dehydrogenase; decoy peptide; thioredoxin; mitochondrial dysfunction; molecular cloning; grants-supported paper; photographs-containing paper; neuroregeneration

Research Highlights
(1) This study first inserted the amyloid β peptide binding alcohol dehydrogenase (ABAD) decoy peptide (DP) into the active sites of human thioredoxin (TRX) to construct the peptide aptamer TRX1-ABAD-DP-TRX2. Adeno-associated virus was used to allow its stable expression, which avoids multiple application of ABAD-DP and reduces synthesis cost.
(2) In vitro studies demonstrated that the peptide aptamer TRX1-ABAD-DP-TRX2 had an inhibitory effect on amyloid β peptide (Aβ) 42 toxicity, which lays the foundation for ABAD-DP gene therapy in Alzheimer’s disease treatment.
(3) The findings confirmed the possibility to construct the TRX-based peptide aptamer using ABAD-DP, indicating that human TRX can stabilize the particular conformation of ABAD-DP and facilitate ABAD-Aβ interaction.
(4) ABAD-Aβ interaction may be important for potential Aβ-mediated mitochondrial and neuronal injury. Inhibition of their interaction may be a strategy for the prevention and treatment of Alzheimer’s disease.

Abbreviations
AD, Alzheimer’s disease; Aβ, amyloid β peptide; ABAD, amyloid β peptide binding alcohol dehydrogenase; TRX, thioredoxin; rAAV, recombinant adeno-associated virus
INTRODUCTION

Alzheimer’s disease (AD) is a neurodegenerative disease mainly characterized by cognitive dysfunction and memory impairment. Amyloid β peptide (Aβ) has been shown to be the key factor in AD causation and progression. Aβ of 40 and 42 amino acids (Aβ40, Aβ42) are pathologically significant, and Aβ42 has a strong cytotoxic effect. Previous studies indicate that the extracellular Aβ can directly damage surrounding neurons and synapses, leading to a series of pathological changes, including oxidative stress, intracellular calcium overload, immunoinflammatory reactions, enhanced glutamic acid toxicity, cell apoptosis, and ultimately AD[1-2]. However, intracellular Aβ accumulation and toxicity occurs earlier than extracellular Aβ-induced AD pathological changes, including senile plaque and neurofibrillary tangle formation, which challenges the classic amyloid cascade hypothesis[3-7].

Mitochondrial dysfunction occurs in early stages of AD, as manifested by energy dysmetabolism, increased free radicals, and intracellular calcium overload[8-9]. Mitochondrial Aβ accumulation was found in the brain of AD patients and mutant amyloid precursor protein (mAPP) transgenic mice[10-11]. Aβ may be produced in mitochondria or enter the mitochondria through translocase of the outer membrane import machinery[12-13]. Aβ in mitochondria can directly damage mitochondrial electron transport chain activity, damage mitochondrial energy metabolism, increase reactive oxygen species production, destroy calcium balance and finally induce neuronal apoptosis[14]. It is of importance that Aβ in mitochondria can bind amyloid β peptide binding alcohol dehydrogenase (ABAD) and upregulate ABAD activity, and inhibiting or antagonizing Aβ cytotoxicity[15, 21]. However, synthesized peptide ABAD-DP is very expensive, and because of its small molecular mass it is easily degraded in cells; the half-life is very short, resulting in the need of long periods of application[22]. In addition, application of ABAD-DP alone cannot guarantee its conformation of L_o loop in cells, affecting the binding efficiency to Aβ. Therefore, application of synthesized ABAD-DP cannot effectively antagonize Aβ.

Aptamers are oligonucleic acids or peptide molecules that bind to a target molecule with high affinity and high specificity; similar, even superior, to antibodies. Thus, they have been recently used in studies of tumors, infection and diseases of the immune system, as well as diagnosis and treatment of nervous system diseases[23-24]. Peptide aptamer is a recombinant protein that can bind to a specific target protein in cells and alter the biological function of the target protein. The typical structure of peptide aptamer is to insert the peptide into a constant scaffold protein, which presents the peptide with a specific conformation, increasing the binding affinity and specificity to the target protein[25]. In contrast to previous studies, we used molecular biology based on peptide aptamers to insert cDNA encoding ABAD-DP into the activated site of the human thioredoxin (TRX), constructed the TRX-ABAD-DP-TRX (T-A-T) fusion gene with human TRX as a scaffold protein, and used adeno-associated virus to allow its stable expression. On one hand, ABAD-DP was presented with a stable LD loop using human TRX scaffold protein function to maximize ABAD-DP and Aβ interaction; on the other hand, gene therapy avoids complex peptide synthesis in vitro and is cost-effective. The present study sought to investigate the possibility of constructing human-TRX-based peptide aptamers using ABAD-DP to explore the T-A-T location in cells, to determine its neuroprotective effect and lay a foundation for AD gene therapy. Moreover, we aimed to verify that the ABAD-Aβ interaction is important for potential Aβ-mediated mitochondrial and neuronal injury and that it may be a target for the prevention and treatment of AD.

RESULTS

Successful construction of T-A-T fusion gene cDNA
Target fragments TRX1, TRX2 and ABAD-DP were obtained by PCR and inserted into the multiple clone
site of the adeno-associated virus shuttle plasmid pSSHG-CMV to obtain the T-A-T fusion gene. The sequencing length of TRX1, ABAD-DP and TRX2 was 114 bp, 93 bp, and 235 bp, respectively. The sequencing results showed that the nucleotide sequences were consistent with our design (Figures 1A–C). The recombinant adeno-associated virus vector pSSHG-CMV/T-A-T was digested with EcoRI and BamHI and confirmed that the T-A-T fusion gene was 435 bp (Figure 1D), consistent with the expected size. These results indicate successful construction of T-A-T fusion gene cDNA.

Stable expression of the T-A-T peptide aptamer
The recombinant adeno-associated virus (rAAV) was used to express T-A-T. Real-time quantitative PCR showed that the rAAV/T-A-T titer was $3.45 \times 10^8$/mL. Anti-human TRX antibody was used to detect T-A-T peptide aptamer expression. As human TRX is highly expressed in human neurons, we used mouse fibroblast NIH-3T3 to assess T-A-T peptide aptamer expression and co-localization with Aβ42 to prevent false positives. After transduction of NIH-3T3 cells with rAAV/T-A-T, immunofluorescent staining showed that 90% of cells expressed TRX (green fluorescence; Figure 2), demonstrating that rAAV/T-A-T can effectively transduce cells and express the fusion gene T-A-T.

Co-expression of the T-A-T peptide aptamer and Aβ
To verify whether the T-A-T peptide aptamer can bind Aβ$_{42}$, we introduced rAAV/T-A-T that expressed T-A-T and the recombinant retrovirus rL42SN that expressed Aβ$_{42}$ into NIH-3T3 cells. Immunofluorescent staining showed T-A-T (green, fluorescein isothiocyanate-labeled secondary antibody) and Aβ$_{42}$ (red, tetramethyl rhodamine isothiocyanate-labeled secondary antibody) in the cytoplasm (Figure 3), indicating that similar to ABAD-DP, T-A-T can bind Aβ$_{42}$ in cells.

**Figure 1** Sequencing and restriction enzyme digestion of constructed TRX1-ABAD-DP-TRX2 fusion gene cDNA. The nucleotide sequence of TRX1 (A), ABAD-DP (B) and TRX2 (C) was measured using Sanger single chain termination. The sequence lengths were 114 bp, 93 bp, 235 bp, respectively, consistent with expected size.

(D) The recombinant adeno-associated virus vector pSSHG-CMV/T-A-T was digested with EcoRI and BamHI and confirmed that the T-A-T fusion gene was 435 bp, consistent with the expected size. (a) HBI 1.0 kb plus DNA ladder; (b) pSSHG-CMV/TRX1-ABAD-DP-TRX2; (c) pSSHG-CMV/TRX1-ABAD-DP-TRX2 following EcoRI and BamHI digestion.

TX: Thioredoxin; ABAD-DP: amyloid β peptide binding alcohol dehydrogenase decoy peptide.
To further verify whether the T-A-T peptide aptamer can competitively antagonize binding between Aβ and ABAD, we assigned SH-SY5Y cells to four groups: normal control, T-A-T, Aβ and T-A-T + Aβ groups. Morphology and MTT assay at 72 hours after incubation (Figure 4).

Results showed that cell morphology was normal, with high density, clear outline and regular borders in the normal control group; T-A-T group cells exhibited similar
morphology with the normal control group; a large number of apoptotic and necrotic cells were observed in the Aβ group, with low density, vague outlines, and irregular borders, with some round, floating cells; cell condition in the T-A-T + Aβ group was significantly better than that in the Aβ group. These findings indicate that T-A-T can suppress Aβ cytotoxicity (Figure 4A–D). MTT results showed that cell viability was significantly reduced in the Aβ group when compared with the normal control group ($P < 0.05$), indicating that recombinant retrovirus rL42SN expressed Aβ42 has significant cytotoxicity. There was no significant difference in cell viability between the T-A-T and normal control groups ($P > 0.05$), suggesting that the T-A-T peptide aptamer itself is atoxic and cannot promote cell proliferation. Cell viability was significantly enhanced in T-A-T + Aβ group when compared with the Aβ group ($P < 0.05$), demonstrating that the T-A-T peptide aptamer has a strong neuroprotective effect (Figure 4E).

**DISCUSSION**

The mitochondrion is the main site where ATP and reactive oxygen species are produced in cells and plays an important role in regulating calcium homeostasis, oxidative damage and cell apoptosis. Mitochondrial dysfunction is associated with age-related neurodegenerative diseases, such as AD, Parkinson’s disease and amyotrophic lateral sclerosis 

Recent studies have shown that Aβ-induced mitochondrial structure abnormalities and dysfunction are involved in AD progression 

In the brain of AD transgenic mice and AD patients that overexpress the amyloid precursor protein, Aβ progressively accumulates in the mitochondrial matrix, and directly induces toxicity to mitochondria 

Aβ can function at the mitochondrial outer membrane, inner membrane, intermembrane space and matrix to exert toxicity through various targets, including translocase of the outer membrane-translocase of the inner membrane, balance between fusion and fission proteins, electron transport chain, Cyclophilin D, mitochondrial DNA mutation, resulting in mitochondrial oxygen consumption disorder, reduced enzyme activity associated with respiratory chain complex III and IV, and opening of the mitochondrial permeability transition pore 

Other studies demonstrated that mitochondrial Aβ-ABAD interaction results in accumulation of harmful intermediate metabolites, aggravating mitochondrial injury, inhibiting ABAD activity, increasing reactive oxygen species production, promoting caspase-3 activity, inducing mitochondrial energy dysmetabolism, impairing memory and learning, and causing neurological dysfunction in AD transgenic mice 

ABAD activity reduction also affects estrogen metabolism, damages neuroprotection of estrogen, and aggravates Aβ toxicity 

Thus, blocking of Aβ-ABAD interaction may be a potential treatment strategy for AD.

ABAD-DP contains a region that can bind Aβ and competitively antagonize binding between ABAD and Aβ. Therefore, intracellular transduction of ABAD-DP can block Aβ-induced mitochondrial injury 

Lustbader et al fused HIV Tat with ABAD-DP to enable the peptide to cross the cell membrane. This fusion peptide inhibited Aβ-induced toxicity in cultured neurons (from wild type, Tg-ABAD and Tg-ABAD/mAPP mice), protecting mitochondrial function by reducing cytochrome c release, reactive oxygen species production, DNA fragmentation and lactate dehydrogenase production. In vivo studies further demonstrated the efficacy of ABAD-DP antagonizing Aβ 

Proteomics studies showed that peroxiredoxin II and endophilin I are biological markers of AD. They were significantly increased in the cerebral cortex of AD patients, as well as Tg-mAPP and Tg-ABAD/mAPP mice. The cell membrane transduction domain of HIV Tat, and the mitochondrial targeting sequence mito, were fused with ABAD-DP to prepare the TAT-mito-ABAD-DP fusion peptide 

Following intraperitoneal injection of this fusion peptide, peroxiredoxin II levels were reduced in Tg-mAPP mice and endophilin I levels reduced to baseline in Tg-ABAD/mAPP mice. Recent evidence indicates that TAT-mito-ABAD-DP antagonized ABAD-Aβ complex formation in the mitochondrion, improved mitochondrial respiratory chain-related enzyme activity, enhanced spatial memory, and increased mitochondrial Aβ catabolic enzyme PreP activity in Tg-mAPP and Tg-mAPP/mito-ABAD mice.

ABAD-DP may be a potential therapeutic agent for AD treatment, but there are some issues with regards to the synthesis of ABAD-DP. (1) As the molecular mass is too small, it is easily degraded by endopeptidases in cells, so long periods of administration are required; (2) peptide synthesis is costly, limiting large scale production and clinical application; (3) administration of ABAD-DP alone cannot guarantee the maintenance of its particular conformation in cells, so the binding efficiency of ABAD-DP and Aβ is not stable. Thus, it is necessary to produce ABAD-DP with stable expression in cells and high binding efficiency to Aβ for AD prevention and treatment.
To solve the above-mentioned issues, we used aptamer because it has several specific advantages when compared with antibodies, such as high specificity and affinity\cite{24, 39-43}, a small molecular mass, high efficiency to synthesize, low or no immunogenicity, a wide range of target molecules, multiple functions by chemical modifications, and a specific antidote. Thus, aptamers have exhibited promising application in biological and chemical studies, disease diagnosis, and drug development. Peptide aptamers are artificial recombinant proteins that can specifically bind and influence biological functions of target proteins in cells. They contain a constant scaffold protein and a short peptide. The scaffold protein displays the peptide by special conformation, beneficial to binding to the target proteins\cite{25}. Peptide aptamer is more stable than free proteins and can be easily transported into cells. The most commonly used scaffold protein is Escherichia coli TRX, containing 109 amino acids, which can assist protein folding, the formation of active sites, and is highly and stably expressed in Escherichia coli or yeast cells\cite{44, 45}. However, it is not optimal for therapeutic purposes because it aggregates during purification and is probably immunogenic in humans\cite{46}. Human TRX of 105 amino acids, 30% sequence homologous with Escherichia coli TRX, has identical overall structure and a similar location of active sites. In 2007, we fused ABAD-DP cDNA to the human TRX 3’ end to construct the fusion gene TRX-ABAD-DP, which can co-express ABAD-DP and recombinant TRX, containing 109 amino acids, 30% sequence homologous with Escherichia coli TRX for glycine, which prevents intermolecular multimerization of recombinantly expressed proteins, assists protein insertion, and benefits conformation maintenance.

Different from previous study designs, based on peptide aptamers, we inserted ABAD-DP into active sites of the human TRX. This method increased ABAD-DP molecular mass, prevented rapid degradation of ABAD-DP, displayed stable L0 loop structure of ABAD-DP using scaffold protein function, and maximized the interaction between ABAD-DP and Aβ. In addition, mutation of the two cysteines, Cys\textsuperscript{32} and Cys\textsuperscript{35}, of Trp-Cys-Gly-Pro-Cys in human TRX for glycine\cite{46} prevented intermolecular multimerization of T-A-T, and increased the stability of T-A-T. Moreover, adeno-associated virus was used to allow its stable expression. Co-localization and MTT results showed that the T-A-T peptide aptamer can specifically bind the Aβ\textsubscript{42} peptide in the cytoplasm to inhibit Aβ\textsubscript{42}-induced neurotoxicity and protect human neuroblastoma SH-SY5Y cells. We concluded that T-A-T has similar protective effects to ABAD-DP, and is expressed in the cytoplasm, indicating that human TRX can confer solubility and effectively stabilize short peptide conformation. Of course, this method is not appropriate to all short peptide sequences and conformation. Therefore, further studies are needed to investigate the applicable conditions. To demonstrate the efficacy of the T-A-T peptide aptamer, our group has been studying its neuroprotective effect in Tg-mAPP mice. However, mitochondrial function and oxidative stress were not assessed in the present study.

The T-A-T peptide aptamer was successfully constructed, which can inhibit intracellular Aβ toxicity. This demonstrates the possibility to construct peptide aptamer using ABAD-DP based on human TRX as a scaffold. This study lays the foundation for ABAD-DP gene therapy for AD, provides novel thoughts for AD treatment and insights into human TRX-based active peptide aptamers. The neuroprotection of the T-A-T peptide aptamer against Aβ also verified that ABAD-Aβ interaction is important in Aβ-mediated mitochondrial and neuronal injuries. Thus, inhibition of ABAD-Aβ interaction may become a strategy for AD prevention and treatment.

MATERIALS AND METHODS

Design
A parallel controlled in vitro study using genetic engineering.

Time and setting
T-A-T was constructed and expressed by Xi’an Huaguang Bioengineering Company from September 2009 to December 2010. The in vitro experiments were performed in the laboratory of the Department of Neurology, First Hospital of Jilin University, China from January 2011 to June 2012.

Materials
The plasmid pLENT/TRXABAD and recombinant retrovirus rL42SN were constructed by Dr. Xin Yang, Department of Neurology, First Hospital of Jilin University\cite{22}. The adeno-associated virus shuttle plasmid pSSHG-CMV, adenovirus helper plasmid pHelper and adeno-associated virus packaging plasmid pAAV/Ad were provided by Xi’an Huaguang Bioengineering.
Methods

Preparation of ABAD-DP cDNA
According to the ABAD-DP sequence in GenBank, the BamHI site (GGA TCC) was added to the 5’ end and the Smal site (CCCGGG) was added to the 3’ end of the sequence, with the upstream primer 5’-GGG ATC CGC AGG CAT CGC GGT GGC TAG-3’ and the downstream primer 5’-GCC CGG GAC ATC AAG AAC TCG CTG GAA G-3’. Moreover, an A–C same-sense mutation at 3’ end was performed. pLENT/ TRXABAD was used as the template for PCR using the following conditions: 94°C pre-denaturation for 300 seconds, 94°C denaturation for 60 seconds, 57°C annealing for 60 seconds, 72°C extension for 70 seconds, for 30 cycles in total. PCR products were inserted into the pGEM-T Easy vector (Promega Corporation, Madison, WI, USA) and used the Sanger chain termination method to determine all nucleotide sequences.

Preparation of TRX1 and TRX2 cDNA
According to the human TRX sequence in GenBank and a previous study, human TRX was divided into TRX1 and TRX2. The EcoRI site (GAATTC) was added to the 5’ end of TRX1 and the BamHI site (GGATCC) added to the 3’ end, with the upstream primer 5’-CGA ATT CAT GGT GAA GCA GAT CGA GAG C-3’ and the downstream primer 5’-CGG ATC CCG GAC CGC CCC ACG TGG CTG AGA AGT CAA C-3’. Plasmid pLENT/TRXABAD was used as a template for PCR using the following conditions: 94°C pre-denaturation for 300 seconds, 94°C denaturation for 60 seconds, 58°C annealing for 60 seconds, 72°C extension for 70 seconds, for 30 cycles in total. TRX1 PCR products were inserted into the pGEM-T vector (Promega Corporation) and TRX2 PCR products were inserted into the pGEM-T Easy vector. All nucleotide sequences were determined using the Sanger chain termination method.

Construction of T-A-T fusion gene cDNA
Restriction enzyme digestion was conducted according to the recombinant plasmid construction strategy (Figure 5), and TRX1, ABAD-DP and TRX2 fragments were subcloned into the adeno-associated virus shuttle plasmid pSSHG-CMV to construct the pSSHG/T-A-T plasmid.

Production and titer determination of rAAV/T-A-T
According to a previously described method, polyethyleneimine (Polysciences Inc., Warrington, PA, USA) was used to mediate transfection of adenovirus helper plasmid pHelper, packaging plasmid pAAV/Ad and constructed pSSHG-CMV to construct the pSSHG/T-A-T plasmid. The virus was retrieved after transfection for 72 hours. Titers of recombinant rAAV/T-A-T virus were determined using the real-time quantitative PCR method. The sequence of the amplification primer of the CMV promoter (primers were synthesized by Shanghai Generay Biotech Co., Ltd., Shanghai, China) was as follows: upstream 5’-CAA GTA CGC CCC CTA TTG AC-3’; downstream 5’-AAG TCC CGT TTA TTG TG-3’. The recombinant
framework plasmid template carrying the CMV promoter after multiple proportion dilution served as the standard sample, and the titer of the recombinant virus was determined.

**Cell culture**

HeLa cells were cultured in DMEM (Gibco, Paisley, UK) containing 5% fetal bovine serum (Gibco) for rAVV package. NIH-3T3 cells (ATCC cell bank) and SH-SY5Y cells (ATCC cell bank) were cultured in DMEM containing 10% fetal bovine serum, respectively, for immunofluorescent staining and MTT test. All cells were incubated in 5% CO₂ at 37°C.

**Immunofluorescent staining for T-A-T and Aβ₄₂**

NIH-3T3 cells were transduced with rAAV/T-A-T at a multiplicity of infection of 10 for 72 hours, fixed in acetone, followed by indirect immunofluorescent staining. Briefly, cells were treated with 0.5% (v/v) Triton X-100, blocked with 20% (v/v) calf serum for 30 minutes, followed by incubation with the following antibodies: goat anti-TRX polyclonal antibody (1:100; R&D, Minneapolis, MN, USA) at 37°C for 2 hours; secondary antibody, fluorescein isothiocyanate-labeled rabbit anti-goat IgG (1:100; Beijing Biosynthesis Biotechnology, Beijing, China) at 37°C for 1 hour. Fusion gene T-A-T expression in cells was observed using a fluorescence microscope (QG2-32, Olympus, Tokyo, Japan). Ten non-overlapping fields of view were randomly selected under 400 × magnification, and green fluorescent-positive cells and cells under dark field were quantified. The percentage of green fluorescent-positive cells out of the total number of cells counted under bright field was calculated.

In addition, NIH-3T3 cells were transduced with rAAV/T-A-T and recombinant retrovirus rL42SN to detect the co-localization of T-A-T and Aβ₄₂ in cells. After transduction for 72 hours, indirect immunofluorescent staining was performed. T-A-T molecules were visualized using the above-mentioned antibodies. In addition, the Aβ₄₂ peptide was visualized using the primary antibody mouse anti-Aβ₄₂ polyclonal antibody (1:100; Beijing Biosynthesis Biotechnology) at 37°C for 2 hours and the secondary antibody, tetramethyl rhodamine isothiocyanate-labeled rabbit anti-mouse IgG (1:100; Sigma, St. Louis, MO, USA), at 37°C for 1 hour.

Co-expression between T-A-T and Aβ₄₂ in cells was observed using a fluorescence microscope (Olympus).

**MTT assay for cell viability**

SH-SY5Y cells were assigned to normal control (no treatment), Aβ (recombinant retrovirus rL42SN that expressed Aβ₄₂), T-A-T (rAAV/T-A-T), and T-A-T+Aβ (rAAV/T-A-T and rL42SN) groups. The multiplicity of infection was 10, and cells were incubated for 72 hours. Cell morphology was observed by microscopy (QG2-32; Olympus). Cells were mixed with 5 mg/mL MTT (Sigma) at 37°C for 4 hours, followed by dimethyl sulfoxide to dissolve the formazan product. Absorbance at 490 nm was determined using the enzyme-linked immunosorbent assay reader (Bio-Tek, Winooski, VT, USA). Cell viability was represented by the absorbance ratio of each treatment group to the normal control group × 100%.

**Statistical analysis**

Data were expressed as mean ± SD and analyzed by SPSS 13.0 software (SPSS, Chicago, IL, USA). Intergroup comparisons were conducted using one-way analysis of variance, and paired comparisons were conducted using the Student-Newman-Keuls test. A value of P < 0.05 was considered statistically significant.

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**Author contributions:** Xu Wang conducted the experiments, performed statistical analysis, analyzed the experimental data, and wrote the manuscript. Yu Yang and Yi Yang conceived and designed the study. Mingyue Jia provided experimental data. Chi Ma integrated the experimental data. Mingyu Wang and Lihe Che assisted with experiments. Jiang Wu was in charge of funds and revised the manuscript. All authors approved the final version of the paper.

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