SPANDIDOS PUBLICATIONS EXPERIMENTAL AND THERAPEUTIC MEDICINE 19: 2149-2154, 2020

Extracellular production of recombinant *sus scrofa* trefoil factor 3 by *Brevibacillus choshinensis*

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Received June 27, 2019; Accepted December 12, 2019

DOI: 10.3892/etm.2020.8477

Abstract. Trefoil factor 3 (TFF3) is involved in cell adhesion, motility and apoptosis, regulates mucosal immunity and maintains the functional integrity of intestinal epithelia. The upregulation of TFF3 expression in the weaning rat intestine attracted our interest. The present study hypothesized that TFF3 may serve a role in preventing diarrhea in weaning piglets, which is an important consideration in the pig farming industry. Previous recombinant TFF3 protein expression yields obtained from *Escherichia coli* were too low and the bioactivity of the protein was poor. Hence, this expression system was unsuitable for industrial applications. The present study explored the production of recombinant *sus scrofa* TFF3 in a *Brevibacillus choshinensis (B. choshinensis)* expression system, aiming to enhance the expression level of bioactive protein. To achieve this, the *sus scrofa* TFF3-encoding gene fragment was fused into an *E. coli-Brevibacillus* shuttle vector pNCMO2. High levels of TFF3 (30 mg/l) were produced and secreted into the *B. choshinensis* culture medium in soluble form with a molecular mass of 13.6 kDa and high immunoreactivity in western blotting. Thus, *Brevibacillus* may be used to produce useful mucosal factors for biochemical analyses and mucosal protection, and in industrial applications to produce novel inhibitors of diarrhea.

Introduction

The family of Trefoil factor (TFF)-peptides, previously known as P-domain peptides, consists of 3 members (TFF1, 2 and 3) (1). TFF-peptides are small proteins secreted by a number of mucosal epithelial cells (2). TFFs possess a common trefoil domain structural motif, forming characteristic disulfide bonds through six conserved cysteine residues, and are highly gastric acid and protease-resistant (2). TFFs help maintain mucosal barrier integrity by protecting the gastrointestinal (GI) mucosa against injury and improving repair following injury (3-5). The pit cells in gastric mucosa primarily secret TFF1, whereas TFF2 is synthesized by the neck cells of the gastric mucosa and the Brunner's glands of the duodenal submucosa (6). The distribution of TFF1 and TFF2 is confined to the proximal GI tract, whereas TFF3 is produced by the goblet cells and secreted abundantly throughout the intestinal tract (3). TFF3 protects and repairs the gastrointestinal mucosa, maintains the tight junction barrier integrity and restores normal intestinal permeability during inflammatory bowel disease pathogenesis (7). TFF3 is also present in the neural lobe of the porcine pituitary gland (8,9).

The GI tract acts as a barrier against bacteria and toxins preventing digestive disorders. Weaning is an important process during development of the pig (10). The structure and function of the digestive system of a growing pig begins immediately to change when weaning begins (10). Weaning involves extensive exposure to novel antigens, improving the redistribution of the microbial community in the small intestine (10). Piglets are susceptible to disease after weaning. When the GI mucosal barrier function is impaired, the ecological balance among gut microbes is destroyed leading to enterogenous infection, resulting in diarrhea (11). Diarrheal piglets are of significant concern to the pig industry (10). Previous studies have revealed that TFFs can maintain the integrity of the intestinal epithelia in mice, heal wounds in humans and participate in cell adhesion, motility and apoptosis *in vitro* (12-17). TFFs are involved in mucosal immune modulation by regulating leukocyte recruitment and repairing tissue to ensure healthy mucosal surfaces (18). In addition, the level of TFF3 expression is increased in the intestine of weaning rats (19). Thus, the present study hypothesized that TFF3 may serve an important role in preventing diarrhea in weaning piglets.

In previous studies, TFF3 has been expressed in *Escherichia coli* in an intracellular and soluble form (20-22). However, this method of production is not ideal because of the low yield and bioactivity of the produced TFF3 (20-22).

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Key words: protein expression, trefoil factor 3, pNCMO2, *Brevibacillus choshinensis*

The present study developed a recombinant expression system for *sus scrofa* TFF3 fused with a 6xhis-tag in a strain of *B. choshinensis*. *B. choshinensis* is a Gram-positive bacterium, which has the excellent advantage of secreting various extracellular proteins with high efficiency (23). The spore forming ability of *B. choshinensis* has been removed by genetic engineering. In addition, this expression system is very powerful for producing proteins with native structures, even when they contain disulfide bonds (23). pNCMO2 is a *B. choshinensis-E. coli* shuttle vector (24). The pNCMO2 vector includes the P2 promoter, which drives the transcription of cell wall protein but has no role in *E. coli* (24). The objective of the present study was to explore the production of *sus scrofa* TFF3 by *B. choshinensis in vitro*.

Materials and methods

Bacteria, plasmids and media. *B. choshinensis* strain HB116 (Takara Bio, Inc.) was used in this study. *E. coli DH5α* competent cells (Sangon Biotech Co, Ltd.) were used for DNA manipulation. pNCMO2 (Takara Bio, Inc.) and pMD19-T (Takara Bio, Inc.) were used as the vector and subcloning plasmid, respectively. Milk-Tween (MT) medium containing 2% yeast extract, 10% glucose, 10% polypeptone, 5% meat extract, 0.01 % $FeSO_4 \cdot 7H_2O$, 0.001% $ZnSO_4 \cdot 6H_2O$ and 0.01% $MnSO_4 \cdot 4H_2O$ was used to culture strain HB116. *E. coli DH5α* cells were cultured in Luria Broth medium (Oxoid; Thermo Fisher Scientific, Inc.). NaOH was used to adjust the pH of all media to 7.0. Neomycin (20 μg/ml; Beijing Solarbio Science & Technology Co, Ltd.) was added to the media used to culture bacteria containing pNCMO2 and derivatives.

RNA extraction and PCR. Total RNA from *sus scrofa* spleen tissue (preserved in our laboratory) was isolated using a TRIzol® reagent kit (Takara Bio, Inc.) according to the manufacturer's instructions. cDNA synthesis was performed using a PrimeScript Reverse Transcriptase kit (Takara Bio, Inc.). Primers for the *sus scrofa* TFF3 gene were designed using Primer v.5.0 software (Sangon Biotech. Co. Ltd.), according to the gene sequence in the GenBank database (accession no. NM_001243483). mRNA specific primers (Sangon Biotech Co, Ltd.) were: TFF3 forward, 5'-GCATGGAGGCCAGGATGT-3' and reverse, 5'-CGGTTAGAAGGTGCATTCT-3'. The PCR program to amplify the TFF3 gene from cDNA was 95˚C for 5 min, followed by 35 cycles of 95˚C for 30 sec, 55˚C for 20 sec and 72˚C for 30 sec with a final extension step at 72˚C for 10 min. PCR products were separated by 2% agarose gel electrophoresis. Purified PCR fragments were retrieved using a PCR gel recovery kit (Takara Bio Inc.). Bands were visualized using the GelDoc XR+ (Bio-Rad Laboratories, Inc.) gel imaging system through nucleic acid staining. Densitometric analysis was performed using Gel-Pro Analyzer 4.0 software (Media Cybernetics, Inc.).

Construction of pNCMO2-TFF3-6xhis. According to the manufacturer's instructions of the pMD19-T vector kit (Takara Bio, Inc.), the TFF3-encoding gene and 6xhis tag were linked to the T vector to generate pMD19-T-TFF3-6xhis and transformed into *E. coli DH5α*. Positive clones were identified by colony PCR, where a single bacterium is used as a template, and can quickly identify whether the colony is a positive colony with the target gene. Plasmids extracted from the positive clones were sequenced by Takara Bio, Inc. The primers were forward, 5'-GCgtcgacATGGAGGCCAGGATGT-3' and reverse, 5'-CGGggtaccTTAGTGATGTGATGGTGATGGAAGGTGCATTCT-3'; lower-case letters denote the enzyme restriction sites for *Sal*I and *Kpn*I and the underlined bases denote the nucleotide sequence encoding the 6xhis tag. To construct the *sus scrofa* TFF3 expression vector pNCMO2-TFF3-6xhis, the fused TFF3-6xhis combined fragment from pMD19-T-TFF3-6xhis was amplified by PCR. The following thermocycling conditions were used for the PCR: initial denaturation at 95˚C for 10 min; followed by 35 cycles of 95˚C for 30 sec, 56˚C for 30 sec and 72˚C for 30 sec; and a final extension 72˚C for 10 min. Digestion with *Sal*I and *Kpn*I followed by ligation at 16˚C overnight was used to clone the amplified fragment into the pNCMO2 vector to generate pNCMO2-TFF3-6xhis. DNA sequencing was performed to confirm the cloned DNA sequence (Takara Bio, Inc.).

B. choshinensis transfection. Competent cells of *B. choshinensis* HB116 were prepared by inoculating 1 ml bacterial solution in 100 ml MT medium (Shanghai Kemin Biotechnology Ltd.), which were then cultured at 37˚C for 18 h. Bacteria were then collected by centrifugation at 4,000 x g for 5 min at room temperature, and suspended in 50 mmol/l Tris-HCl (pH=8.5; Beijing Dingguo Changsheng Biotechnology Co, Ltd.). Bacteria were then transfected with vector DNA using electrophoretic transfer according to the manufacturer's instructions (Takara Bio, Inc.). The empty pNCMO2 vector was transfected as a negative control. A MicroPulser (Bio-Rad Laboratories, Inc.) was used with the Ec2 program.

Protein expression of recombinant sus scrofa TFF3. *B. choshinensis* containing pNCMO2-TFF3-6xhis or pNCMO2 was cultured in liquid MT medium containing 20 mg/l neomycin at 30˚C for 60 h. Once the bacteria reached the logarithmic growth period at 37˚C, isopropyl β-D-1-thiogalactopyranoside (final concentration 1 mmol/l) was added to the bacteria. Following induction, the collected bacteria were disintegrated by ultrasound (JY88-II Ultrasonic Cell Disruptor; Bio-Equip) and lysed in PBS (pH=7.4). The bacterial precipitate and supernatant were subsequently used in SDS-PAGE. Supernatants and cells were separated by centrifugation at 12,000 x g for 20 min at 4˚C. Proteins were detected by reducing 14% SDS-PAGE stained with Coomassie Brilliant Blue, and the amount of secreted proteins was evaluated. Gel-Pro Analyzer 4.0 software (Media Cybernetics, Inc.) was used to scan and measure the density of bands on gels to evaluate the expression level of the target protein. For western blotting, protein samples were boiled in SDS/b-mercaptoethanol loading buffer (192 mM glycine and 25 mM Tris; pH 8.3; Beijing Dingguo Changsheng Biotechnology Co, Ltd.) and subsequently electrophoresed. A total of 10 μg protein/lane was separated via SDS-PAGE on a 14% gel. Proteins in the gel were transferred onto PVDF membranes (Merck KGaA) by electrophoretic transfer. Next, the membrane was blocked for 1 h in 5% skim milk at room temperature. TBST [20 mM Tris (pH 8.0), 200 mM NaCl and 0.1% Tween 20] was used to dilute the mouse anti-6xhis-tag

monoclonal primary antibody (Abcam; cat. no. ab18184; 1:2,000). The membrane was incubated overnight with the primary antibody at 4˚C and washed with TBST three times for 15 min. Horseradish peroxide-conjugated secondary antibody (Abcam; goat-mouse IgG; cat. no. ab150113) was diluted 1:3,000 with TBST and incubated with the membrane for 1 h at room temperature. Washing with TBST was subsequently performed three times for 15 min. ECL development methods and X-ray film exposure were used to visualize the bands (Merck KGaA).

Results

Cloning of the TFF3 gene. The coding DNA sequence (CDS) region of the *sus scrofa* TFF3 gene (NM_001243483) was searched for in the NCBI database. The length of the TFF3 CDS is 243 bp (25). In the present study, the TFF3 CDS was cloned and a ~250 bp PCR product was obtained (Fig. 1). Sequencing confirmed the nucleotide sequence and length of the target band (data not shown).

Verification of pNCMO2-TFF3-6xhis protein fragment. pNCMO2-TFF3-6xhis was generated by cloning the TFF3 CDS into pNCMO2. pMD19-T-TFF3-6xhis was verified by a restriction enzyme digest, which produced two fragments of the expected sizes 273 and 2,692 bp (Fig. 2). pNCMO2-TFF3-6xhis was verified by PCR using the total extracted DNA as the template (Fig. 3).

Verification of recombinant B. choshinensis. pNCMO2-TFF-6x his was electroporated into the *B. choshinensis* strain HB116. The resulting strain HB116-pNCMO2-TFF3-6xhis was verified by double enzyme digestion using extracted plasmid DNA as the template, *Sal*I and *Kpn*I were used as restriction enzymes to digest plasmid. The size of target band and plasmid were 273 and 5,200 bp, respectively (Fig. 4).

Analysis of the recombinant fusion protein. Protein samples from the culture medium of HB116 cells, which were transformed with the pNCMO2-TFF3-6xhis plasmid, were detected using SDS-PAGE. A band with a molecular weight of ~55 kDa in the supernatant (lane 1; Fig. 5) and precipitate (lane 2; Fig. 5) of the lysate of positive control vector-amylase, while a recombinant protein with a molecular weight of ~13 kDa was secreted by the supernatant and precipitate of HB116-pNCMO2-TFF3-6xhis (lane 5 and 6, respectively; Fig. 5). In western blotting, no immunoreactive band was present in the negative control lane, in which vector-free bacterium were induced. However, bacteria transfected with pNCMO2-TFF3-6xhis plasmid after induction displayed high immunoreactivity for the his-tag antibody (Fig. 6).

Discussion

TFF3 is a small secreted peptide and potential cytokine involved in protecting the GI mucosa (3). TFF3 can alleviate injuries in the GI mucosa caused by numerous types of stimulus and quicken repair of damaged mucosa (2,3,7). TFF3 enhances the integrity of the mucosal epithelial surface and promotes the process of reconstitution through cell migration (7). The interaction between TFF3 and mucins has also been reported to protect intestinal epithelia from injuries, maintaining mucosal barrier function (26). Furthermore, mitogenic effects of TFF3 have been identified *in vitro* in primary cell culture, and TFF3 has been demonstrated to inhibit apoptosis, induce cellular invasion, act as an inflammatory modulator and exhibit pro-angiogenic activity (27-32). A protective or healing effect of TFF3 has also been reported in a series of experiments, such as cells *in vitro*, mice, rats and human (12-17). In addition, a previous study in TFF3-deficient mice demonstrated that the intestinal mucosa was damaged and apoptosis increased in the colon of the model mice compared with TFF3-competent controls (6).

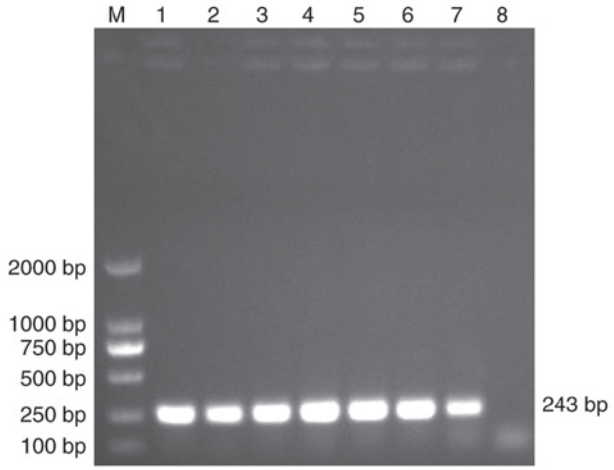

Figure 1. Electrophoretic analysis of amplified products for *sus scrofa* TFF3 gene. Lane 1-7, PCR products of TFF3 gene; lane 8, negative control, the PCR product without template DNA; M, DNA marker; TFF3, trefoil factor 3.

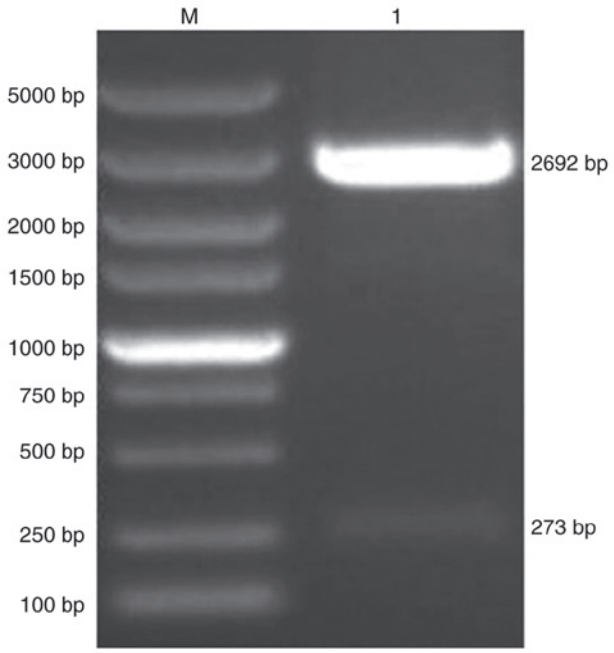

Figure 2. Identification of double enzyme digestion for pMD19-T-TFF3-6xhis plasmid. Lane 1, vector pMD19-T-TFF3-6xhis digested by restriction enzyme *Sal* I and *Kpn* I; M, DNA marker; TFF3, trefoil factor 3.

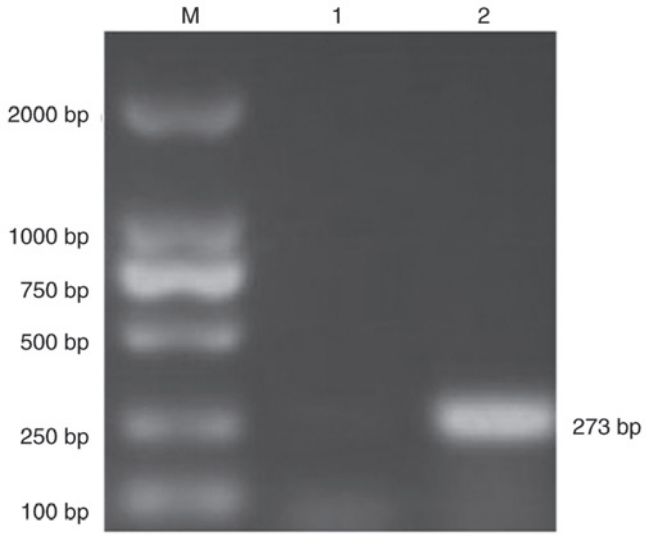

Figure 3. Electrophoretic analysis of amplified products for the TFF3-6xhis gene. Lane 1, negative control, the PCR product without template DNA; lane 2, PCR products with pNCMO2-TFF3-6xhis vector; M, DNA marker; TFF3, trefoil factor 3.

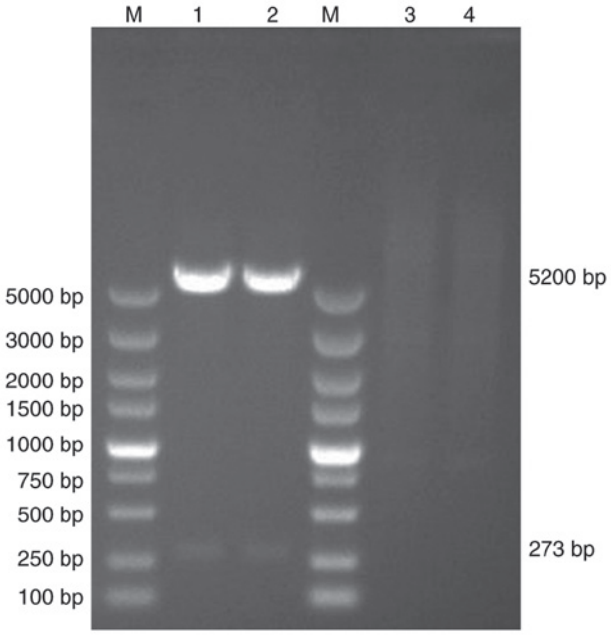

Figure 4. Identification of double enzyme digestion of the pNCMO2-TFF3-6xhis plasmid. Lane 1-2, pNCMO2-TFF3-6xhis plasmid digested by restriction enzymes *Sal* I and *Kpn* I; lane 3-4, double digestion without plasmid; M, DNA marker; TFF3, trefoil factor 3.

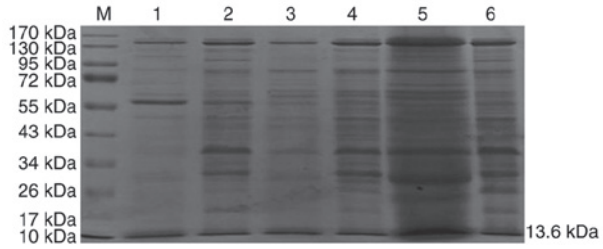

Figure 5. SDS-PAGE of *Brevibacillus choshinensis* HB116 transformants with pNCMO2 and pNCMO2-TFF3-6xhis plasmid. Lane 1, supernatant of the lysate of the position control vector-amylase after induction; lane 2, precipitate of the lysate of the position control vector-amylase after induction; lane 3, supernatant of the lysate of the pNCMO2 vector after induction; lane 4, precipitate of the lysate of the pNCMO2 vector after induction; lane 5, supernatant of the lysate of the pNCMO2-TFF3-6xhis vector after induction; lane 6: precipitate of the lysate of the pNCMO2-TFF3-6xhis vector after induction; M, protein marker; TFF3, trefoil factor 3.

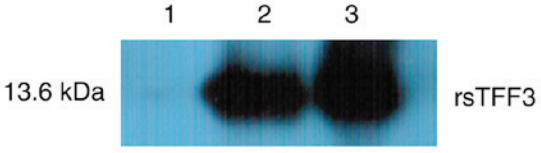

Figure 6. Western blot analysis of TFF3-6xhis fused protein. Lane 1, negative control, induction of vector-free bacteria; lanes 2 and 3, supernatant protein of bacteria transfected with pNCMO2-TFF3-6xhis plasmid after induction. rsTFF3, recombinant secreted trefoil factor 3.

TFF3 is mainly secreted by the small intestine and colon goblet cells. However, it is very difficult to directly isolate natural TFF3 from these tissues (20-22). Thus, it is important to use genetic engineering strategies to produce large amounts of recombinant TFF3 for biochemical and biomedical applications. *B. choshinensis* has an exceptional capacity to produce heterologous proteins (23,24,33-35). Target proteins can be produced and secreted efficiently with a high yield by *Brevibacillus* expression systems (33). The *Brevibacillus* expression system is suitable for producing eukaryotic proteins (23,24,33,34). Compared with traditional *E. coli* expression systems, the genus *Brevibacillus,* including thermophilic, alkalophilic, psychrophilic, acidophilic and halophilic strains, use more carbon for heterotrophic or autotrophic growth (34). *Brevibacillus* expression systems can produce certain secretory or cytoplasmic proteins that *E. coli* expression systems fail to produce (35). *Brevibacillus* expression systems possess a good protein folding environment, lack proteases and have a convenient downstream processing model, e.g. cell separation and purification of secreted proteins from the culture medium (36). Thus a number of genetically engineered enzymes and heterologous proteins including cytokines, antigens and antibody fragments are expressed using *Brevibacillus* systems (33). Extracellular proteins, including several bacterial and mammalian proteins (with yields ranging between 10-1,250 mg/l) have been produced using *Brevibacillus* systems (37-41). Recombinant TFF1 was secreted extracellularly with a high yield by the *Brevibacillus* system and had better wound healing capability compared with TFF1 produced by *E. coli* (37).

In the present study, the *sus scrofa* TFF3 gene was cloned into the pNCMO2 shuttle vector. *B. choshinensis* was used as a host bacterium to express *sus scrofa* TFF3 and produced 30 mg/l protein fused with a 6xhis-tag. Protein production was confirmed by SDS-PAGE and western blot analyses. The obtained fusion protein exhibited good antigenicity and specificity. Thus, *Brevibacillus* may be used to produce useful mucosal factors, which can be analyzed in terms of protein structure, bioactivity and kinetics as well as for their mucosal-protection activities. This expression system may be used in industrial applications to produce novel inhibitors of diarrhea.

Acknowledgements

Not applicable.

Funding

This work was supported by grants from the National Key Research and Development Program of China (grant no. 2017YFD0501100) and the Colleges and Universities Key Research Plans in Henan (grant nos. 18A230009 and 19B230013).

Availability of data and materials

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

Authors' contributions

YYW, HPL, CMX, BYW and KZ designed the study. YYW, HPL, CMX, BYW, KZ, YZZ, LPF and YDC performed the experiments. GMZ, XYJ, GYY and AQL analyzed the data. HPL, KZ and YYW wrote the manuscript. AQL drafted and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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