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Preparation of recombinant myoglobin and investigation of the liquid antigen stability for quality control materials

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ABSTRACT

Myoglobin (Mb) has been used as a biomarker for acute myocardial infarction. This study aimed to evaluate the stability of liquid Mb as quality control materials for Mb determination. Mb protein was expressed in *Escherichia coli* system and purified using Ni^{2+} chelate affinity chromatography. The purity of purified recombinant Mb reached to 95 %. The immunoreactivity of Mb was investigated using Mb assay kits. The coefficient of determination (R^2) of the curve fitted with dilution ratio and Mb concentration as variables was greater than 0.95, which indicated that Mb had good immunoreactivity. The concentrations per gradient measured using different kits had no significant difference (p-value>0.05), which indicated that the reactivity between the Mb antigen and Mb antibodies with different epitopes was good. The effects of different storage buffer, storage temperature and storage times on the stability of liquid Mb were investigated by detecting the concentration changes. At 2–8 °C for two months, Mb concentration in buffer B (Tris-HCl, pH 7.8, containing 1 % BSA and 0.05 % NaN_3) decreased within 10 % compared with the initial concentration. The long-term storage stability was investigated by the thermal acceleration experiment. At 37 °C for one week, Mb concentration decreased by less than 15 %, indicating that the Mb had good long-term storage stability. The prepared liquid Mb had good immunoreactivity and stability, avoiding storage in freeze-dried powder. It was a promising alternative as the quality control material for Mb detection.

1. Introduction

Acute myocardial infarction (AMI) is myocardial necrosis caused by acute, persistent ischemia and hypoxia in the coronary arteries [1]. Myoglobin (Mb) expressed in cardiac muscle and skeletal muscle cells is an oxygen-binding protein composed of globin and iron porphyrin, with a molecular weight of 16.7 kDa [2,3]. When AMI happened, the concentration of Mb in human blood increases rapidly due to the myocardial cell damage and persists for a long duration [4]. The assay of Mb as the cardiac biomarker is recommended for routine early detection of the AMI in the absence of definitive electrocardiogram [5,6]. The Mb content could be detected by some clinical testing methods, including chemiluminescent immunoassay (CLIA) [7], fluorescence immunoassay [8], immunoturbidimetry [9] and microfluidic chips [10]. The quality control (QC) materials are used to evaluate the reliability and accuracy of the analytical data in clinical laboratories [11]. QC materials can be tailored to the specific value of interest, and they must be easily prepared, homogenous and sufficient stability for longitudinal use [12]. Mb proteins are usually used as the quality control materials for the daily

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testing. Although the natural Mb protein could be extracted from human blood or animal muscle, it has the disadvantages of limited sources, low yield and high cost [13,14]. Mb proteins have been successfully expressed using different recombinant strategies, such as in *Escherichia coli* [15], *Komagataella Phaffii* [16] and *Saccharomyces cerevisiae* [17]. The expressions of recombinant Mb proteins have been an alternative to the extraction from animal blood or tissues [18]. The recombinant antigen usually is prepared as freeze-dried products to increase the stability, but freeze-dried products increase energy consumption and operational burden of reconstitution before use.

In this study, an alternative method for the preparation of stable liquid Mb protein as QC material was investigated. We planned to prepare the Mb protein expressed in *Escherichia coli* expression systems instead of the extraction from blood sources. The Mb protein for QC material was tailored in liquid state rather than the lyophilized powder, which would decrease costs, complexity and time handing freeze-dried powder. It is a promising technology for the development of myoglobin diagnostic reagents and quality control products.

2. Materials and methods

2.1. Materials

Restriction enzymes Xho I, Nde I and T4 DNA ligase were purchased from Takara Bio (Osaka, Japan). The plasmid extraction kit was purchased from Axygen (Silicon Valley, USA), BL21(DE3) competent cell, pET-30a(+) plasmid, kanamycin, isopropyl β-D-thiogalactoside (IPTG) and bovine serum albumin (BSA) were purchased from Sangon Biotech (Shanghai, China). Ni-NTA Resin was purchased from Smart Lifesciences (Changzhou, China). Myoglobin test kits were obtained from BIOSINO Bio (Beijing, China), BSBE Bio (Beijing, China), AIOLOS Bio (Hebei, China) and Roche Diagnostics (Mannheim, Germany). All other chemicals were of analytical grade.

2.2. Construction of recombinant expression engineering bacteria

The gene sequence of human myoglobin protein was obtained from NCBI database, while the restriction endonuclease sites of Nde I and Xho I were modified at ends of the gene sequence. The modified gene sequences with the His purification tags added to the COOH-ends were synthesized. The Mb gene fragment and pET-30a plasmid were digested with NdeI and XhoI restriction endonuclease respectively, and then linked with T4 DNA ligase. The recombinant expression vector of Mb-pET30a was obtained. The recombinant vectors were transformed into Top10 competent cells, which were coated with kanamycin resistant LB medium plate and cultured overnight at 37 °C. The colonies were selected for kanamycin resistance and plasmid DNA was characterized by colony PCR and restriction enzyme digestion. The gene sequences of the positive clones were confirmed by Sanger sequencing method in the sequencing service company.

2.3. Expression of recombinant protein

The Mb-pET30a recombinant plasmids were transformed into *E.coli* BL21(DE3) competent cells, and coated with kanamycin resistance LB medium plate. The single colonies were selected after overnight culture and inoculated into LB liquid medium containing Kanamycin resistance for culture at 37 °C. When OD600 of the liquid medium was about 0.8, isopropyl β-D-thiogalactoside (IPTG) with a final concentration of 0.4 mM was added. It was cultured for another 4h. The bacteria were centrifuged and extracted using ultrasonic crushing. The intracellular cell-free extracts would be separated and purified.

2.4. Purification of recombinant protein

The bacteria expressed Mb protein were suspended in PBS buffer (10 mM, pH 7.4) and then centrifuged after ultrasonic crushing, while the supernatant was collected. Ni^{2+} chelate affinity interaction chromatography was used for the protein purification from the supernatant, and the eluent containing the target protein was dialyzed into Tris-HCl buffer (20 mM, pH7.8).

2.5. Mb antigen performance verification

(1) Immunoreactivity verification

Mb test kits were used to measure the concentration of myoglobin in human blood by immunoassay. The immunoreactivity of Mb was verified by measuring the absorbance change value of the reaction process between the purified Mb protein (antigen) and the Mb antibody included in test kit. The Mb protein was diluted in a multiple ratio gradient, and the concentrations were measured using the test kits. The curves with dilution ratio and measured concentration as variables were fitted by the least-squares method, and the coefficient of determination (R^2) indicated the reactivity between the Mb antigen and Mb antibody. Different Mb test kits from different manufacturers, including latex immunoturbidimetric assay and chemiluminescent assay, were used to investigate the reactivity between the Mb antigens and different Mb antibodies with different epitopes.

(2) Storage stability verification

The Mb antigen in different storage buffers was diluted to four gradient concentrations, and placed at different temperature respectively. After some variable storage time, the change of antigen concentration was detected by Mb test kit, which was used to verify the storage stability of Mb antigen in different buffers and at different storage temperature. Accelerated stability testing is a stability study designed to increase the rate of chemical or physical degradation of an IVD reagent by using exaggerated environmental conditions (eg, light, temperature, humidity) [19]. That was used to estimate the long-term stability of the Mb antigen. In the study, the conditions of 37 °C for one or two weeks were chosen for the accelerated stability testing.

3. Results

3.1. Construction of recombinant expression bacteria

The Mb gene fragment and pET-30a plasmid were digested with Nde I and Xho I restriction endonuclease respectively, and then linked with T4 DNA ligase at 4 °C overnight (as shown Fig. 1). It could be found that there was a specific fragment of about 500 bp in agarose gel, which was consistent with the theoretical value of Mb gene fragment (465bp). The recombinant expression vectors of Mb-pET30a were transformed into Top10 competent cells, which were coated with kanamycin resistant LB medium plate and cultured overnight at 37 °C. The gene sequences of the positive clones were confirmed in the sequencing service company. Furthermore, the recombinant expression vectors of Mb-pET30a were transformed into BL21(DE3) competent cells, cultured with kanamycin-LB medium at 37 °C. When OD600 of the bacterial fluid was 0.8, IPTG with a final concentration of 0.4 mM was added, and then cultured for 4h. The cultured bacteria were collected, from which the recombinant Mb protein would be purified in future.

3.2. Purification of Mb protein

The bacteria expressed Mb protein were suspended in PBS buffer (10 mM, pH7.4) and then centrifuged after ultrasonic crushing, while the supernatant was collected. Ni^{2+} chelate affinity chromatography was used for the protein purification from the supernatant by gradient elution with different concentrations of imidazole solution. The purity of purified Mb protein was analyzed by 12 % SDS-PAGE, and the best purity of the partition eluted by 200 mM imidazole solution was greater than 95 % as shown in Fig. 2. The eluent containing the target protein was dialyzed in Tris-HCl buffer (20 mM, pH7.8), which would be evaluated as the quality control material in future.

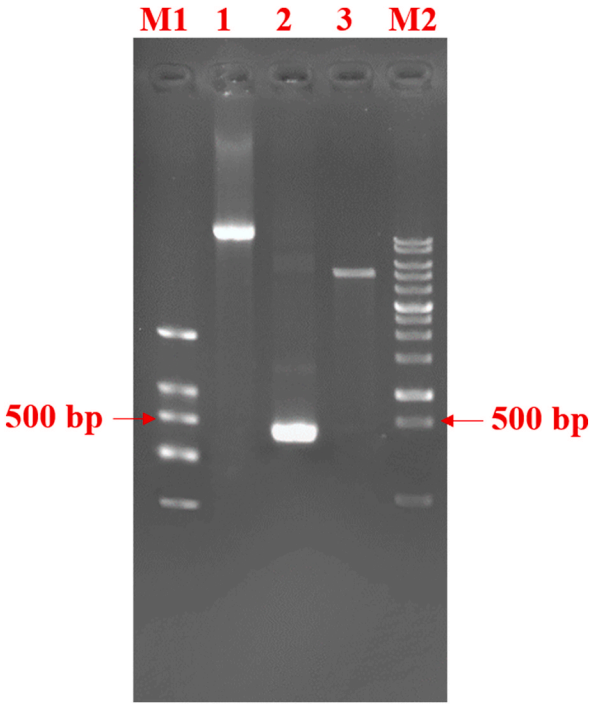

Fig. 1. Restriction analysis of recombinant plasmid Mb-pET30a. (Lane M1 and M2: DNA ladder markers with different size range; Lane 1and 2: The pET-30a plasmid and Mb gene fragment digested with Nde I and Xho I restriction endonuclease respectively; Lane 3: Mb-pET30a linked with T4 DNA ligase).

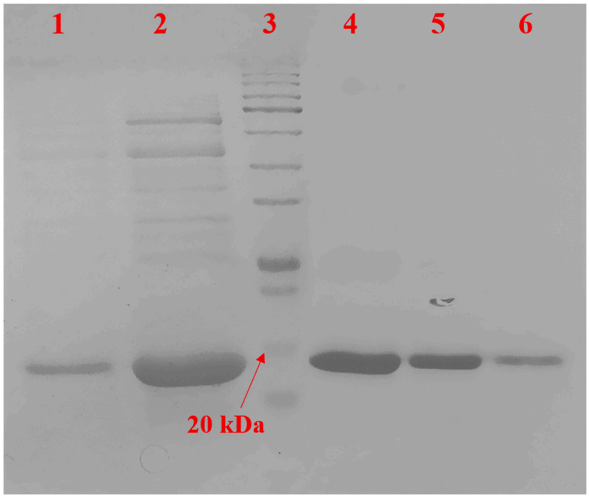

Fig. 2. SDS- PAGE profile of the purified Mb protein. (Lane 1, 2, 4, 5 and 6: the eluents by 50 mM, 150 mM, 200 mM, 300 mM, 500 mM imidazole solution; Lane 3: protein ladder markers with different size range).

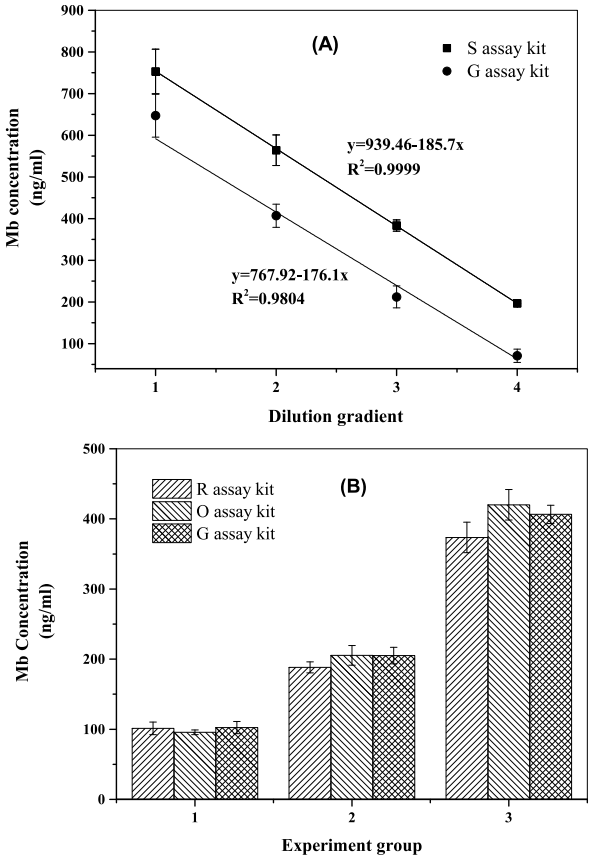

Fig. 3. (A) The linear fitting for the Mb dilution gradient and measured concentration as variables; (B) The reactivity between the Mb antigens and different Mb antibodies included in different kits. S, O and G assay kits form different manufacturers were latex immunoturbidimetric assay. R assay kits was chemiluminescent assay.

3.3. Verification of the Mb antigen immunoreactivity

Two patches of purified Mb proteins were successively diluted to prepare 4 concentration gradients, and the corresponding concentrations were measured using two Mb test kits from different manufacturers in the same lab. The linear fitting was performed by using the Mb dilution gradient and measured concentration as variables (as shown in Fig. 3(A)). The coefficients of determination (R^2) for the fitting lines were both greater than 0.95, which indicated that the purified Mb protein reacted well with the Mb antibodies in the test kits. Besides, one patch of purified Mb proteins was successively diluted to prepare 3 concentration gradients, and the corresponding concentrations were measured using three different Mb test kits in different labs respectively (as shown in Fig. 3(B)). The concentrations per gradient measured using different kits had no significant difference (p-value>0.05), which indicated that the reactivity between the Mb antigens and different Mb antibodies with different epitopes was good. Therefore, the prepared Mb protein had good antigenic immunoreactivity.

3.4. Verification of the Mb antigen storage stability

In the study, the purified Mb antigen would be used as the quality control materials for the Mb test kit in clinical lab. The storage stability of Mb antigen was the key for its clinical application, so the effect of different storage buffer, storage temperature and storage time on the storage stability would be investigated.

3.4.1. Effect of different storage buffers on the Mb stability

Two kinds of buffers, buffer A (20 mM Tris-HCl, pH 7.8, containing 1 % BSA) and buffer B (20 mM Tris-HCl, pH 7.8, containing 1 % BSA and 0.05 % NaN_3), were used as the storage buffer for Mb protein. The Mb protein was diluted to four gradient concentrations corresponding to the four experiment groups: Test 1, Test 2, Test 3 and Test 4. The stability of Mb in different storage buffer was investigated for different storage time and the results are shown as Fig. 4. When stored at 2–8 °C for 1 week, the change range of Mb concentration in buffer A and B was both within ±10 % compared with the initial concentration. When stored at 2–8 °C for 1 month, Mb concentration in buffer A decreased significantly more than 20 % compared with the initial concentration, while the change range of Mb concentration in buffer B was still within 10 %. Therefore, buffer B was the better choice as the storage buffer for Mb protein and would be investigated further.

3.4.2. Effect of different storage time on the Mb stability

The effect of different storage time on the stability of Mb in the buffer B at 4–8 °C was investigated. The Mb protein was diluted to four gradient concentrations corresponding to the different experiment groups: Test 1, Test 2, Test 3 and Test 4. The results are shown as Fig. 5. When stored for 1 week, 1 month and 2 months, the change range of Mb concentration was all within ±10 % compared with the initial concentration. It indicated that the liquid Mb in buffer B at 2–8 °C had good stability. The effect of long-term storage on the stability of Mb would be carried out through the thermal acceleration experiment.

3.4.3. Effect of the thermal acceleration on the Mb stability

The accelerated stability testing was carried out to investigate the stability of Mb with long-term storage. The Mb antigen was diluted to four gradient concentrations corresponding to the Test 1, Test 2, Test 3 and Test 4. The results are shown as Fig. 6. Although the concentration decreased more than 20 % at 37 °C for two weeks, the concentration of Mb decreased by about 10 % compared with the initial concentration at 37 °C for one week. It indicated that Mb protein would have good long-term storage stability at the storage temperature of 2~8 °C, and could be used as one promising quality control product for the Mb test kit in clinical lab.

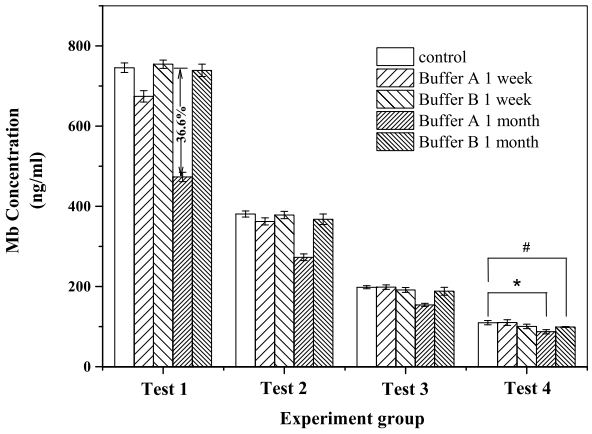

Fig. 4. Stability of Mb protein in different storage buffers at 2–8 °C. Values depicted are mean n = 3, ±SD. * p-value <0.05, #p-value >0.05, by one-way ANOVA. The concentrations were detected using the S assay kit.

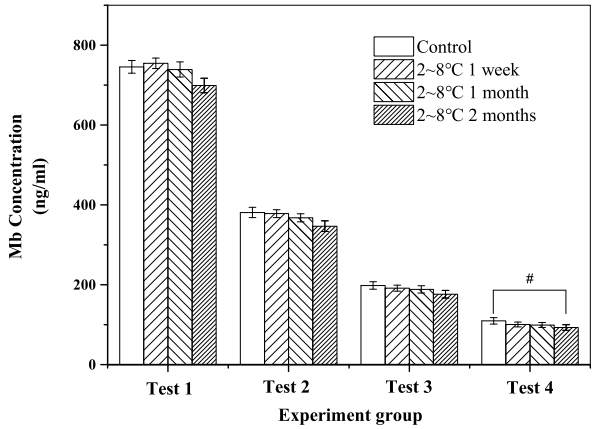

Fig. 5. Stability of Mb protein for different storage times at 2–8 °C. Values depicted are mean n = 3, ±SD. #p-value >0.05, by one-way ANOVA. The concentrations were detected using the S assay kit.

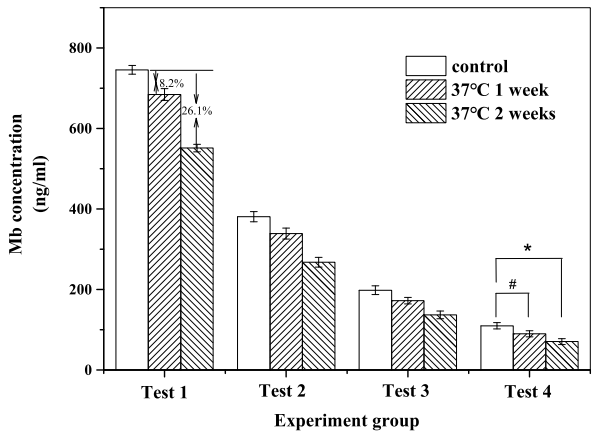

Fig. 6. Stability of Mb protein at 37 °C for different times. Values depicted are mean n = 3, ±SD. * p-value <0.05, #p-value >0.05, by one-way ANOVA. The concentrations were detected using the S assay kit.

4. Discussion

Quality control (QC) of the myocardial infarction marker is essential to obtain accurate and precise examination results. This work describes the evaluation of the liquid Mb proteins as the QC materials for the Mb detection in the clinical labs. We present the workflow for the preparation of the recombinant Mb materials with good antigenic immunoreactivity, and how to successfully optimize the storage conditions of the materials with an accepted QC error. The quality materials for the internal quality control (IQC) just requires the antigenic immunoreactivity and stability, while that for the external quality assessment (EQA) requires the traceability to the real value [20]. In the study, Mb protein was prepared to be used as the quality materials for the IQC, so we have paid more attention for the antigenic immunoreactivity and stability of the Mb protein.

Two kinds of proteins, the natural proteins and the recombinant proteins, could be prepared for the QC materials. Although the natural proteins extracted from the blood or tissue [11,12] have better antigenic immunoreactivity due to its real modification folding and spatial structure, the recombinant proteins expressed in prokaryotic or eukaryotic organisms [13–15] have been more chosen with the advantage of high yield, obtainable and low cost. Mb protein has no complex spatial folding and modification, so the prokaryotic organisms, *Escherichia coli BL21(DE32)*, was chosen for the expression of the recombinant Mb protein in the study. The purity of the Mb proteins purified using Ni^{2+} chelate affinity interaction chromatography could reach to 95 %, which was beneficial for the protein storage due to the removal of the impurities such as protease. Besides, the antigenic immunoreactivity of the Mb protein was investigated by reacting with the Mb antibody included in the Mb test kits. The Mb proteins were diluted in a multiple ratio gradient, and the corresponding concentrations were measured using the test kits. The coefficient of determination (R^2) of the fitting lines with dilution ratio and Mb concentration as variables presented the tendency of reaction between Mb antigen and antibody, indicating that the purified Mb protein had good antigenic immunoreactivity. Different Mb test kits from different manufacturers have different Mb antibodies with different epitopes. The concentrations per gradient measured using different kits had no significant difference, which indicated that the reactivity between the Mb antigens and Mb antibodies with different epitopes was good. Therefore, the prepared Mb

antigens used for QC materials were commutable in different lab.

Three storage types of QC materials could be prepared: stabilized liquid sample [21], frozen sample [22] and lyophilized sample [23]. Lyophilized samples and frozen samples are widely used in clinical labs, which have a relatively prolonged stability [18]. However, freeze-dried products and frozen samples increase energy consumption and operational burden of reconstitution before use. Stabilized liquid sample is the much better type for QC materials due to avoiding freeze-dried and frozen process. In this study, the storage stability of liquid Mb antigen for the clinical application was investigated, especially the effect of the composition of storage buffer. The binding of globin and heme consisting of Mb protein is unstable under acidic conditions [24]. The alkaline storage buffer including the protein protectant and preservative was more conducive to the stability of Mb protein. The buffer B (Tris-HCl, pH 7.8, containing 1 % BSA and 0.05 % NaN_3) was chosen for storage buffer. The change range of Mb concentration was about within ±10 % compared with the initial concentration for long-term storage at 2–8 °C. The percentage change was an accepted QC error according the CLSI EP25-A [25]. Therefore, the prepared liquid Mb protein provides an alternative for QC material instead of the freeze-dried product.

5. Conclusion

The human myoglobin was expressed in the prokaryotic expression system and purified using Ni^{2+} chelate affinity interaction chromatography with the purity of 95 %. The Mb antigen immunoreactivity was investigated using Mb test kits, the coefficient of determination (R^2) of the fitting lines with dilution ratio and Mb concentration as variables was greater than 0.95, which indicated that the purified Mb had good antigenic immunoreactivity. The effects of different storage buffer, storage times and storage temperature on the stability of liquid Mb were investigated by detecting the concentration changes. The change range of Mb concentration in buffer B (Tris-HCl, pH 7.8, containing 1 % BSA and 0.05 % NaN_3) was within 10 % for 2 months at 2–8 °C. The results of the thermal acceleration experiment shown that the concentration of Mb decreased by 8 %–15 % compared with the initial concentration at 37 °C for one week. It indicated that the liquid Mb protein had good storage stability to be used as the quality control material for the Mb test kit in clinical lab.

CRediT authorship contribution statement

Yu-Hui Wang: Writing – review & editing, Methodology, Conceptualization. **Xi-Feng Sun:** Visualization, Formal analysis. **Chun-Xin Xu:** Investigation, Data curation. **Feng-Qiang Sun:** Writing – review & editing, Validation. **Rong-Rong Wang:** Validation, Resources. **Xiao-Kun Bian:** Writing – review & editing. **Zhan-Zhao Wang:** Writing – original draft. **Qiang Wu:** Writing – review & editing, Writing – original draft, Project administration, Funding acquisition.

Declaration of competing interest

The authors have declared that there is no conflict of interest.

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Data availability

Data will be made available on request.

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