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Development of a novel extracellular matrix membrane with an asymmetric structure for guided bone regeneration

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ABSTRACT

A novel asymmetric membrane based on small intestinal submucosa (SIS) was fabricated. The microstructure, physicochemical properties, cytological characterization and effects on bone defect repair *in vivo* were investigated. Compared with a traditional SIS-based dense membrane, the asymmetric membrane had a bilayer structure with dense and loose layers, and better mechanical properties and wettability. The loose layer was favorable for the three-dimensional proliferation of human bone mesenchymal stem cells, and thus led to better osteogenic effects *in vivo*. Given its extensive material sources and simple preparation process, the asymmetric SIS membrane is expected to be a promising candidate for guided bone regeneration.

1. Introduction

Barrier membranes are widely used in guided bone regeneration (GBR) [1]. One trend in GBR membrane research is the development of bionic layered membranes with an asymmetric structure to meet the different functional needs of the target [2,3]. The dense layer towards the soft tissue acts as a barrier to prevent fibroblasts from invading bone defects. The porous layer facing bone defects possesses a highly specific surface and porosity, which can stabilize blood clots and facilitate osteoblast adhesion [4,5]. Given this, asymmetric membranes are expected to trigger increased bone formation compared to monolayer membranes [6].

Decellularized natural extracellular matrix (ECM) has attracted attention as an excellent biomaterial because it provides the most *in vivo*-like microenvironment for cell growth [7]. Small intestinal submucosa (SIS) is a common ECM material. More than 90% of the SIS is collagen; the remaining components are bioactive factors, including glycosaminoglycans, glycoproteins, and growth factors [7]. This natural bioactivity makes SIS an ideal membrane material.

Commercial SIS-based membranes are formed by pressing together multiple layers of SIS to form a compact structure and ensure a barrier effect [8]. Compared with commercial collagen membranes, these dense SIS membranes have better mechanical and degradation properties, which ensure reliable barrier function [9]. However, considering the important role of a three-dimensional porous structure in osteoblast adhesion, proliferation, and differentiation [10], an asymmetric structure is needed.

Accordingly, we developed a novel asymmetric SIS membrane composed of dense and loose layers. Physicochemical properties inspections, in vitro tests, and preliminary animal studies were conducted to evaluate the potential of the asymmetric membrane for GBR.

2. Materials and methods

2.1. Asymmetric SIS membrane fabrication and characterization

Decellularized SIS was ground to a powder by a freezer mill (6770, SPEX, USA) and dissolved in deionized water containing pepsin (0.1% w/v) and acetic acid (3% v/v) at 1% and 10% w/w concentrations. The 1% SIS solution was lyophilized using a freeze dryer (FreeZone, Labconco, USA) in a silicone mold to format the SIS sponge, and then bonded with a dense SIS membrane (Datsing Biological Technology Co., Ltd., Beijing, China) to produce an asymmetric membrane. The adhesion was achieved by coating a thin film of 10% SIS solution between the two layers. After freeze-drying, the membrane was cross-linked with 1-ethyl-3-(3-dimethylamino propyl) carbodiimide/N-hydroxysuccinimide (EDC/NHS; 50 mM/25 mM) in 95% ethanol for 24 h, washed with

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deionized water to remove the crosslinker and freeze-dried to harvest the final asymmetric SIS membrane.

The morphology of the membranes was observed by an environmental scanning electron microscope (ESEM; Quanta 200F, FEI, USA). The porosity was determined by the liquid displacement method [11]. The wettability of its loose layer and the wet tensile strength were respectively characterized using contact angle measurement device (GBX, Digidrop, France) and universal testing machine (336, Instron, USA). For degradation test, samples were incubated with type I collagenase solution (12.5U/mL) at 37 °C for 14 days, and the solution was replaced with fresh solution every other day. At specified times, the samples were washed with deionized water and weighed after being lyophilized to calculate the degradation rate. All the above experiments were carried out with the dense SIS membrane as the control group and repeated 4 times.

2.2. In vitro cell culture

Human bone mesenchymal stem cells (hBMSCs) were implanted onto the loose layer of the asymmetric SIS membranes at a density of 2×10^4 cells. After 1, 3, 5 and 7 days, a Cell Counting Kit (CCK)-8 Assay Kit (Donjindo, Japan) was used to measure cell proliferation (n = 3). The dense SIS membranes were detected as a control. Besides, the morphology and distribution of hBMSCs seeded onto the loose layer were observed by confocal laser scanning microscopy (CLSM; Leica, Germany) at the specified time (n = 3). Before observing, the cytoskeletons were stained with FITC-Phalloidin and the nuclei were stained with 4′,6-diamidino-2-phenylindole.

2.3. Preliminary animal study

Calvarial defects were established in Sprague-Dawley rats to evaluate osteogenic effects. The ethics committee of Peking University's Medical Department approved this experiment. The rats (300–350 g, male, 6 weeks old) were randomly divided into three groups (n = 4): blank control, asymmetric SIS membrane, and dense SIS membrane. After anesthetizing, two full-thickness defects (5 mm in diameter) were created using a trephine drill on both sides of the skull. No treatment was applied to the blank control group. In the other two groups, the bone defects were

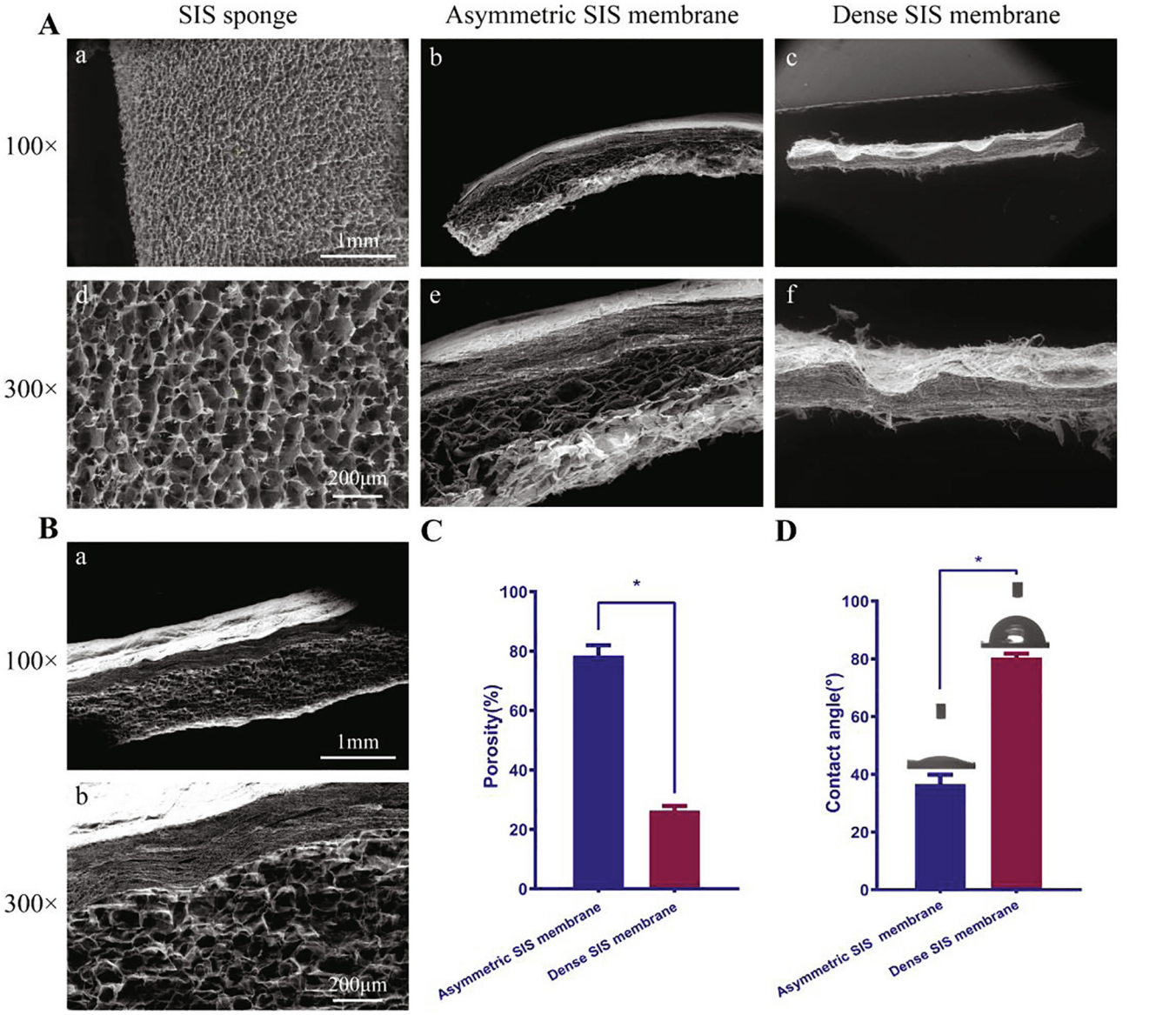

Fig. 1. A) ESEM images: The SIS sponge (a and d), asymmetric SIS membrane (b and e) and dense SIS membrane (c and f). B) ESEM images of the asymmetric SIS membrane in wet state. C) The porosities (n = 4). D) The contact angles (n = 4). * $P < 0.05$.

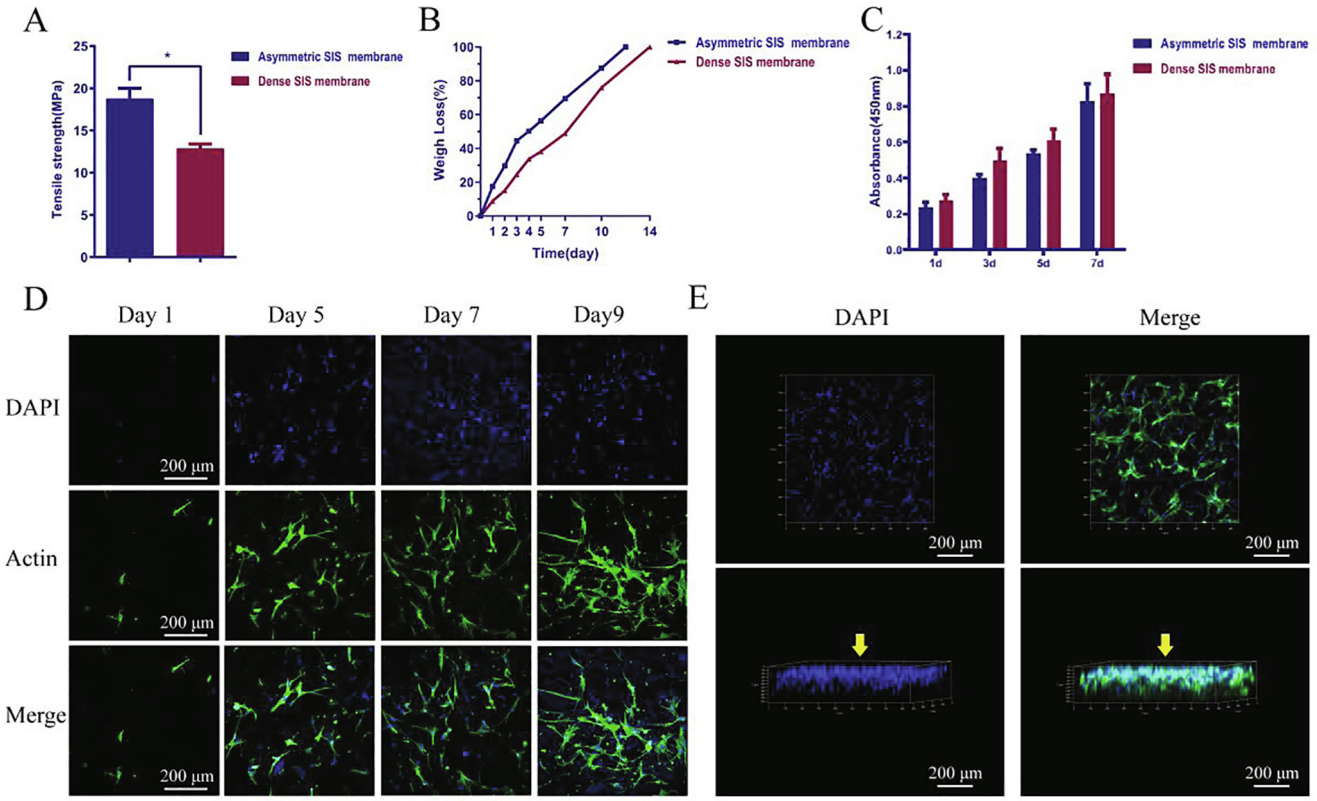

Fig. 2. Results obtained from A) the tensile strength test (n = 4), B) in vitro degradation test (n = 4), C) CCK-8 assay (n = 3), D) observation by CLSM of the asymmetric SIS membrane, E) observation of hBMSCs' distribution on loose layer on day 9 (yellow arrows indicated the upper surface of the loose layer). * $P < 0.05$.

covered with the appropriate membranes. The periosteum and skin were then sutured with 5–0 sutures. After 4 weeks, the animals were sacrificed by CO_2 asphyxiation and the defect areas were harvested and fixed in 10% formaldehyde for histological evaluation.

2.4. Statistical analysis

Statistical analyses were performed using SPSS 23.0 software. Independent-samples *t*-tests were used for comparisons. $P < 0.05$ was considered statistically significant.

3. Results and discussion

As shown in Fig. 1A, the SIS sponge possessed a uniform porous structure. In the asymmetric membrane, the bi-layer structure was distinct. The dense layer was similar to the dense SIS membrane; the non-porous structure ensured its cell barrier effect [12]. The loose layer was highly porous; it quickly reverted to a porous morphology after hydration (Fig. 1B). Thus, the preparation process (e.g., bonding and cross-linking) had no significant effect on pore structure.

In accordance with the ESEM results, the porosity (Fig. 1C) of asymmetric SIS membrane (79.102 ± 6.897%) was significantly higher than that of the dense SIS membrane (26.298 ± 2.705%) ($P < 0.05$), which is essential for nutrient diffusion and osteogenesis [11].

Compared with the dense SIS membrane, the contact angle of the loose layer in the asymmetric SIS membrane was significantly reduced ($P < 0.05$) (Fig. 1D), which indicated that it is more conducive for cell adhesion and proliferation [10].

Sufficient mechanical properties are crucial for maintaining the space for bone formation [13]. The asymmetric SIS membrane had greater wet tensile strength (18.755 ± 1.242 MPa) than the dense SIS membrane (12.843 ± 0.569 MPa) (Fig. 2A), which might be due to cross-linking [4] or/and the bonding interface formed by high concentration SIS solution. However, further studies are needed to confirm their respective effects.

The degradation rate of the asymmetric SIS membrane was slightly faster than that of the dense SIS membrane (Fig. 2B), possibly due to its better wettability and higher specific surface area. Good wettability makes it easier for enzyme solutions to penetrate, while a high specific surface area means more contact area with enzyme solutions [14].

Cell proliferation was evaluated by the CCK-8 assay. There was no significant difference between the two groups ($P > 0.05$) (Fig. 2C), though the OD value of the asymmetric SIS membrane was slightly lower than that of the dense membrane. This might be due to the introduction of a cross-linking agent during the preparation process. EDC/NHS is usually used to crosslink collagen [15], but it may have low cytotoxicity [16]. Therefore, alternative cross-linking methods such as dehydrothermal cross-linking should be found [17].

The cell morphology and distribution of hBMSCs in the loose layer of the asymmetric SIS membrane were observed by CLSM. Obvious cell proliferation was seen, and vivid cytoplasmic extensions and intercellular communication among the hBMSCs were gradually established (Fig. 2D). hBMSCs could be observed in the range of 200 μm from the surface to the interior on day 9 (Fig. 2E). The cells produced long extensions and many cells were anchored on the material. This demonstrates that the interconnected macro-porous structure of the loose layer ensured good adhesion and migration of hBMSCs, and provided sufficient space for their nutrient metabolism and growth.

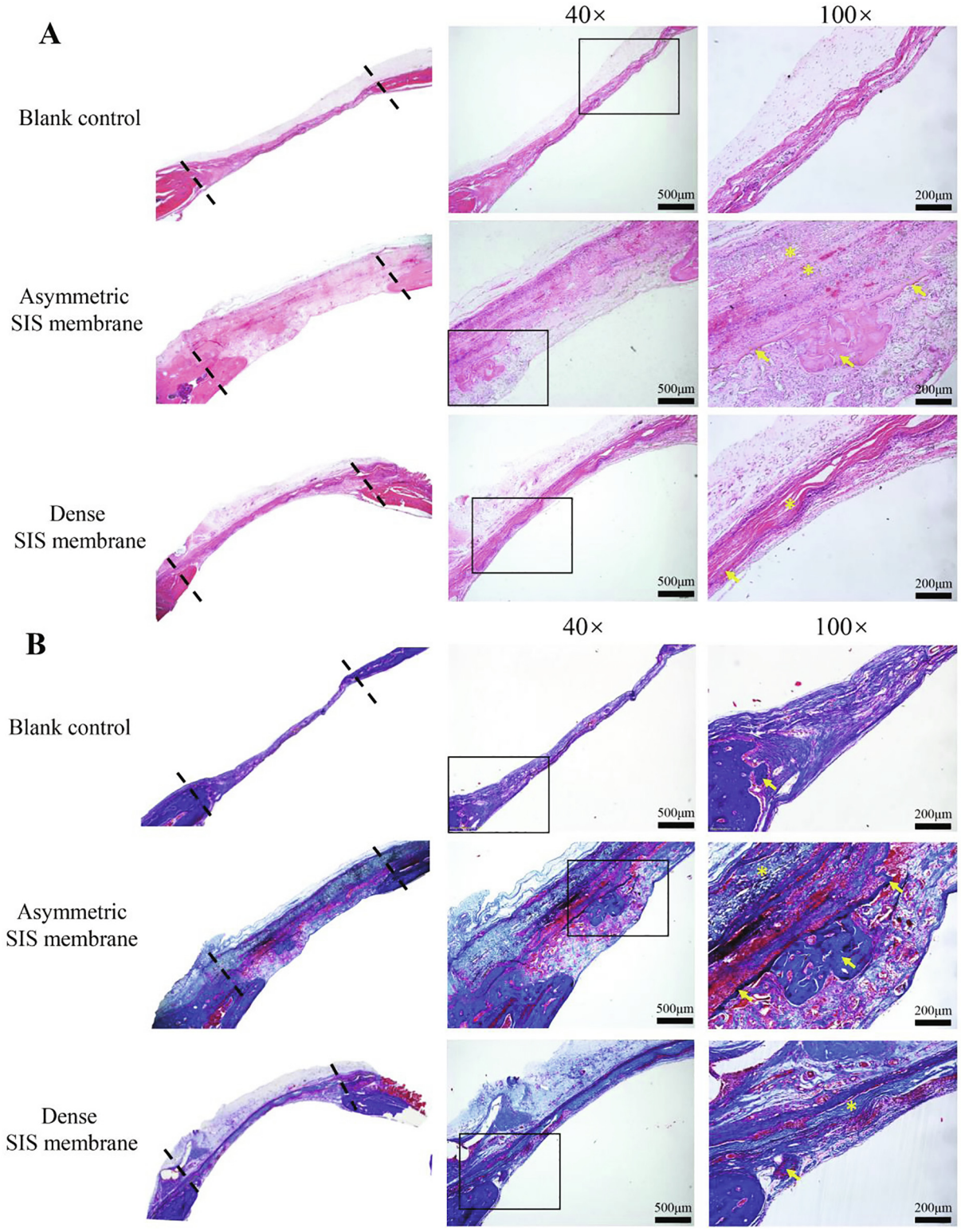

Fig. 3. A) Hematoxylin and eosin staining. B) Masson's trichrome staining. (n = 4, yellow asterisk showed the remaining membranes, and yellow arrows pointed to the nascent bone).

As shown in Fig. 3, the two membrane types showed no significant degradation after 4 weeks, indicating that they could provide barrier function for at least 4 weeks to ensure temporary matrix formation [18]. In the blank control and dense SIS membrane groups, thin fibrous connective tissue occupied the defect area, with only a little new bone seen at the edge of the defect. However, in the asymmetric SIS membrane group, the membrane was well integrated with the adjacent tissue; new bone appeared not only in the vicinity of the old bone edge, but also under the membrane. Interestingly, new bone islands and strips of bone-like tissue were

noted along the loose layer, and cubic osteoblasts could be seen in a dense arrangement. Thus, compared with the dense membrane, the asymmetric membrane promoted osteoblast adhesion and function and subperiosteal osteogenesis. This is particularly important for the regeneration of skull bone with an insufficient blood supply.

4. Conclusions

We developed a novel asymmetric SIS membrane, with a dense layer acting as a cell barrier and a loose layer providing space for bone regeneration. The *in vivo* tests showed that this asymmetric membrane possessed satisfying biological properties. In the animal study, the asymmetric SIS membrane seemed to stimulate more bone formation than the dense SIS membrane. Despite its preliminary character, this study can clearly indicate its potential for practical application. However, animal studies with larger sample size and longer observation period are still necessary to explore its effect and mechanism in GBR therapy.

CRediT authorship contribution statement

Bowen Li: Methodology, Validation, Formal analysis, Resources, Writing - original draft. **Yuhua Liu:** Conceptualization, Supervision, Writing - review & editing. **Yongsheng Zhou:** Supervision, Writing - review & editing. **Pengyue You:** Investigation, Data curation. **Mei Wang:** Investigation, Formal analysis. **Lin Tang:** Investigation. **Yi Deng:** Investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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