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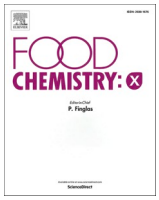

Plant-derived leaf vein scaffolds for the sustainable production of dog cell-cultured meat

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ABSTRACT

Animal cell culture technology in the production of slaughter-free meat offers ethical advantages with regards to animal welfare, rendering it a more socially acceptable approach for dog meat production. In this study, edible plant-derived scaffold was used as a platform for cell expansion to construct cell-cultured dog meat slices. Primary dog skeletal muscle satellite cells (MSCs) and adipose stem cells (ASCs) were isolated and cultured as seed cells, and 3D spheroid culture in vitro promoted MSCs and ASCs myogenic and adipogenic differentiation, respectively. Natural leaf veins (NLV) were produced as edible mesh scaffolds to create 3D engineered dog muscle and fat tissues. After MSCs and ASCs adhered, proliferated and differentiated on the NLV scaffolds, and muscle and fat slices were produced with cultured dog muscle fibers and adipocytes, respectively. These findings demonstrate the potential of plant-derived NLV scaffolds in the production of cultured dog meat.

1. Introduction

The increasing global population has led to a growing demand for meat, resulting in expanded livestock production and subsequent environmental pollution, water scarcity, increased greenhouse gas emissions, land degradation, and animal diseases(van Dijk, Morley, Rau, & Saghai, 2021). Therefore, there is an urgent need for sustainable and efficient methods of meat production. Cell cultured meat offers a promising solution by utilizing cell culture techniques to create products that resemble traditional meat in terms of appearance, texture, taste, and nutritional composition without relying on animal breeding(Jahir, Ramakrishna, Abdullah, & Vigneswari, 2023).

The tradition of consuming dog meat has been preserved in East Asia (including China, Japan, and South Korea), Southeast Asia, Pacific Island countries, as well as certain countries and regions in the Americas for an extended period of time to cater to diverse consumer demands. The population of pet dogs is expanding with the advancement of society, while the conflicts arising from dog consumption are becoming increasingly conspicuous(Smith et al., 2019). In recent years, dog meat consumption has become a subject of controversy due to concerns regarding animal ethics and other related issues. Additionally, the consumption of dog meat is associated with potential food safety risks such as the transmission of rabies, leptospirosis, filariasis, trichinosis, and other pathogens (Cui & Wang, 2001; Ekanem et al., 2013; Ndiana et al., 2023; Wang, Cui, & Shen, 2007; Wiwanitkit, 2014). The cultivation of dog meat in cell culture may provide an optimal solution to address these issues and inconsistencies.

Cultured meat consists of fat cells and muscle cells, with its composition and structure resembling genuine muscle tissue(Hong, Shin, Choi, Do, & Han, 2021). The rapid advancement of large-scale cell culture technology has enabled researchers to successfully fabricate cultured meat tissues derived from various animals such as chicken (Pasitka et al., 2023), cattle(Skrivergaard, Rasmussen, Therkildsen, & Young, 2021), pig(Li et al., 2022), fish(Xu et al., 2023), and other species. However, large-scale production of cell cultured meat still faces many challenges, among which the acquisition and large-scale culture of high-quality seed cells and the development of 3D edible scaffolds are two major technical bottlenecks that need to be solved in the process of cell cultured meat industrialization(Chen et al., 2022). Embryonic stem cells, induced pluripotent stem cells, mesenchymal stem cells, and satellite cells are commonly used as preferred seed cells for generating muscle fibers or adipose tissue(Lee et al., 2023). 3D scaffolds are typically employed to mimic the extracellular matrix, ensuring effective cell adhesion, proliferation, and differentiation(Bomkamp et al., 2022).

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In recent times, conventional scaffolds for cultivated meat production, such as collagen scaffolds, are derived from animals. Collagen, a major component of Extracellular matrix (ECM), was widely used in the early development of cultured meat at the laboratory stage. However, collagen needs to be extracted from animals, and its use is limited when it comes to the large-scale commercialization of cell-cultured meat. Therefore, researchers have shifted their focus to porous scaffolds made from plant-based polysaccharides and protein materials in cell-cultivated meat research due to their advantages in terms of cost and scalability(Wang et al., 2022). For instance, soy protein-based three-dimensional scaffolds have been employed for the fabrication of bovine muscle tissue(Ben-Arye et al., 2020). Building upon these advancements, we tried to use natural vein (NLV) as a novel 3D culture scaffold. This represents a significant departure from traditional collagen scaffolds and existing plant-based alternatives.

Currently, there has been relatively little research on seed cells and scaffold materials for the cultivation of canine muscle tissue and adipose tissue from two-dimensional to three-dimensional. In this study, canine muscle satellite cells (MSCs) and adipose stem cells (ASCs) were isolated, and NLV were used as 3D culture scaffolds for MSCs and ASCs to construct canine cell cultured tissue meat slices (Fig. 1).

2. Methods

2.1. Cell isolation and culture

Skeletal muscle tissue and abdominal subcutaneous adipose tissue of 6 dogs (4 females and 2 males) were collected aseptically in Animal Hospital Affiliated to Foshan University. All experiments were performed in accordance with the Guangdong Province Administrative Regulation of Experimental Animals. The study has been approved by the Animal Ethics Committee of Foshan University and all methods are reported in accordance with ARRIVE guidelines.

After removing the fascia tissue through PBS cleaning, the muscle tissue was sectioned into 1 mm^3 blocks and subjected to digestion with 1 mg/ml type I collagenase (Solarbio, China) for 90 min. Subsequently, it was further digested with 0.25% trypsin (Gibco, USA) for 20 min. After filtration using a 70 μm cell strainer (JET BIOFIL, China) cells were centrifuged at 300*g* for 5 min, then resuspended in α-MEM (BDBIO, China) containing 10% fetal bovine serum (FBS, ExCellBio, China), 20 ng/ml bFGF (Longtime, China), and 1× Penicillin-Streptomycin (PS, Macklin, China), seeded in 100 mm cell culture dish and cultured at 37 °C with 5% CO2. After 24 h, the medium was transferred to a new dish for continued culture to remove miscellaneous cells. Relatively pure muscle satellite cells (MSCs) could be obtained after serial passage.

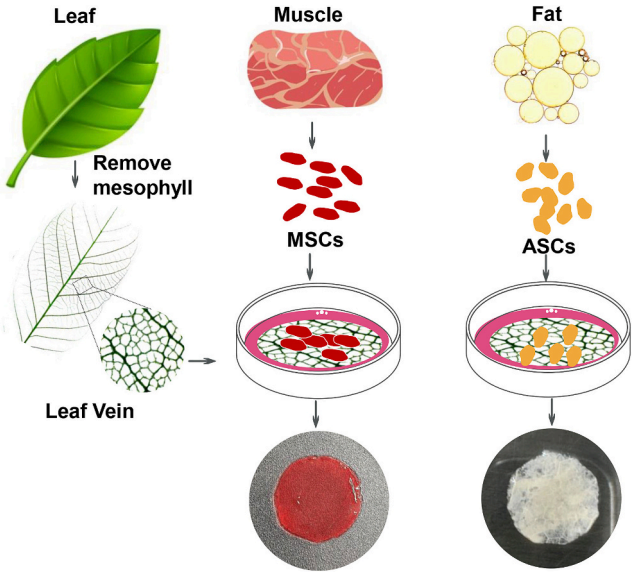

Fig. 1. Schematic illustration of dog cultured meat platform. The mesophyll of the fresh leaves was removed to obtain the veins to prepare the scaffolds. The primary isolated dog MSCs and ASCs were seeded on scaffolds, and after adhesion, proliferation and differentiation, tissue-like muscle slices and fat slices were finally produced. The graphic was made with assets from Freepik.com.

Adipose-derived stromal cells (ASCs) were isolated and culture as previously described(Luo, Li, Chen, Wang, & Chen, 2021; Zhang et al., 2018). Briefly, adipose tissues were minced, digested with collagenase type I at 37 °C for 1–2 h, filtered through a 70 μm cell strainer, and then cultured with adipose mesenchymal stem cell complete culture medium (Oricell, Cyagen Biosciences, China) at 37 °C with 5% CO2. When the cells reached 80% confluence, subculture process was performed.

2.2. Myogenic differentiation

MSCs were expanded to 80% cell confluence in complete medium and then differentiated by replacing with DMEM medium containing 2% horse serum (HS, Biosharp, China), and the medium was renewed every 3 days.

2.3. Adipogenic differentiation

Upon ASCs reached 80% confluency, the medium was changed to adipose mesenchymal stem cell adipogenic induction differentiation medium (OriCell, Cyagen Biosciences, China) to initiate differentiation.

2.4. Cell identification

MSCs and myotubes were characterized by immunofluorescence staining. After fixed, permeabilized, and blocked, cells were incubated in MyoD1, Pax7, or Desmin mAbs (Wanleibio, China) in the dark overnight, followed by incubation with FITC or PE-labeled secondary antibodies for 2 h, after which DAPI was added to stain the nucleus for 5 min and photographed under a fluorescence microscope (Olympus, Japan).

ASCs were characterized using flow cytometry. The cells were dissociated and resuspended in flow cytometry buffer at a concentration of 1 × 10^6 cells/ml, then incubated with fluorescently labeled monoclonal antibodies (mAbs, Biolegend, USA) specific for CD29, CD45, CD73, and CD90 for 30 min on ice in the dark. After washing to remove unbound antibodies, the cells were resuspended in 500 μL of buffer for analysis. The CytoFLEX flow cytometer (Beckman Coulter, USA) was utilized for data acquisition, ensuring the collection of at least 10,000 events per sample.

2.5. Oil red O staining

Adipocytes were fixed using 4% paraformaldehyde (PFA, Aladdin, China) at a volume of 1 ml per well in a 24-well plate for 30 min at room temperature. Following fixation, cells were washed with 1× Phosphate-Buffered Saline (PBS, pH 7.4) for a duration of 5 min, with a total volume of 2 ml per well to ensure complete removal of fixative. Subsequently, the cells were stained with Oil Red O to visualize intracellular lipid droplets.

2.6. Sphere-Forming Culture

MSCs and ASCs were cultured in 3D-conditions to generate cell spheroids using ultra-low adherent cell culture plates (Costar, Corning, USA) and used for subsequent experiments after 12 h of 3D culture.

2.7. Preparation of leaf veins

Fresh green leaves were boiled in a 5% NaOH (Aladdin, China) solution for 15–30 min, resulting in their transformation into a yellowish

hue and acquiring a softened texture. Subsequently, the boiled leaves are removed and rinsed in clean water. Mesophyll tissues were gently brushed with a soft toothbrush, while carefully exposing the intricate network of veins. Finally, the vein tissue was bleached overnight in 2% hydrogen peroxide.

2.8. DNA quantitative analysis

DNA was extracted from plant tissues using the cetyltrimethylammonium bromide (CTAB, Beyotime, China) Kit, following the manufacturer's instructions. The concentration and purity of the extracted DNA were assessed using a spectrophotometer (NanoDrop, Thermo Fisher, USA), which utilizes wavelength frequencies of 260 nm for DNA quantification and 280 nm to determine protein contamination, ensuring the integrity and purity of the DNA samples.

2.9. Scanning electron microscope (SEM)

The morphologies of leaf vein scaffolds were characterized by scanning electron microscope (SEM, Hitachi, Japan) after coating with platinum.

2.10. Live/dead staining

Cell viability was using Calcein/PI Live/Dead Viability/Cytotoxicity Assay Kit according to the manufacturer's instructions (Beyotime, China). The cells were washed twice with PBS, then stained with Calcein AM/PI detection solution for 30 min in the dark, and then washed with PBS for 5 min. The staining effect was observed under a fluorescence microscope (Olympus, Japan). Calcein AM showed green fluorescence, and PI showed red fluorescence.

2.11. Immunofluorescence staining

After cell spheroids and cells on the scaffold were fixed and permeabilized, the cytoskeleton was stained with FITC or TRITC labeled phalloidin (Yeasen, China) for 45 min. Adipocytes were stained with 5 μM BODIPY 493/503 (MCE, USA) for 30 min. The samples were visualized using a fluorescence microscopy.

2.12. Quantitative real-time PCR

RNA was isolated using a total RNA extraction kit (RNAsimple Total RNA Kit, Tiangen, China) according to the manufacturer's instructions. RNA was reverse transcribed by HiScript® III RT SuperMix for qPCR (+gDNA wiper) (Vazyme, China). Quantitative PCR was performed using ChamQ Universal SYBR qPCR Master Mix (Vazyme, China) to detect with primer pairs detailed in Supplementary Table S1.

2.13. Statistical analysis

Data are presented as mean ± standard deviation (SD). The unpaired *t*-test was used to explain the differences between the groups. $P < 0.05$ was considered statistically significant. All analyses were performed using GraphPad Prism 8.0 (GraphPad Software, USA).

3. Results

3.1. Isolation, proliferation, and differentiation for dog MSCs and ASCs

The muscle cells were obtained from dog skeletal muscle tissue through enzymatic hydrolysis using a combination of type 1 collagenase and trypsin (Fig. 2A). After filtration, primary cultures were established. The adherent cells consisted predominantly of fibroblasts and satellite

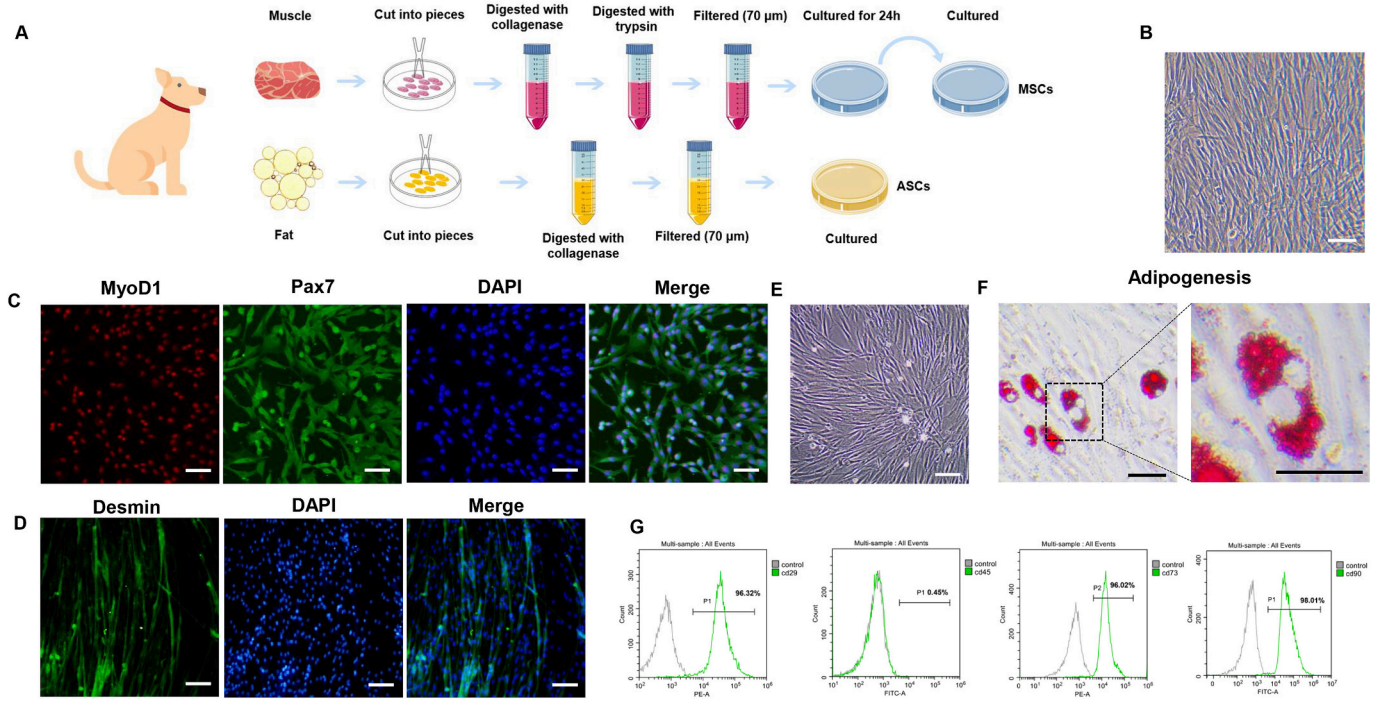

Fig. 2. Proliferation and differentiation of dog MSCs and ASCs.
(A) Schematic diagram of isolation process of dog primary MSCs and ASCs. The graphic was made with assets from Freepik.com. (B) The morphology of dog MSCs at passage 3. Scale bars: 100 μm. (C) Representative images of immunofluorescence staining of dog MSCs at passage 3. Green: Pax7; Red: MyoD1; Blue: DAPI for nuclear staining. Scale bars: 100 μm. (D) Representative images of fluorescence staining of dog MSCs during myogenic differentiation at day 5. Green: Desmin; Blue: DAPI; Scale bars: 100 μm. (E) The morphology of dog ASCs at passage 3. Scale bars: 100 μm. (F) Representative image of oil red O staining during adipogenic differentiation of ASCs at day 7. Red: Lipid droplets. Scale:100 μm. (G) Flow cytometry of dog ASCs with positive expression of CD29, CD73, and CD90 makers, and negative expression of CD45. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cells. Pre-plating is a method that effectively separates fibroblasts from muscle satellite cells by exploiting their differential adhesion properties (Yun et al., 2023). The pure MSCs were obtained through multiple rounds of pre-plating (Fig. 2B, Fig.S1). By optimizing the combination of various media, serum ratios, and growth factors, the final medium for MSCs proliferation was determined to be MEM-α supplemented with 10% fetal bovine serum (FBS) and 20 ng/ml bFGF. MSCs demonstrated a robust proliferation ability with a doubling time of <24 h within 15 passages. The cells at passage 3 were identified through immunofluorescence staining, revealing a dual positive expression of Pax7 and MyoD1(Fig. 2C). These data indicate that the isolated cells have the properties of satellite cells and can be used as seed cells for dog meat production. To evaluate the differentiation potential of MSCs, satellite cells were induced for myogenic differentiation after multiple passages using a medium supplemented with 2% horse serum (HS). Within 2 to 3 days, long myotubes started to emerge, while on day 5, striated myotubes resembling skeletal muscles were observed. Immunofluorescence staining revealed the presence of for desmin-positive multinucleated myotubes (Fig. 2D).

The isolation and culture of ASCs have been effectively established through our previous successful method(Luo et al., 2021). Adipose tissue was digested with type I collagenase to obtain nucleated cells and seeded in cell culture dishes. After 48 h of culture, the vast majority of the cells adhered to the culture plate with a fibroblast-like morphology, and after 4–5 days the cells reached 80% confluence and were passaged for the first time (Fig. 2E, Fig.S1). OriCell® adipose mesenchymal stem cell adipogenic induction differentiation kit was used to induce the adipogenic differentiation to confirm the characteristics of ASCs. After 7 days of induction, oil red O staining showed that lipid droplets of different sizes appeared on the cell surface (Fig. 2F). The flow cytometry analysis revealed the expression of CD29, CD73, and CD90 on ASCs, while the expression of CD45 was absent (Fig. 2G). These results suggested that the isolated cells exhibit characteristics consistent with those of ASCs.

3.2. Three-dimensional spheroids of dog MSCs and ASCs

After being seeded on ultra-low attachment surface, both MSCs and ASCs were able to spontaneously form three-dimensional spheroids floating in the culture medium (Fig. 3A, B). In 3D culture, the cell viability of MSCs was 96.09% ± 0.48% (Fig. 3C), while that of ASCs was 95.95% ± 0.73% (Fig. 3D). In comparison, in 2D culture, the cell viability was higher with MSCs at 98.95% ± 0.29% and ASCs at 98.49% ± 0.4%. MSCs and ASCs exhibited a consistently high viability exceeding 95% under both 2D and 3D culture conditions. In 2D culture, high cell density promoted contact inhibition of proliferation, while in 3D culture, MSCs and ASCs proliferated in greater numbers (Fig. 3E, F). To investigate the potential of spherical culture in enhancing the differentiation capacity of MSCs and ASCs, we subjected the cells to various induction medium. Notably, when exposed to myogenic induction differentiation medium, spherical MSCs exhibited a significantly higher proportion of multiple multinucleated myotubes compared to those derived from continuous monolayer culture (Fig. 3G, I). Similarly, following 7 days of culture in an adipogenic induction differentiation medium, three-dimensional (3D) cultured ASCs exhibited a higher expression of lipid droplets compared to the monolayer culture (Fig. 3H, I).

3.3. Preparation and characterization of natural leaf vein (NLV) scaffolds

Leaf vein consists of highly oriented and closely arranged vascular bundles of varying thickness, forming an optimized multilevel network

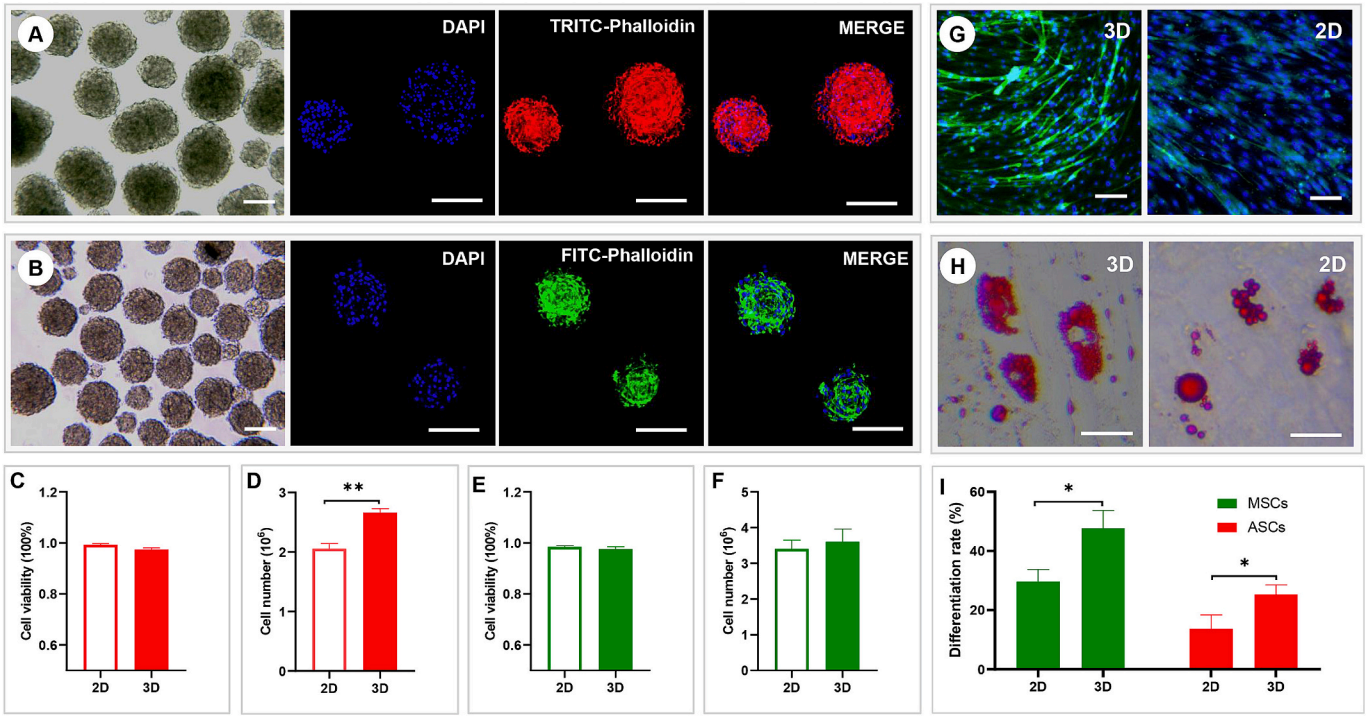

Fig. 3. 3D-spheroid culture of dog MSCs and ASCs. (A and B) Representative images of bright-field and fluorescence staining of MSCs and ASCs spheroids. Red: TRITC-Phalloidin; Green: FITC-Phalloidin; Blue: DAPI for nuclear staining; Scale bars: 100 μm. Cell viability (C) and numbers (D) of MSCs in 2D and 3D culture conditions. Cell viability (E) and numbers (F) of ASCs in 2D and 3D culture conditions. (G) Representative images of fluorescence staining during myogenic differentiation of MSCs in 2D and 3D culture conditions. Scale bars: 100 μm. (H) Representative image of oil red O staining during adipogenic differentiation of ASCs in 2D and 3D culture conditions. Scale bars: 100 μm. (I)Efficiency of myogenic and adipogenic differentiation of MSCs and ASCs in 2D and 3D culture conditions, respectively. * $P < 0.05$, ** $P < 0.01$, $n = 3$, "n" was represented different biological replicates. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

structure(Pierantoni, Brumfeld, Addadi, & Weiner, 2019). As a special structure of transmission and support function in plant leaves, leaf vein has water and nutrient transmission channels, and plays a supporting role, so that the leaves can unfold and ensure the normal physiological metabolism process smoothly. It can be called the “blood vessels” of plants(Sack & Scoffoni, 2013).

This networked vein is a mass-produced, edible, porous and cellulose-rich natural 3D scaffold. It can be easily obtained by bleaching the mesophyll after NaOH corrosion (Fig. 4A). The quantitative analysis of DNA revealed a significantly lower presence of DNA in the vein compared to normal leaves of the same size (Fig. 4B). The vein was cut into a circle of appropriate size and placed in the ultra-low suction cell culture plate (Fig. 4C). It was observed that the obtained vein network structure was clear, the non-vein tissue was less and the vein was complete under the optical microscope (Fig. 4D, E). We examined six different types of leaf veins from poplar, oak, chestnut, banyan and camphor trees (Fig. S2). Through comparative analysis, we discovered that the leaf vein of poplar exhibited the smallest pore diameter and highest density, suggesting a potential advantage for cell adhesion and growth. The structure of the poplar vein scaffold was observed using scanning electron microscopy (SEM). The results revealed that the scaffold exhibited a porous nature and possessed ample surface area (Fig. 4F-I), facilitating cell adhesion for proliferation and differentiation. Moreover, the scaffold material is cost-effective and does not necessitate further modifications, rendering it highly suitable for large-scale production of cultured meat.

3.4. MSCs and ASCs adhesion and proliferation on NLV scaffolds

MSCs and ASCs were inoculated on NLV scaffolds to evaluate the compatibility of scaffolds with dog cells. It is crucial that the adhesion proliferation ability of MSCs and ASCs on NLV scaffold for the effective construction of cell culture meat model. Due to the pore size of the NLV scaffold being much larger than the diameter of cells, after single-cell seeding, gravity causes cells to easily fall through the scaffold pores and result in poor adhesion between cells and scaffold. We enhanced this process by inducing the spontaneous formation of 3D cell spheroids, which closely match the diameter of the scaffold aperture. Subsequently, upon seeded, these spheroids effectively adhered to the NLV scaffold surface and promoted cellular adhesion (Fig. 5A, Fig. S3A). We found that the adhesion efficiency of cell spheres (82% and 84% for MSCs and ASCs, respectively) was much higher than that of single cells (36% and 40% for MSCs and ASCs, respectively) (Fig. S3B). After fluorescent staining with fluorescent labeled phalloidin (cytoskeleton, red for MSCs, green for ASCs), the morphology of the cells growing on the NLV scaffold was observed by confocal microscopy. The cells initially attach to the surface of the scaffold fibers and grow along the fibers, forming a circle in the pore of the scaffold. Subsequently, MSCs and ASCs continue to proliferate until they spread throughout the pore region, forming a dense network of cells and filling all the empty surfaces (Fig. 5B). According to the results of live/dead assay, MSCs and ASCs showed a tendency of rapid proliferation during the whole culture process after attaching to NLV scaffolds (Fig. 5C). The cells showed high proliferative activity within 5 days, and the cell viability remained above 90% (Fig. 5D).

3.5. MSCs and ASCs differentiation on NLV scaffolds

To validate the myogenic and adipogenic potential of cells on NLV scaffold, following 5 days of proliferation, MSCs and ASCs were subjected to differentiation in specific myogenic and adipogenic induction media, respectively, for a minimum duration of 7 days (Fig. 6A). The myogenic and adipogenic differentiation of MSCs and ASCs were detected by FITC-Desmin and BODIPY 493/503 immunofluorescence

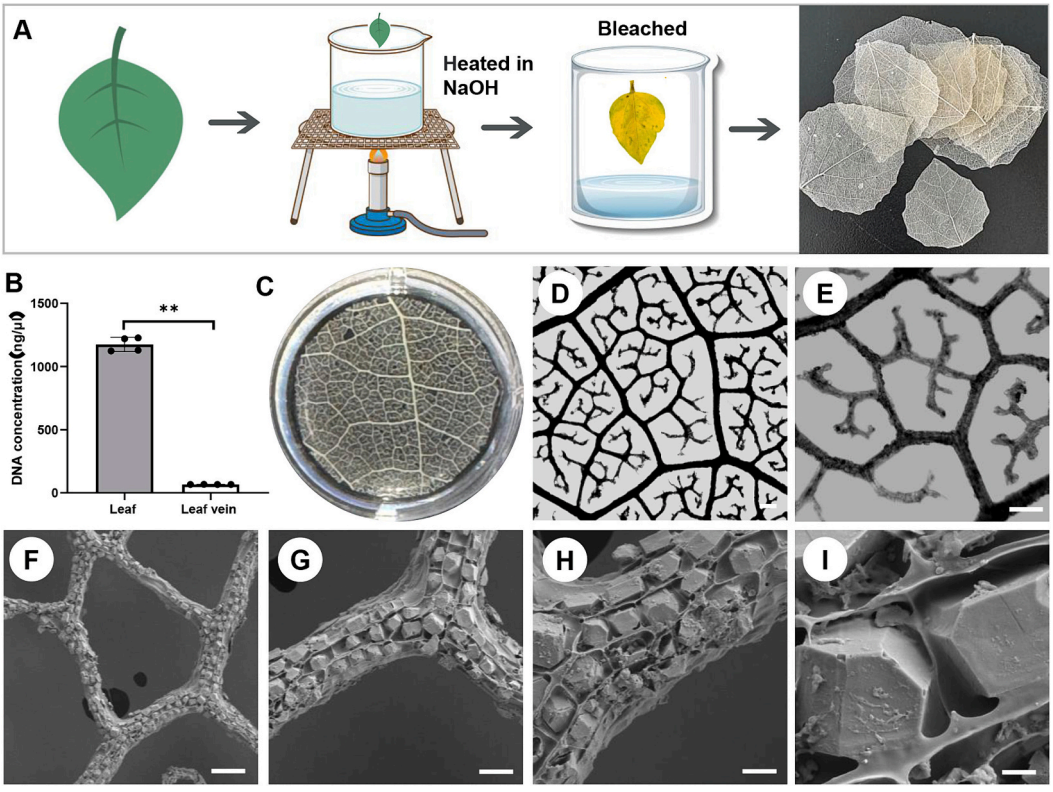

Fig. 4. Characterization of natural leaf vein (NLV) scaffolds. (A) Schematic diagram of preparation process of natural leaf vein scaffold. The graphic was made with assets from Freepik.com. (B) The DNA content was measured by OneDrop, ** $P < 0.01$, $n = 4$, “n” was represented different biological replicates. (C) The NLV scaffold was cut into small round sheets and placed in the cell culture plate. (D, E) The morphology of the scaffold under an optical microscope. Scale bars: 100 μm. (F—I) Scanning Electron Microscopy (SEM) images of NLV scaffolds. Scale bars: (F)50 μm; (G)20 μm; (H)10 μm; (I)2 μm.

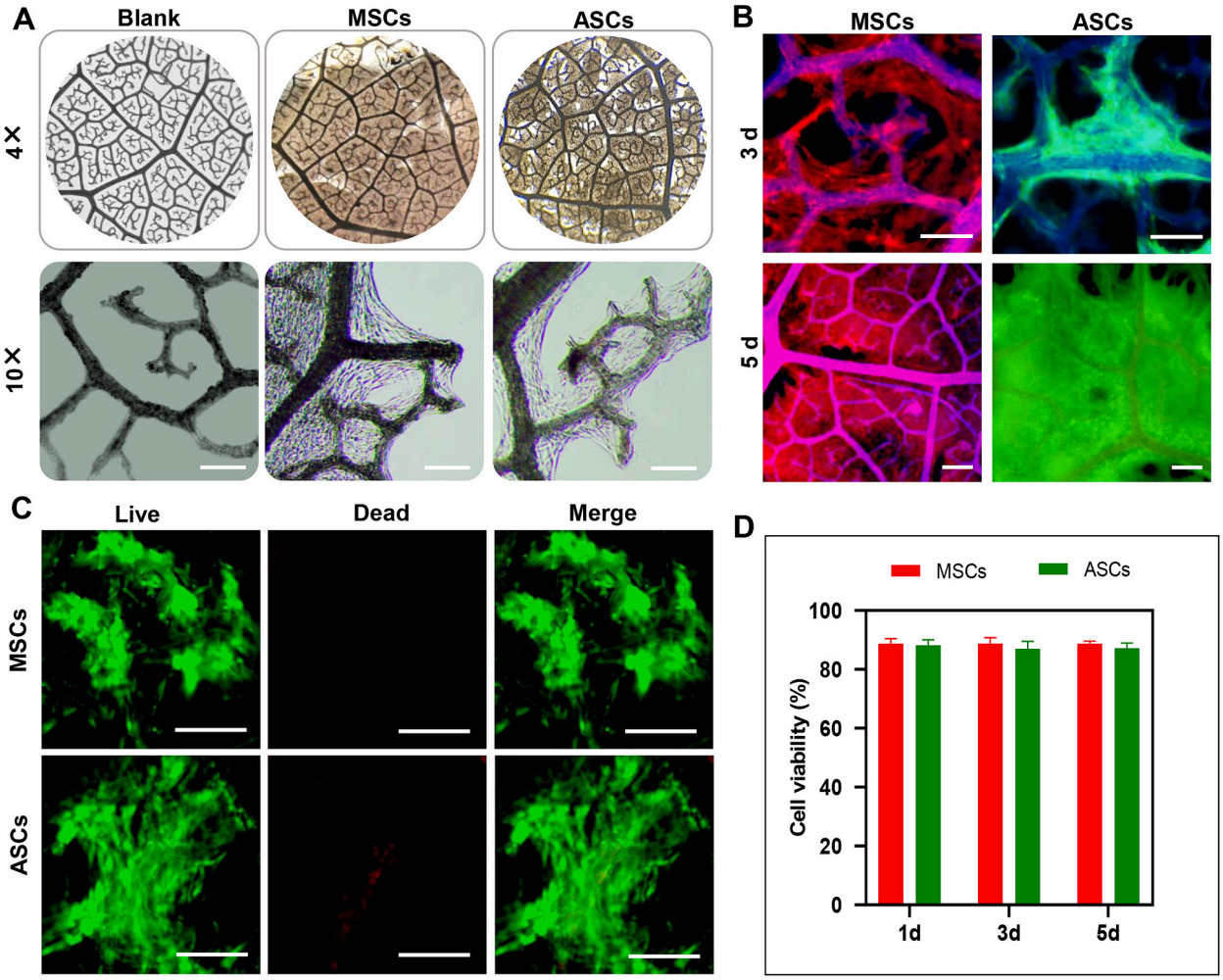

Fig. 5. Adhesion and proliferation of MSCs and ASCs on NLV scaffolds. (A) The morphology of MSCs and ASCs adhered on NLV scaffolds. Scale bars: 100 μm. (B) Representative images of immunofluorescence staining of MSCs and ASCs on scaffolds after 3 and 5 days of culture. Red: TRITC-Phalloidin; Green: FITC-Phalloidin; Blue: veins. Scale bars: 100 μm. (C) Live/dead staining of MSCs and ASCs on NLV scaffolds after a 5-day period of culture. Green: live cells with Calcein; Red: dead cells with PI. Scale bars: 100 μm. (D) Cell viability was obtained from the Live/Dead assay of MSCs and ASCs on NLV scaffolds for 1–5 days. No statistically significant differences. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

staining, respectively. After 7 days of serum starvation culture (the conventional method for inducing myoblastic differentiation), Desmin protein-positive muscle fibers were observed (Fig. 6B). As expected, RT-qPCR analysis confirmed significant upregulation of genes associated with myogenic differentiation in the induced cells (Fig. 6D). Lipogenic differentiation required a longer duration, and following a 14-day treatment with lipogenic differentiation medium (ADM), significant accumulation of lipid droplets in ASCs was observed through staining with Bodipy (Fig. 6C) and oil red O (Fig. S4). In addition, PCR results showed significant up-regulation of adipose-related genes after induced differentiation (Fig. 6E).

3.6. Assessment of Cultured Meat Model

Finally, the concept of developing dog cultured meat using NLV scaffolds was validated by constructing meat slices through cell seeding on the scaffold and subsequent cultivation and differentiation. MSCs were expanded on the scaffold for 5 days, differentiated for 7 days, and the reddish meat slices were constructed using beet extract as a food colorant (Fig. 6F). ASCs were amplified on the scaffold for 5 days, differentiated for 14 days, and spontaneously formed into white, grease-like fat slices (Fig. 6G). The colour and texture of these meat pieces of cultured meat closely resemble that of real meat. These results further validate the great potential of the proposed natural plant-derived vein scaffolds for cultured meat production.

4. Discussion

As the pet culture has thrived, the practice of dog slaughter for meat consumption has faced widespread criticism(Meng et al., 2016). Cell cultured meat technology is an emerging technology for future food production. It is a feasible method to obtain dog meat protein by cell culture technology(Zhang et al., 2019). The successful production of cell cultured meat from various animal species, including cattle(Zagury, Ianovici, Landau, Lavon, & Levenberg, 2022), pig(Zhu et al., 2022), chicken(Pasitka et al., 2023), fish(Xu et al., 2023), and rabbit(Chen et al., 2023), has been reported. Additionally, we have established the dog cultured meat model for the first time.

In the process of cultured meat production, acquiring seed cells constitutes the initial step(Reiss, Robertson, & Suzuki, 2021). The quality and quantity of seed cells exert a substantial influence on subsequent stages of proliferation and differentiation. Seed cells need to have a good proliferation ability in vitro, and can produce muscle fibers, adipocytes, matrix proteins and other mature somatic cells that constitute muscle tissue(Gershlak et al., 2017). Primary seed cells include embryonic stem cells, mesenchymal stem cells, satellite cells, fibroblasts

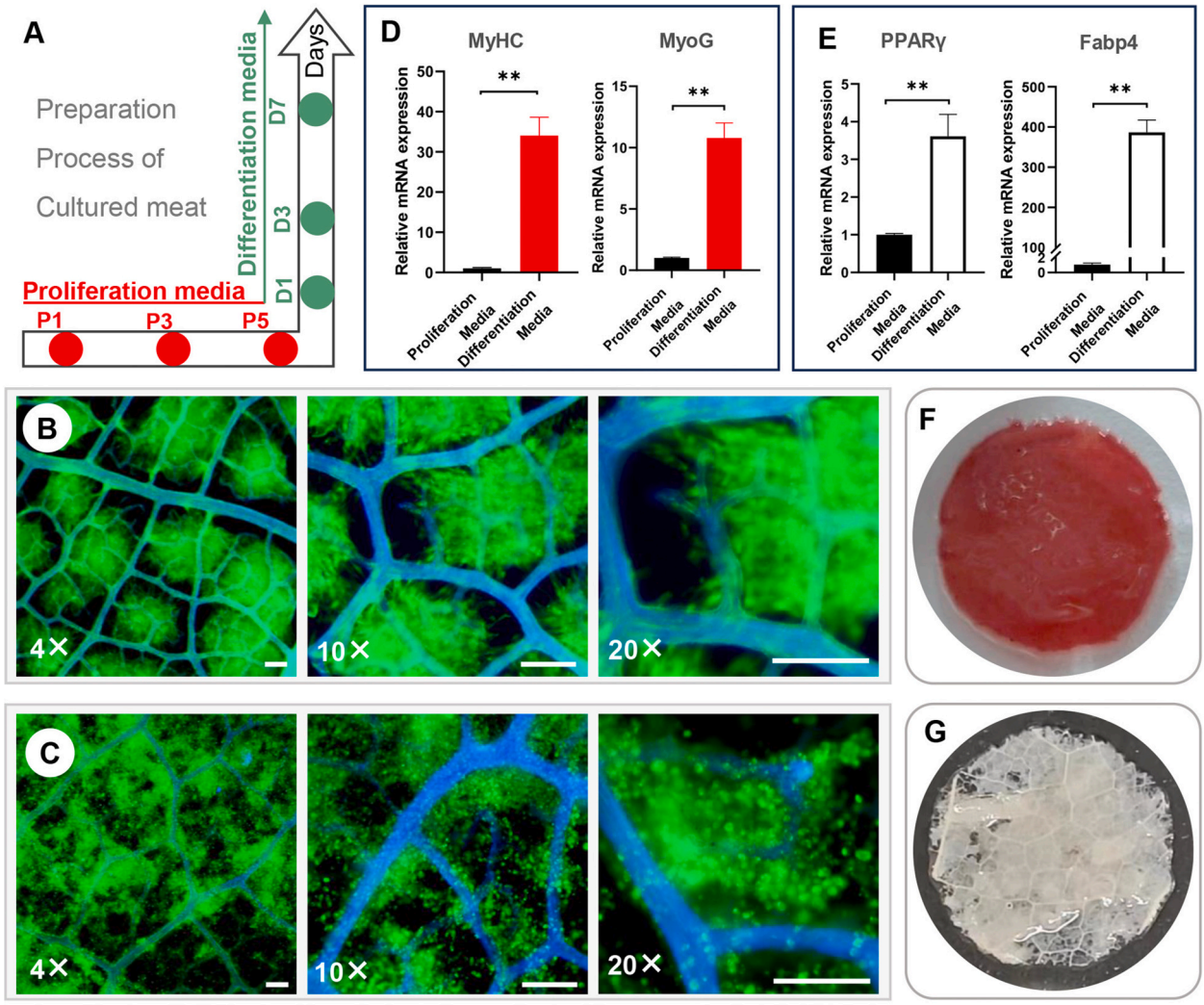

Fig. 6. Differentiation of MSCs and ASCs on NLV scaffolds and example of dog muscle and fat slices. (A) Experimental schematic of MSCs and ASCs cultured in differentiation media for 7 days after proliferation for 5 days on NLV scaffolds. (B) Representative images of immunofluorescence staining of MSCs-derived myotubes on NLV scaffolds after 7 days in differentiation media. Green: Desmin; Blue: Veins. Scale bars: 100 μm. (C) Representative images of immunofluorescence staining of ASCs-derived adipocytes on NLV scaffolds after 14 days in differentiation media. Green: BODIPY; Blue: Veins. Scale bars: 100 μm. (D) Expression of genes associated with myogenic differentiation of MSCs on NLV scaffolds in proliferation media and differentiation media, as measured by RT-qPCR. (E) Expression of genes associated with adipogenic differentiation of ASCs on NLV scaffolds in proliferation media and differentiation media, as measured by RT-qPCR. ** $P < 0.01$, n = 3, "n" was represented different biological replicates. (F) Appearance of cultured dog muscle slices with treatment by food colorant. (G) Appearance of cultured dog fat slices. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and so on(Knezic, Janjusevic, Djisalov, Yodmuang, & Gadjanski, 2022).

Although methods for the isolation and cultivation of canine adipose-derived mesenchymal stem cells and canine skeletal muscle satellite cells have been established(Luo et al., 2021; Michal et al., 2002), there still remain significant challenges in terms of their large-scale cultivation as well as their adhesion, proliferation, differentiation, and fusion on 3D scaffolds. In this study, based on the texture of dog muscle tissue, muscle fibers and adipocytes were loaded with plant scaffolds to prepare tissue-like cultured meat slices and fat slices.

MSCs and ASCs isolated from dog leg muscles and abdominal adipose tissue demonstrated efficient proliferative capacity and possessed the potential to differentiate into myotubes and adipocytes, respectively, albeit with limited differentiation efficiency. In order to restore the original growth state of the cells and improve the differentiation efficiency in vitro, we conducted 3D cell spheroids, and found that 3D cultured MSCs and ASCs had stronger ability of myogenic and lipogenic differentiation in vitro. This enhancement may be attributed to the strengthened interactions between cells and the increased components of the extracellular matrix (ECM) under 3D culture conditions, which provide an environment more closely resembling physiological states (Son et al., 2021). In 3D culture, cell-cell contact and signaling promote the maturation and differentiation of cells, which, compared to 2D culture, are more conducive to the formation and accumulation of intracellular lipid droplets(Hoefner et al., 2020). Additionally, subsequent study revealed an increased propensity for adhesion between the 3D cell aggregates and the scaffolds.

Another key step for the production of cultured meat is the construction of 3D biological scaffolds which provide spatial structure for the adhesion, proliferation and differentiation of seed cells (Seah, Singh, Tan, & Choudhury, 2022).

Plant proteins, with their advantages of low cost, easy availability, and processability, will become important raw materials for 3D scaffolds of cultured meat(Ianovici, Zagury, Redenski, Lavon, & Levenberg, 2022). Researchers used an edible porous textured soy protein as a novel cultured meat scaffold to support cell attachment and proliferation and created a 3D engineered bovine muscle tissue(Ben-Arye et al., 2020). A yeast-free "soda bread" scaffold with controllable porosity and mechanical properties which was stable over several weeks in culture with

fibroblasts, myoblasts and pre-osteoblasts. Importantly, myoblasts were also able to differentiate into myotubes (Holmes et al., 2022). C2C12 and porcine skeletal muscle satellite cells could efficiently adhere, proliferate, differentiate, and fuse into tissue structures on 3D-printed zein/hordein and zein/secalin scaffolds(Su et al., 2023).

Decellularized scaffold biomaterials are easy preparation and biocompatible, which could potentially compensate for the shortcomings of synthetic bio-scaffold materials(Lu, Ying, Shi, Liu, & Chen, 2022). Decellularized spinach porous scaffold has a unique microvascular-like structure, and researchers have been able to successfully cultured beating heart cells with it(Gershlak et al., 2017). In addition, decellularized spinach porous scaffold was successfully applied to primary bovine muscle satellite cell culture as a three-dimensional scaffold for cultured meat. After 14 days of culture, the results showed that the survival rate of satellite cells was as high as 99% (Jones et al., 2023). A natural decellularized grass scaffold retained natural striated topography and supported the attachment, proliferation, alignment and differentiation of C2C12 myoblasts, without the need for additional functionalization(Allan et al., 2021).

Based on the above results, we tried to prepare porous scaffolds with leaf veins, compared with decellularized leaf scaffolds, leaf veins have a larger void structure to accommodate more seed cell growth. Additionally, the preparation process of leaf veins is relatively simple and does not require the use of potentially harmful chemicals such as Triton X-100 and sodium dodecyl sulfate (SDS)(Bilirgen et al., 2021). Scanning electron microscopy (SEM) was used to characterize the surface of the vein, revealing different topographic patterns and highly porous structures. 3D spheroids of MSCs and ASCs ere easily attached to NLV scaffolds, showed good proliferation and differentiation potential, and could spontaneously form cell slices. This study showed the potential use of NLV in developing lab-grown meat. But there is still a lot of work to be done before the technology is ready for commercial use. For example, how to achieve large-scale cultivation of MSCs and ASCs, as well as large-scale preparation of NLV scaffolds.

5. Conclusion

In conclusion, our research successfully utilized natural leaf vein scaffolds to cultivate dog MSCs and ASCs, which has laid the groundwork for an alternative to traditional dog meat production methods. Given the ethical controversies surrounding the slaughter of dogs for meat, our study presents a potentially humane and sustainable approach to meet cultural dietary preferences while avoiding the ethical concerns associated with animal slaughter. The findings from our experiments indicate that these plant-based scaffolds could support the growth and differentiation of cells into muscle and fat tissues, which closely resemble their natural counterparts. As we look to the future, we anticipate that this technology could be adapted to other animal cells, further expanding the possibilities for ethical cultured meat production. However, we acknowledge that additional research is required to optimize the culture systems and to assess the broader implications of this technology.

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CRediT authorship contribution statement

Huina Luo: Writing – original draft, Software, Resources, Methodology, Data curation. **Huimin Ruan:** Software, Methodology, Data curation. **Cailing Ye:** Methodology. **Wenkang Jiang:** Methodology. **Xin Wang:** Methodology. **Shengfeng Chen:** Writing – review & editing, Project administration, Investigation, Formal analysis, Conceptualization. **Zhisheng Chen:** Writing – review & editing, Resources, Formal analysis, Data curation, Conceptualization. **Dongsheng Li:** Writing – original draft, Visualization, Supervision, Software, Resources, Project administration, Methodology, Data curation, Conceptualization.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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