Research Article

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Anthocyanin extract from black rice attenuates chronic inflammation in DSS-induced colitis mouse model by modulating the gut microbiota

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Abstract: There is substantial evidence for the probiotic activity of anthocyanins, but the relationship between anthocyanins involved in the regulation of microbiota and intestinal inflammation has not been fully elucidated. The aim of this study was to investigate the regulatory effects of black rice anthocyanin extract (BRAE) on intestinal microbiota imbalance in mice with dextran sulfate sodium (DSS)-induced chronic colitis. DSS was added into drinking water to induce a mouse model of chronic experimental colitis, and BRAE was given by gavage (200 mg/kg/day) for 4 weeks. Body weight, fecal viscosity, and hematochezia were monitored during administration. After mice were sacrificed, the serum concentrations of TNF-α and IL-6 were detected by enzyme-linked immunosorbent assay, and the composition of intestinal flora was analyzed by 16S rDNA sequencing. The results showed that BRAE significantly suppressed DSS-induced colonic inflammatory phenotypes and maintained colon length in mice. In addition, BRAE reduced intestinal permeability and improved intestinal barrier dysfunction in mice with colitis. Gut microbiota analysis showed that BRAE significantly improved the imbalance of intestinal microecological diversity caused by DSS, inhibited the increase in the relative abundance of inflammatory bacteria, and promoted the abundance of anti-inflammatory probiotics including Akkermansia spp.

Keywords: black rice anthocyanin, inflammation, inflammatory bowel disease, microbiota

1 Introduction

Inflammatory bowel disease (IBD) is a chronic and recurrent inflammatory disease that occurs in the digestive tract and is classified as Crohn’s disease (CD) and ulcerative colitis (UC) [1]. Usually, the main drugs for the treatment of inflammatory diseases are sulfasalazine and corticosteroids, which mainly work by inhibiting the immune response of the body, but there are many side effects and poor compliance [2–4]. Rich natural products from food and herbs have a wide range of biological activities, including anti-inflammatory, antibacterial, and antioxidant activities [5–8]. Effective and evidence-based anti-inflammatory alternative therapy is currently an emerging field of IBD.

The maintenance of intestinal homeostasis depends on the balance between gut microbiota, on the intestinal epithelial barrier, and on the immune system [9–11]. Recently, the role of the microbiome and intestinal mucosal homeostasis has been elucidated in animal models of IBD [12]. A key factor in the development of IBD is the destruction of the mucus layer and intestinal epithelium as the first line of defense against microbial invasion [13]. The maintenance of intestinal homeostasis is based on an intact, microbially impermeable mucosal barrier, and disruption of this barrier increases the risk of colitis [14,15].

IBD patients have some common microbial patterns, such as reduced microbial diversity, lower relative abundance of Firmicutes, and more of Proteobacteria [16–18]. Biodiversity loss may lead to increased inflammation and immune responses, resulting in loss or reduction of key functions required by the host immune system to...
maintain gut barrier integrity and function [19]. On the other hand, the overproliferation of pathogenic bacteria leads to the degradation of the mucosal barrier, thus allowing more pathogens to break through the intestinal mucosal barrier and enter the intestinal tissue [20].

Anthocyanins are a kind of common secondary metabolite, belonging to flavonoids, widely existing in fruits, vegetables, and grains. There is ample research evidence that anthocyanins have many health benefits, including anti-inflammatory, antioxidant, anti-obesity, anti-cancer, anti-diabetes, and immunomodulatory properties [21]. Notably, most studies focus on its complex bioavailability, ignoring the fact that the primary site of absorption of anthocyanins is the colon. Experimental evidence in models of intestinal inflammation suggests that anthocyanins may have therapeutic potential in mice with dextran sulfate sodium (DSS)-induced colitis [22].

Most previous studies on the anti-colitis activity of anthocyanins have focused on berries derived from unacylated anthocyanins, rather than crops that contain predominantly heat-resistant acylated anthocyanins, such as black rice anthocyanin (BRA) [23]. There is sufficient evidence that intestinal flora imbalance associated with intestinal epithelial integrity leads to IBD in humans. Given the potential for interaction between intestinal flora and anthocyanins, we hypothesize that anthocyanin’s response to colitis is mediated by the regulation of intestinal flora, which in turn leads to host response to inflammation and oxidative stress. In this study, the palliative effect of oral anthocyanins extracted from black rice was tested for the first time in a mouse model of chronic colitis induced by DSS.

2 Materials and methods

2.1 Preparation of black rice anthocyanin-rich extract (BRAE)

According to the method of Bae et al. [24], a certain amount of crushed and sifted black rice powder was put into a flask, and 50% of ethanol solution (pH 2–3, adjusted with acetic acid solution) was added at the ratio of 1:8, and ultrasonic extraction was carried out for 30 min. After filtration, vacuum distillation, and concentration, the BRAE was obtained after freeze drying. The yield of BRAE was 1.89 mg/g of black rice. The BRAE powder was stored at −20°C prior to use.

2.2 Quantitation of anthocyanins in BRAE

High liquid chromatography-diode array detector (HPLC-DAD) was used to determine the purity of cyanidin 3-glucoside (C3G) in BRAE. The C3G of analytical standard (Shanghai Aladdin Biochemical Technology Co., Ltd., Cat No. C135886) or BRAE powder was dissolved in acidic methanol (1 mol/L HCl: methanol = 85:15, v/v) and loaded on Agilent Zorbax SB-C18 column (2.1 mm × 50 mm, 1.8 μm). The mobile phase of HPLC system consisted of eluent A (10% formic acid aqueous solution, v/v) and eluent B (methanol). The gradient elution procedure was 0–1 min, 2% B; 1–5 min, 10% B; and 5–20 min, 10–60% B. The column temperature was 25°C, the flow rate was 0.2 mL/min, and the wavelength was 520 nm measured by photodiode array detector.

2.3 Chronic colitis mice model and BRAE administration

Male C57BL/6 mice (8 weeks of age) obtained from Beijing Weitonglihua Experimental Animal Technology Co., Ltd., were randomly assigned to three groups as follows: control group (CON), chronic colitis model group (DSS), and black rice anthocyanidin treatment group (DSS + BRAE), with ten mice in each group, and adaptive feeding for 1 week before the experiment. The normal control group was given pure water during the whole process, and the remaining two groups were induced chronic colitis by repeated and periodical treatment of drinking water containing DSS. Briefly, drinking water with 2.5% DSS (MP Biomedicals, Cat No. 216011080) for 4 days, followed by drinking pure water for 6 days, formed a cycle lasting three times in total. The BRAE treatment group was given 0.2 mL of BRAE solution (20 mg/mL) every day, and the normal group and the chronic UC model group were given 0.2 mL of pure water, respectively, until the end of the experimental period. After the mice were given drinking water containing DSS for modeling, the body weight of the mice was measured every day, and the disease activity index (DAI) for IBD of the mice was evaluated until the end of the animal experiment. There was a good correlation between DAI score and pathological results of DSS-induced IBD model [25]. DAI is based on a composite score of weight loss, stool consistency, and bleeding as described previously [26]. Mice were exsanguinated under terminal anesthesia, and serum was collected by centrifuge phase separation. Colon was isolated, and its length was measured. About 1 cm of the distal...
colon was collected and fixed in 4% of paraformaldehyde solution for histopathological examination. The remaining colon tissue was rapidly frozen with liquid nitrogen and stored at −80°C. Ileocecal valve contents were collected and placed in a sterile centrifuge tube and stored at −80°C for subsequent microbiota analysis (Figure 1).

2.4 Pathological test

Colon tissue was collected, fixed in 10% formalin solution with PBS as buffer for 48 h, and then dehydrated with concentration gradient alcohol, and tissue blocks were placed in xylene to replace the alcohol in tissues and embedded in paraffin. Tissue sections with a thickness of 4 μm were prepared by the continuous section method and stained with hematoxylin–eosin (H&E) solution. The degree of lesion was compared according to ulcer size, inflammatory infiltration, and tissue damage degree.

2.5 Cytokine assay

According to the kit instructions, ELISA was used to detect the concentrations of IL-6 and TNF-α in the serum of each group of mice. Mouse IL-6 ELISA kit (Cat. No. ABS520004) and TNF-α ELISA kit (Cat. No. ABS520010) were purchased from Absin Bioscience Inc.

2.6 RT-qPCR

Total RNA was extracted from 10 mg frozen colon tissue by the Trizol method (RNApure Tissue&Cell Kit, CoWin Biosciences, Cat No. CW0560), and the reverse transcription synthesis of cDNA was carried out according to the instructions of the reverse transcription kit (Super RT cDNA Synthesis Kit, CoWin Biosciences, Cat No. CW0741). Hieff® qPCR SYBR Green Master Mix (Yeasen Biotechnology, 11201ES08) was used to amplify reverse transcription products from different samples. The primers used are shown in Table 1.

2.7 Fecal 16S rDNA amplicon sequencing

The genomic DNA of the fecal flora was extracted from mouse feces by the SDS method, and DNA purity and concentration were detected by agarose gel electrophoresis. We take an appropriate amount of DNA and dilute to

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1 ng/μL with sterile water in a centrifuge tube. The V3–V4 region of the 16S rDNA gene was amplified using the bar-coded universal primer 338F/806R using the diluted genomic DNA as a template. PCR was performed with Phusion® high-fidelity PCR Master Mix and high-fidelity enzymes purchased from New England Biology Laboratories to ensure amplification efficiency and accuracy.

PCR products were detected by 2% agarose gel electrophoresis. PCR-qualified products were mixed in equal amounts according to the PCR product concentration, purified by magnetic beads, and quantified by microplate labeling. After thorough mixing, the PCR products were tested by 2% agarose gel electrophoresis, and the target bands were recycled using the gel recovery kit provided by QIAGEN.

Libraries were constructed using TruSeq® DNA PCR-free Sample Preparation Kit. The library was quantified by Qubit and Q-PCR and sequenced on NovaSeq 6000 by Beijing Nuohe Zhiyuan Bioinformation Co., Ltd, China.

2.8 Bioinformatics analysis of 16S rDNA sequencing data

According to the barcode and primer sequence, data of each sample were obtained from the off-machine sequence. After truncating the barcode and primer sequences, the reads of each sample were spliced using FLASH (v1.2.7). The QIIME (v1.9.1) process was used for quality control and data processing.

The Uparse algorithm (v7.0.1001) was used for all samples with all valid cluster labels, and the sequences defaulted to 97% consistent clusters as operational taxonomic units (OTUs). At the same time, the OTU sequence is selected, which has a certain representativeness. The sequence with the highest frequency in the OTU is selected as the representative sequence of the OTU according to the algorithm principle. Species annotation of bacterial OTUs sequences was performed using the mothur method and the SSUrRNA database of SILVA138 to obtain taxonomic information. For analysis, multiple sequence alignments were performed using MUSCLE (version 3.8.31) software to obtain all OTUs representing systematic sequences. Finally, using the sample with the least amount of data as the homogenization standard, the data of each sample are homogenized for subsequent analysis.

2.9 Statistical analysis

The data are expressed as mean ± SD. Group-to-group comparisons were performed using one-way ANOVA. A P value ≤0.05 was considered statistically significant. All statistical analyses were performed using Prism software.

3 Results

3.1 Content of C3G in BRAE

The content of anthocyanins in BRAE was determined using an externally calibrated HPLC peak area integral. As shown in Figure 2b, two anthocyanins were detected in BRAE. The content of total anthocyanins in BRAE is 30.25%, while C3G accounts for 90.15% of all anthocyanins. Therefore, C3G is the main anthocyanins in BRAE. Crude protein, crude fat, and total carbohydrate of BRAE were analyzed by conventional laboratory techniques. In addition to anthocyanins, BRAE also contained water (7.86%), crude protein (4.22%), crude fat (5.13%), total carbohydrate (17.52%), and other components (35.02%).

3.2 Effect on general condition of mice with UC

Oral DSS can damage the integrity of the colon epithelial barrier, making it permeable to bacterial influx and thus stimulating inflammation. The body weight of the mice in the UC model induced by 2.5% of DSS water for three consecutive cycles was significantly decreased with obvious blood stools, while the symptoms of body weight loss were alleviated by BRAE intervention, with a statistically significant difference compared with the model group (Figure 3a).

The colon length of mice with DSS-induced colitis was shorter than that of the control mice, and the BRAE significantly shortened the colon length (Figure 3c and d). Oral DSS water can disrupt the integrity of the colonic epithelial barrier, allowing it to penetrate bacterial influx, thereby stimulating the inflammatory response. Splenomegaly often occurs in patients with IBD, and spleen weight is defined as an indicator of a systemic inflammatory phenotype. DSS induces splenomegaly in mice with colitis. As expected, BRAE significantly reversed splenomegaly in colitis mice (Figure 3b).
DAI reflects the degree of colitis injury. In the experiment, DSS was used to induce the mouse model of chronic UC and 200 mg/kg BRAE was used to treat it. As shown in Figure 4, the DAI score of BRAE-treated mice was significantly lower than that of the model group.

Histopathological images of the distal colon (Figure 5) showed significant inflammatory response in the DSS group with goblet cell loss, hyperemia, and mucosal damage. BRAE significantly improved crypt structure and reduced edema, mucosal damage, and inflammatory cell infiltration.

3.3 BRAE inhibits the release of pro-inflammatory cytokines

Cytokines play an important role in the etiology of IBD. To investigate systemic inflammatory pathways induced by intestinal inflammation, we examined serum levels of two major pro-inflammatory cytokines, IL-6 and TNF-α. After DSS treatment, serum levels of IL-6 and TNF-α were significantly upregulated, while BRAE significantly inhibited the production of these two pro-inflammatory cytokines (Figure 6).

The colorectal mucosa of patients with IBD is characterized by an increased number of T helper cell-17 (Th-17). We further detected the gene expression levels of Il-17a, Il-23a, and Il-6 in mouse colon tissues by fluorescence quantitative PCR and found that DSS treatment significantly increased the gene expression levels of Il-17a, Il-23a, and Il-6, while BRAE inhibited the expression of related genes (Figure 7).

3.4 Overall changes in gut microbiota

After high-throughput sequencing, the OTU petal diagram of each sample was drawn according to different groups. Different samples were represented by different colors. The petal part was OUT unique to each sample,
and the flower center part was OUT common to all samples. As shown in Figure 8, there were 681 OTUs in the normal group, 603 OTUs in the model group, 605 OTUs in the BRAE group, and 448 OTUs in the same OTUs among the three groups, indicating that there was a great difference in the bacterial community structure among the groups, and the total OTUs of the bacterial community decreased after DSS induction and increased after BRAE administration.

The sparse curve can directly reflect the rationality of the amount of sequencing data and indirectly reflect the richness of the species in the sample. This sparse curve (Figure 8b) tends to be flattened, indicating that the amount of sequencing data is progressive and reasonable, and the more the data, the fewer the new OTUs generated. At the same sequencing depth, by comparing OTU numbers of different samples, the species abundance in the modeling group was lower than that in the normal group, while the species abundance increased after the BRAE treatment.

3.5 Changes in beta diversity of gut microbiota

In the beta diversity analysis, PCoA analysis and metric multidimensional NMDS analysis for dimensionality
reduction were used to compare the differences between groups. The results in Figure 9 showed that the samples in each group could be significantly distinguished, indicating that modeling and BRAE treatment had a certain impact on the intestinal microflora structure of mice. The PCoA analysis showed that modeling factors had the greatest impact on intestinal microflora. Compared with the model group, it was obvious from the NMDS analysis chart that

Figure 5: Representative pictures of H&E-stained colon tissue.

Figure 6: Serum concentrations of inflammatory cytokines TNF-α (a) and IL-6 (b). Data represent mean ± SD (n = 10), ***P < 0.001.

Figure 7: BRAE alleviates colonic inflammation gene expression in DSS-induced colitis mice.
the gut microbiota structure of the treatment group had a
certain deviation, and the flora structure of the BRAE treat-
ment group had a trend of regression to the normal control
group.

3.6 Species annotation and difference analysis of gut microbiota

By comparing the OTU representative sequences with the
microbial reference database, the species classification
information corresponding to each OTU can be obtained,
and then, the community composition of each group at
the phylum and genus levels can be counted, and the
species abundance tables at different taxonomic levels
can be generated to evaluate the differences in species
composition of each group at the phylum and genus
levels. The results are shown in Figure 10a and b, from
the phylum-level species distribution map. The dominant
phyla mainly include Bacteroidota, Verrucomicrobiota,
Firmicutes, Proteobacteria, Campylobacterota, Desulfoba-
terota, and Cyanobacteria. Other bacteria accounted for
a small proportion. Compared with the normal group,

Figure 8: BRAE inhibited DSS-induced decrease of gut microbiota diversity in mice. (a) OTU petal diagram, the petal part is OTU unique to
each group, and the flower center is the OTU shared by all samples; (b) sample sparsity curve.

Figure 9: Beta diversity inter-group matrix. (a) PCoA bray_curtis and (b) NMDS bray_curtis.
the abundance of *Bacteroidota* and *Firmicutes* decreased in the model group, while the abundance of *Proteobacteria* increased. Compared with the model group, the abundance of *Verrucomicrobiota* in the BRAE treatment group increased while that of *Proteobacteria* decreased.

The map of the subordinate horizontal species shows that at the genus level, the dominant species were *Lachnospiraceae NK4A136 group*, *Akkermansia*, *Bacteroides*, *Parasutterella*, *Herbaspirillum* and *Rikenellaceae RC9 gut group*, *Lactobacillus*, and *Desulfovibrio*. Compared with the normal group, the abundance of *Bacteroides*, *Parasutterella*, and *Herbaspirillum* in the model group increased, while the abundance of *Lactobacillus* and *Desulfovibrio* decreased. Compared with the model group, the abundance of *Akkermansia* and *Candidatus Saccharimonas* increased in the BRAE group, while the abundance of *Bacteroides*, *Lachnospiraceae NK4A136 group*, and *Parasutterella* decreased.

The LEfSe software was used to compare and analyze the species diversity of the gut microbiota of the three groups of mice. First, non-parametric Kruskal–Wallis (KW) rank sum test was used to detect the species with significant abundance differences between different groups. Then, Wilcoxon rank sum test of groups was used to judge the difference between groups. Finally, linear discriminant analysis (LDA) was used to evaluate the impact size of significantly different species. The value of LDA score represents the impact size of different species. In this study, LDA score was set to be greater than 3.5, and the LEfSe cladogram of the bacterial community was drawn (Figure 11). The inside-out circle represents the level of classification from the gate to the species. Each small circle represents a level of classification at a different classification level, and the diameter of the small circle is proportional to the relative abundance. Species with no significant differences are yellow, and the next group of biomarkers of different species is yellow. The green node represented the microbial group that played an important role in the normal control group, the blue node represented the microbial group that played a major role in the DSS-induced chronic UC model group, and the red nodes represented the microbiome that played an important role in the BRAE treatment group.

The LEfSe analysis based on LDA found that, at the genus level, *Lactobacillus*, *Eubacterium brachy group*, and *Peptococcus* were relatively abundant in the normal control group. The abundance of *Bacteroides*, *Rikenellaceae RC9 gut group*, and *Parabacteroides* was higher in the chronic colitis model group. However, the abundance of *Akkermansia*, *Defluviitaleaceae UCG-011*, and *Acetitomaculum* was higher in the BRAE group.

### 4 Discussion

IBD has become a global disease, and there is an urgent need to develop effective drugs to treat it. At present, there is no cure for IBD. Aminosalicylate, corticosteroids, biological immunomodulatory drugs are used clinically to reduce inflammation and relieve symptoms of IBD. Long-term medication in patients with IBD is important for the maintenance of efficacy, but long-term side
effects are worrisome [27]. In contrast, alternative medicine therapy has unique advantages in the treatment of UC, especially in the prevention of recurrence [28]. Anthocyanins have significant potential in the treatment of IBD. Due to the very low oral bioavailability, the mechanism of action of anthocyanins in the treatment of IBD is still unclear, which greatly hinders the development of new drugs for the treatment of IBD by anthocyanins in clinical practice [29]. Given how poorly anthocyanins are absorbed in the body, the amount of the drug in feces is much higher than that in mouse plasma, so the gut may be the primary site where anthocyanins work.

As we know, the gut microbiota is important to sustain human health. Under physiological conditions, symbiotic physiological anaerobic bacteria, symbiotic conditional pathogenic bacteria and other harmful bacteria co-exist in the intestinal tract in a stable proportion [30]. Under normal circumstances, microbiota in dynamic equilibrium, once the flora imbalance, will lead to conditional pathogenic bacteria group or pathogenic bacteria group increased to the extent of the disease, and this leads to the development of intestinal diseases. Therefore, the balance of gut microbiota acts as a natural barrier to prevent the invasion of foreign pathogens. It is estimated that trillions of microbes live in a symbiotic relationship with their hosts in the human gut, performing basic functions of healthy individuals such as nutrition, host defense, and immune development [31]. In most healthy individuals, 99% of the gut microbiota consists of Firmicutes, Bacteroidetes, proteobacteria, and actinobacteria, of which Firmicutes and Bacteroidetes together account for about 90% [15]. Intestinal dysregulation occurs when the diversity, composition, or function of the gut microbiome is disrupted, negatively affecting the individual, for example, loss of microbiota homeostasis and inappropriate immune activation [32].

Gut microbiota plays an important role in the pathogenesis, development, and recovery of UC. Related studies have shown that the changes of gut microbiota in UC patients are closely related to the degree of inflammation [33]. The imbalance of gut microbiota is mainly manifested in the increase of pathogenic bacteria. The intestinal toxins secreted by UC patients increase the permeability of intestinal epithelium, and the bacteria and their products in the intestinal lumen enter the lamina propria of intestinal mucosa, resulting in mucosal immune disorders and damage to the intestinal mucosal barrier [34]. Furthermore, it affects and alters the diversity and composition of the gut microbiome.

Figure 11: Species diversity of the gut microbiota analyzed by LEfSe based on LDA score. (a) LDA score for the bacterial taxa differentially abundant (LDA > 3.5); (b) cladograms generated by LEfSe indicating differences in the groups; (c–f) gut microbiota biomarker.
In this study, OTU petal plot, species accumulation curve, beta diversity, and other results showed that DSS modeling significantly reduced the abundance and diversity of gut microbiota in mice, and the imbalance of gut microbiota was an important cause of intestinal mucosal inflammation. After treatment with BRAE, intestinal inflammation may be alleviated by restoring the abundance and diversity of gut microbiota. According to the species annotation and difference analysis results of gut microbiota, there were significant differences in phylum and the genus classification of gut microbiota before and after modeling and treatment. Changes in the abundance of Firmicutes and Bacteroides can be used as an important indicator to measure the structural changes of gut microbiota and can be used to evaluate whether the bacterial structure of UC tends to balance after drug intervention [35]. After treatment, Firmicutes were significantly increased and bacteroidetes were inhibited. Similarly, Fang et al. [36] found in clinical studies that the gut microbiota structure of IBD patients showed a decrease in the proportion of Firmicutes and an increase in the proportion of Bacteroidetes, both of which were consistent with the results of this study.

IBD patients showed decreased biodiversity (mainly firmicutes), decreased stability, and increased proteobacteria, such as Enterobacteriaceae and choleophilia [37]. Reduced microbial diversity results in the loss or reduction of important functions that maintain the integrity of the intestinal barrier and regulate the host immune system. At the same time, mucus-dissolving bacteria and pathogenic bacteria also increase, leading to the damage of the mucosal barrier, allowing more pathogens to invade the intestinal tissue. In this study, 16S rDNA high-throughput sequencing was used to analyze the gut microbiota. The results showed that, similar to clinical IBD patients, the abundance of Bacteroides increased in the DSS-induced colitis mouse model, while the ratio of Firmicutes to Bacteroides decreased. After continuous intervention, BRAE can significantly improve the intestinal microflora disorder associated with colitis, especially increase the relative abundance of probiotics Akkermansia, suggesting that BRAE has certain prebiotic function.

5 Conclusion

The interaction between intestinal flora and intestinal mucosa is essential for maintaining immune system homeostasis. UC occurs due to the atrophy of beneficial bacteria in the gut and the increase of pathogenic bacteria, which leads to mucosal inflammation of the colon. According to the aforementioned experimental results, It is speculated that BRAE achieves the effect of treating UC by adjusting the abundance and diversity of bacteria to restore the normal structure of the bacteria to promote the increase of anti-inflammatory factors and the decrease of pro-inflammatory factors to reduce intestinal inflammatory response and promote the recovery of colonic mucosa.

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Conflict of interest: The authors state no conflict of interest.

Ethical approval: The breeding and the use of animals involved in this study were in accordance with the regulations of the Animal Ethics Committee of Jianghan University and were permitted (license number: JHDXLL2022-085).

Data availability statement: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

References


