Bone marrow-derived mesenchymal stem cells attenuate myocardial ischemia–reperfusion injury via upregulation of splenic regulatory T cells

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Abstract

Background: Myocardial ischemia–reperfusion injury (MIRI) is the main pathological manifestation of cardiovascular diseases such as myocardial infarction. The potential therapeutic effects of bone marrow-derived mesenchymal stem cells (BM-MSCs) and the participation of regulatory T cells (Tregs) in MIRI remains to be defined.

Methods: We used the experimental acute MIRI that was induced in mice by left ascending coronary ischemia, which were subsequently randomized to receive immunoglobulin G (IgG) or anti-CD25 antibody PC61 with or without intravenously injected BM-MSCs. The splenectomized mice underwent prior to experimental MIRI followed by intravenous administration of BM-MSCs. At 72 h post-MIRI, the hearts and spleens were harvested and subjected to cytometric and histologic analyses.

Results: CD25+Foxp3+ regulatory T cells were significantly elevated after MIRI in the hearts and spleens of mice receiving IgG + BM-MSCs and PC61 + BM-MSCs compared to the respective control mice (all p < 0.01). This was accompanied by upregulation of interleukin 10 and transforming growth factor β1 and downregulation of creatinine kinase and lactate dehydrogenase in the serum. The post-MIRI mice receiving BM-MSCs showed attenuated inflammation and cellular apoptosis in the heart. Meanwhile, splenectomy compromised all therapeutic effects of BM-MSCs.

Conclusion: Administration of BM-MSCs effectively alleviates MIRI in mice through inducing Treg activation, particularly in the spleen.

Keywords: Bone marrow-mesenchymal stem cells, T regulatory cells, Ischemia–reperfusion injury, Spleen, Interleukin 10, Transforming growth factor β1

Backgrounds

Although the incidence of myocardial infarction (MI) has decreased in recent years, it remains a major global threat to human health and healthcare costs (1). Mortality from MI has been reduced, however, through the development and use of reperfusion therapy such as pharmacological thrombolytic agents, coronary artery bypass surgeries, and percutaneous coronary intervention (2). Inadvertently, further myocardial damage may occur as a consequence of blood flow restoration, resulting in complications that may affect cardiac function long-term. This phenomenon was termed myocardial ischemia–reperfusion injury (MIRI) by Jennings et al. in 1960 (3). It
was later demonstrated that microstructural, functional, metabolic, and electrophysiological alterations in the myocardium also may occur as part of post-reperfusion tissue damage, and present as clinical manifestations of arrhythmia, myocardial stunning, no-reflow, and sometimes even death (<sup>citation needed here</sup>) (4).

In that preclinical animal model of acute MI, the extent of MIRI is shown to be as high as 50% of the total infarction area in fatal MIRI. Thus, preventive and therapeutic approaches for MIRI have been greatly sought.

Previous studies have demonstrated that regulatory T cells participate in myocardial ischemia–reperfusion injury and play an important role in tissue repair (5–7). The homeostasis of the T cell population was found to be altered after experimentally induced MIRI in mice, and the adoptive transfer of exogenous Tregs attenuated atrial remodeling (6). The PI3K/Akt pathway is critical in the induction of Tregs by N,N-dimethylsphingosine in mice subjected to experimental MIRI by left main coronary artery occlusion (5). In a similar MIRI animal model, activated Tregs exerted their anti-apoptotic properties in a CD39-dependent manner (7). Presently, the therapeutic potential of bone marrow-derived mesenchymal stem cells (BM-MSCs) has been demonstrated in preclinical MIRI studies and shown to enhance left ventricular ejection fraction, myocardial blood flow, and atrial reconstruction (8–10). BM-MSCs have also been shown to exhibit immunomodulatory and anti-inflammatory functions (11–13), where their production of various cytokines, including interleukin 10 (IL-10) and transforming growth factor β1 (TGF-β1), promotes numbers of regulatory T cell (Treg) (12, 13). Furthermore, MSCs rely on a successful splenocyte activity in order to induce Tregs that suppress ischemic-induced tissue inflammation and injury, and splenectomy prevents such a phenomenon (14). To date, the immunomodulatory and Treg promoting capacities of BM-MSCs have not been explored in the context of concurrent MI. The present study aimed to examine changes in the Treg population after administration of BM-MSCs in a murine MIRI model, and to evaluate the roles of Tregs and spleen in the amelioration of MIRI after post-BM-MSCs therapy.

**Methods**

**Mouse MIRI model**

Left anterior ascending coronary artery (LAD) ligation was performed in male C57BL/6 mice weighting 20–25 g prior to surgery to induce acute MIRI according to a protocol published previously (15). In brief, mice were anesthetized by chloral hydrate (10% chloral hydrate, 0.3 ml/100 g mouse weight) administered peritoneally, secured onto the operating surface, and intubated in the supine position. Mice were then connected to a small rodent ventilator and an electrocardiogram throughout the experiment. After exposing the heart by cutting through the intercostal space between the 3<sup>rd</sup> and 4<sup>th</sup> sternal ribs, LAD was temporary blocked by a ligation tightened around a medical latex tube (socket inner diameter, 1.5 mm) placed between the ligature and the LAD to induce myocardial ischemia. After 30 min of successful ischemia confirmed by S-T segment elevation in inferior lead II, the suture and latex tube were withdrawn to reperfuse the myocardium. For mice receiving splenectomy, the spleen was removed according to a previously published protocol (16) hours prior to LAD ligation. The euthanasia procedure was performed according to NIH guideline for euthanasia of rodents using carbon dioxide states that a CO2 fill rate of 30–70% of the chamber volume per minute is to be used when euthanizing rodents. All experimental procedures related to animals were performed in accordance with the Guide for the Care and Use of Laboratory Animals, performed according to ARRIVE guidelines. Experimental facilities and laboratory space was kindly provided by Hangzhou Hibio Biotech Co., Ltd. (Hangzhou, China). The ethical approval was obtained from Hangzhou Hibio Animal Care and Use Committee (IACUC NO. HB2019011904001PXX-A).

**Post-MIRI treatments**

Six hours after the reperfusion, mice were randomly selected into separate groups to receive saline or commercially developed RFP-labeled BM-MSCs (1 × 10<sup>6</sup> cells per mouse; Humanwell Biocell Biotechnology Co., Ltd, Zhengzhou, China) via the tail vein.

**Tissue collection**

Seventy-two hours after MIRI induction, mice were euthanized and their target tissues and whole blood collected. A portion of the heart tissue was saved and processed for histological examination and MI area measurements. Cells were isolated from freshly harvested heart and spleen tissues. For this purpose, tissues were rinsed twice with pre-chilled phosphate buffered saline (PBS), and immersed in pre-chilled RPMI 1640 medium. Cells were subsequently dissociated from tissue samples by mincing and passing through a mesh strainer. Red blood cells (RBC) in the tissue cell suspension were lysed by incubating with RBC lysis buffer (MultiSciences Biotech, Co., Ltd, Hangzhou, China) followed by appropriate washes with PBS. Serum was collected after the blood samples had clotted and stored appropriately for target protein quantification at a later date.
Identification of Tregs
Cell suspensions freshly prepared from the spleen and the heart were triple-stained with anti-CD4-FITC, anti-CD25-APC, and anti-Foxp3-PE antibodies following the instructions and concentration recommended by the manufacturer (Invitrogen eBioscience, catalog No. 88–8111–40). For the identification of Tregs on flow cytometry, a CD4 positive gate was first applied and followed by a CD25–Foxp3 double-positive gate.

ELISA analysis
The activity of serum creatine kinase (CK) and lactate dehydrogenase (LDH), which are biomarkers of myocardial injury, were assessed using commercial biochemical kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer’s instructions. The concentrations of serum IL-10 and TGF-β1 were measured by commercial ELISA kits according to the user’s manual (MultiSciences Biotech, Co., Ltd, Hangzhou, China).

H&E staining
Formalin-fixed, paraffin-embedded 4 μm sections of heart tissues were stained with hematoxylin and eosin (HE; Sigma-Aldrich, St. Louis, MO, USA) and examined by light microscopy.

TUNEL assay
After an appropriate pretreatment of heart tissue sections with proteinase K, the extent of cellular apoptosis in the myocardium was examined using a commercial TUNEL assay (Roche, Basel, Switzerland) following the package insert. Apoptotic cells were counted manually in five non-overlapping fields randomly selected from each tissue section at 200 × magnification.

MI area measurements
The harvested heart tissues were sliced and immersed in 2% 2,3,5-triphenyltetrazolium (TTC; Sigma-Aldrich, St. Louis, MO, USA) solution protected from light. After 15–30 min, the tissue slices were transferred and fixed in 4% acetone for 24 h. The MI area is expressed as the averaged percent of total ventricular area.

Statistical analysis
All data are expressed as mean ± standard deviation (SD). The differences between more than 2 groups were analyzed by one-way ANOVA, while those between 2 groups were analyzed by the least significant difference (LSD) test. P values < 0.05 were considered statistically significant. All statistical analyses were performed by SPSS version 19.0 (IBM, Armonk, NY, USA).

Results
BM-MSCs administration increases numbers of Treg cells in MIRI mice
After treatment with IgG + BM-MSCs, the number of CD25+/Foxp3+ Tregs were significantly increased by almost 2- to three-fold compared with treatment with IgG alone in both the heart and spleen of mice with 72 h post-MIRI (IgG vs IgG + BM-MSCs, p < 0.01) (Fig. 1). Depletion of Tregs by PC61 treatments was observed in both mice receiving antibody alone and in combination with BM-MSCs 72 h after MIRI induction (IgG vs PC61 and IgG + BM-MSCs vs PC61 + BM-MSCs, both p < 0.001) (Fig. 1). Of note, the cardiac and splenic Treg pool depleted by PC61 was significantly replenished by BM-MSCs (PC61 vs. PC61 + BM-MSCs, p < 0.01), so that it was numerically comparable to that by IgG alone (IgG vs PC61 + BM-MSCs, p > 0.05) (Fig. 1).

BM-MSCs treatment increased the serum concentration of immunomodulatory cytokines in MIRI mice
The serum concentrations of IL-10 and TGF-β1 were significantly higher in the group receiving IgG + BM-MSCs compared to those receiving IgG alone 72 h post-experimentally induced MIRI (IgG vs IgG + BM-MSCs, both cytokines p < 0.01; Table 1 and Fig. 2a, b). Mice receiving PC61 alone to deplete Tregs had significantly lower levels of the two immunomodulatory cytokines compared with those receiving IgG alone. (IgG vs PC61, both cytokines p < 0.05 Table 1 and Fig. 2a, b). In contrast, administration of BM-MSCs to the mice to replenish the Treg pool depleted by PC61 showed a concurrent and significant increase in the serum level of IL-10 and TGF-β1 (Table 1 and PC61 vs. PC61 + BM-MSCs, both p < 0.01; Fig. 2a, b) to a similar concentration observed in mice that received isotype and saline control (Table 1 and IgG vs PC61 + BM-MSCs, both cytokines p > 0.05; Fig. 2a, b).

BM-MSCs treatment reduced the level of MIRI-induced biomarkers in the serum
After treatment of BM-MSCs, the serum levels of CK and LDH induced by MIRI was significantly reduced (Table 1 and IgG vs. IgG + BM-MSCs, both biomarkers p < 0.05; Fig. 2c, d). Depletion of the Treg pool by PC61 administration significantly increased CK and LDH in the serum compared with those receiving IgG alone (IgG vs. PC61, both biomarkers p < 0.05; Table 1 and Fig. 2c, d), and this was suppressed by co-administration of BM-MSCs to replenish the Treg pool depleted by PC61 (PC61 vs. PC61 + BM-MSCs, both p < 0.01; Table 1 and Fig. 2c, d). No significant differences were found in the serum levels of CK and LDH between mice receiving PC61 + BM-MSCS and those receiving isotype and saline control (IgG vs. IgG + BM-MSCS, both biomarkers p > 0.05; Table 1 and Fig. 2c, d).
Myocardial injury induced by ischemia–reperfusion was ameliorated by administration of BM-MSCs.

H&E staining of heart tissue sections revealed that cardiomyocytes had been replaced by loose fibro-connective vs. PC61 + BM-MSCs, both biomarkers p > 0.05; Table 1 and Fig. 2c, d).
Table 1  The serum level of IL-10, TGF-β1, CK, and LDH 72 h after MIRI

| Group                        | IgG  | IgG + BM-MSCs | PC61  | PC61 + BM-MSCs | Splenectomy | Splenectomy + BM-MSCs |
|------------------------------|------|---------------|-------|----------------|-------------|-----------------------|
| IL-10 (pg/mL)                | 426.7 ± 18.5 | 569.87 ± 36.25 | 363.71 ± 37.44 | 434.79 ± 46.68 | 378.1 ± 41.8 | 384.32 ± 47.18        |
| TGF-β1 (pg/mL)               | 19,901.1 ± 2387.9 | 26,955.38 ± 2077.85 | 16,287.67 ± 1220.69 | 21,352.49 ± 2018.52 | 18,501.3 ± 1257.9 | 20,137.69 ± 1965.71 |
| CK (U/mL)                    | 1.3 ± 0.1 | 1.03 ± 0.13 | 1.71 ± 0.27 | 1.41 ± 0.08 | 1.29 ± 0.25 | 1.08 ± 0.24          |
| LDH (U/L)                    | 549.8 ± 58.0 | 433.09 ± 50.72 | 754.44 ± 48.51 | 587.17 ± 36.54 | 540.4 ± 54.0 | 495.48 ± 76.70        |

Fig. 2  BM-MSCs treatment increased the level of IL-10 and TGF-β1 cytokines and decreased the level of CK and LDH in the serum of MIRI mice. The level of IL-10 (a), TGF-β1 (b), CK (c), and LDH (d) in the serum of mice treated with IgG, IgG + BM-MSCs, PC61, PC61 + BM-MSCs, splenectomy, and splenectomy + BM-MSCs (n = 5 in each group) are shown. Whole blood was collected 72 h after surgically-induced MIRI, from which the serum was separated. IL-10 and TGF-β1 were measured by ELISA, while CK and LDH were measured by enzyme assays. The bars represent the mean ± standard deviation of target proteins in each indicated group. BM-MSCs, bone marrow-derived mesenchymal stem cells; CK, creatinine; LDH, lactate dehydrogenase; MIRI, myocardial ischemia–reperfusion injury. SEM ± SD, *p < 0.05, **p < 0.01 and ***p < 0.001
tissues after the induction of MIRI, accompanied by fibroblast proliferation and inflammatory cell infiltration (Fig. 3, upper left panel). The administration of BM-MSCs assisted with retaining the structure and distribution of cardiomyocytes, with limited edema and inflammatory foci (Fig. 3, upper right panel). The histologic findings of heart tissue treated with PC61 alone showed clearance of necrotic cardiomyocytes and decreased staining of the endocardial infarct, which was replaced by loose fibro-connective tissues, edema, and numerous infiltrating inflammatory cells (Fig. 3, middle left panel). In contrast, the heart tissue sections of mice treated with PC61 + BM-MSCs exhibited limited infiltration of mononuclear cells and fibrogenesis.

**Fig. 3** Cardiac infiltrating cells and cardiomyocyte disruption were reduced after BM-MSCs treatment in MIRI mice. Images (200×) of HE-stained myocardium sections of representative MIRI mice treated with IgG, IgG + BM-MSCs, PC61, PC61 + BM-MSCs, splenectomy, and splenectomy + BM-MSCs are shown. The hearts were collected 72 h after surgically-induced MIRI. Arrows indicate the mononuclear cells. The scalebar shown in the lower right corner of each image represents 50 μm. BM-MSCs, bone marrow-derived mesenchymal stem cells; MIRI, myocardial ischemia–reperfusion injury.
**Table 2** The myocardial infarction area in each group after myocardial ischemia–reperfusion injury

| Group              | Infarction area (\(\bar{x}\pm SD,\%) |
|--------------------|-------------------------------------|
| IgG                | 31.13±6.82                          |
| IgG+ BM-MSCs       | 20.15±1.22                          |
| PC61               | 54.79±7.65                          |
| PC61+ BM-MSCs      | 39.68±4.12                          |
| Splenectomy        | 57.30±11.091                         |
| Splenectomy + BM-MSCs | 52.53±8.55                         |
dampened inflammatory response (30). In MIRI, Tregs are able to revert M1 polarization to M2, enhance collagen production and angiogenesis, induce anti-inflammatory cytokines, improve ventricular remodeling, and facilitate recovery of the ischemic myocardium (31, 32). In addition, Tregs have been shown to improve post-MIRI cardiac functions via suppressing the activation of matrix metalloproteinase 2 (MMP-2) (26). Through inhibiting the MMP-2 pathway, Tregs were shown to attenuate myocardium cellular apoptosis and extracellular matrix remodeling (33). The findings from that MIRI preclinical study illustrate the importance of IL-10 and TGF-β1 in Tregs and BM-MSCs-attenuated myocardial injury. The participation of the MMP-2 pathway and its

Fig. 4 BM-MSCs treatment decreased the cardiac apoptotic cell numbers after MIRI. Representative images (200 x) of myocardial TUNEL staining of MIRI mice treated with IgG, IgG + BM-MSCs, PC61, PC61 + BM-MSCs, splenectomy, and splenectomy + BM-MSCs are shown (a). The hearts were collected 72 h after surgically-induced MIRI. Arrows indicate the apoptotic cells. The scalebars shown in the lower right corner of each panel represent 50 μm. From the images, the number of apoptotic cells were counted and expressed in mean value ± standard deviation of each indicated group (n = 5) (b). BM-MSCs, bone marrow-derived mesenchymal stem cells; MIRI, myocardial ischemia–reperfusion injury.
anti-apoptotic role are currently under investigation and deserve to be the focus of future studies.

Parekkadan et al. (2011) observed previously that coculturing BM-MSCs cells with splenocytes resulted in increased numbers of Tregs in the presence of IL-2 (16). Additionally, transplantation of the splenocytes into a murine colitis model prolonged animal survival and enhanced the Treg population in the mesenteric lymph nodes, while these effects were eliminated by splenectomy (16). Corroborating the enhanced Treg pool in secondary lymphoid organs, the engraftment of exogenous BM-MSCs in the inguinal, mesenteric, and pancreatic

**Fig. 5** MI area was attenuated by BM-MSCs treatment after MIRI induction. Photographs of TTC stained myocardium tissues of representative MIRI mice treated with IgG, IgG + BM-MSCs, PC61, PC61 + BM-MSCs, splenectomy, and splenectomy + BM-MSCs are shown (a). The hearts were collected 72 h after surgically-induced MIRI. From the micrographs, the area of MI (stained grey by TTC) was calculated and expressed in mean value ± standard deviation of each indicated group (n = 5) (b). BM-MSCs, bone marrow-derived mesenchymal stem cells; MI, myocardial infarction; MIRI, myocardial ischemia–reperfusion injury; TTC, 2,3,5-triphenyltetrazolium
lymph nodes was previously found to occur coincidently with the therapeutic activities of BM-MSCs in streptozotocin-induced diabetic mice (34). Promotion of BM-MSCs’ nurturing of the spleen through splenocyte activity augmented the anti-inflammatory effects of BM-MSCs in mice with colitis induced by dextran sulfate sodium (35). The present study expanded these findings and further demonstrated the importance of the spleen as a Treg nurturing site post-MIRI as illustrated by the findings of splenectomized mice in the present experiments. The cardiac Treg pool induced by the administration of BM-MSCs after MIRI induction was abrogated in the splenectomized mice. Thus, further studies should be conducted to investigate the molecular mechanisms of BM-MSCs-promoted Treg activation in the spleen, which ameliorates MIRI-induced inflammatory and injury.

Ischemic injury is typically accompanied by a deeper grade of myocardial tissue hypoxia, generating increased the production of reactive oxygen species (ROS), which in turn activates maladaptive signaling pathways, leading to myocardial cell death and maladaptive cardiac remodelling (36). Carbonic anhydrases (CAs) are a family of ubiquitous metalloenzymes that are responsible for the rapid conversion of carbon dioxide to bicarbonate and protons and thus are involved in a variety of physiological and pathological processes that involve pH regulation, CO2 and HCO3 transport, ion transport, and biosynthetic reactions (37). The CA inhibitor ethoxzolamide prevents rat cardiomyocyte hypertrophy in vitro and reverses it once it is established (38). Elevated CA-II expression has been detected in rats with spontaneous hypertension and heart failure (39). CA-I myocardial expression regulates with capillary density and endothelial cell death (40), which correlated with myocardial ischemia–reperfusion injury (41). Recent report revealed that the depletion of CA-I increases the activation of Treg cell (42). Taken together, these evidences also suggest that a mechanism similar to that underlying CA-II increase in myocytes may also explain CA-I increase in endothelial cells in myocardial ischemia–reperfusion injury. Certainly, this hypothesis remains highly speculative, and it remains to be properly tested in future studies.

Otherwise, to explore the CD25 + Foxp3 + Treg cells function in myocardial ischemia–reperfusion injury, which drive from BM-MSCs, we defined the significantly effects of adoptive transfer of BM-MSCs. In post-MIRI remodeling, multiple immune cell types infiltrate the infarcted heart, including neutrophils, infiltrating pro-inflammatory monocytes (43). Study reported that CD4+ T cells (including Foxp3 + Tregs) infiltrated the ischemia injury heart and promote wound healing via favorably monocytes and/or macrophage trafficking and differentiation (44). Additional, splenocytes obviously mediated a broad array of pro-inflammatory factors. The current results were demonstrated a robust tissue-injurious cardiосpленic axis in ischemic heart reperfusion injury, which consistent with pervious works. However, we did not transfer splenocytes into the MIRI recipient mice to explore the influence of CD25 + Foxp3 + Treg cells after splenectomy. Still, we did not evaluate the myocardial function after transfer BM-MSCs in MIRI recipient mice. Thus, the clear mechanisms need to investigate in the future. Clinical application may require a large dose of Treg cells, and one challenge is hard to expand and distinguish human Treg cells. Results from Taams et al. suggested that CD4+CD25+Foxp3+Treg cells can polarize macrophage transition in the inflammation site under tissue repairing (45). CD127, an IL-7 has been reported could be as an appropriate marker for natural Treg cell isolation for clinical testing (46). Study demonstrated that total Treg cell number of human body is around $1.3 \times 10^{10}$, while the circulation Treg cells number is around $0.2 \times 10^{9}$ without expansion (47). These studies indicated that the limitation for the application of human-obtained Tregs. Despite these limitation, BM-MSCs exhibited the advantage in treatment of ischemia–reperfusion injury in comparison of Treg cells and provide another therapeutic strategy in clinical application.

Conclusion

In conclusion, BM-MSCs ameliorate MIRI-induced inflammatory and cellular apoptosis in the myocardium via the induction of Tregs and upregulation of IL-10 and TGF-β1. The spleen is the key organ facilitating BM-MSCs-directed promotion of Tregs. However, the precise molecular and cellular components involved in the immunomodulatory mechanism of BM-MSCs warrant more in-depth investigation, especially in the context of MIRI.

Abbreviations

MI: Myocardial infarction; MIRI: Myocardial ischemia–reperfusion injury; BM-MSCs: Bone marrow-derived mesenchymal stem cells; Tregs: Regulatory T cells; IgG: Immunoglobulin G; IL-10: Interleukin 10; TGF-β1: Transforming growth factor β1; CK: Creatinine kinase; LDH: Lactate dehydrogenase; LAD: Left anterior ascending coronary artery; PBS: Phosphate buffered saline; RBC: Red blood cells; H&E: Hematoxylin and eosin; MMP2: Matrix metalloproteinase 2.

Supplementary Information

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Additional file 1. Supplemental Fig 1. BM-MSCs cocultured with splenocytes induced the Treg population and increased the levels of
IL-10 and TGF-β1. Splenocyte (5 × 10^6 cells) were freshly isolated from mice and cocultured with BM-MSCs (1 × 10^5 cells) for 72 h. Representative flow cytometry scatter plots (A) and qualification results (B) confirmed CD25^+Foxp3^+ Tregs from splenocytes and splenocytes+BM-MSCs. The cocultured supernatant were examined IL-10 and TGF-β1 by ELISA. SEM ± SD, **p < 0.01 and ***p < 0.001.

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None.

Authors’ contributions
WJZ, LXP, WW and QL had made substantial contributions to the conception and study design; KZ, HJL and BS contributed for clinical studies RCA and MF collected the data; YWO and YSY analyzed the data; LXP prepared the manuscript, and WJZ critical revision of the manuscript; all authors read and approved the final version for submission.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Declarations
Ethics approval and consent to participate
All experimental procedures related to animals were performed in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Hangzhou Hibo Animal Care and Use Committee (IAALC NO. HB201901 1904001PXK-A) prior to initiation of the study. Moreover, the current article does not contain any human samples or participants performed by any of the authors.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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