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Bone marrow mesenchymal stem cell exosomes improve fracture union via remodeling metabolism in nonunion rat model

Cheng Li[1†], Ming Chen[1†], Lijun Guo[1], Dadong Yu[1], Zhonghai Xu[1], Bin Chen[1] and Zhijian Xiao[1*]

Abstract

Background Nonunion of fractures is a major unsolved problem in clinical treatment and prognosis of orthopedics. Bone marrow mesenchymal stem cell (BMSC) exosomes have been proven to be involved in mediating tissue and bone regeneration in a variety of diseases. However, the role of BMSC exosomes in fracture nonunion is unclear.

Methods BMSC exosomes were injected into a rat model of nonunion fracture, and the fracture-healing site was detected by micro-CT and the serum metabolites were analyzed by LC-MS/MS.

Results The results showed that the exosomes could be successfully isolated from rat BMSCs cultured in an exosome-free medium. Compared with the model group, the fracture site of the exosome-treated rats were healing obviously. Compared with the PBS group, there were 158 up-regulated differential abundance metabolites (DAMs) and 79 down-regulated DAMs in the BMSC-exo group. The DAMs were enriched in 'Th1 and Th2 cell differentiation', 'ErbB signaling pathway', 'PPAR signaling pathway' and 'HIF-1 signaling pathway' that were related to the function of cell proliferation and differentiation. DAMs-PE in HIF-1 signaling pathway were the major metabolite to promote fracture healing.

Conclusions Our study reveals the mechanism by which BMSC-exosome improves the fracture healing process through metabolic reprogramming and provides a reference for the treatment of fracture nonunion.

Keywords Exosomes, Fracture nonunion, HIF-1 signaling pathway, Bone marrow mesenchymal stem cell, Metabolites

†Cheng Li and Ming Cheng contributed equally to this work and shared the first authorship.

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Introduction

Fracture nonunion is a common orthopedic complication. Nearly 5–10% of fractures result in delaying union or nonunion [1], and the life quality of patients is reduced because of suffering repeated treatments [2]. The healing of bone fractures is a regenerative process that involves complex physiological processes and can be roughly separated into two overlapping stages, which were named as metabolic stage and the biological stage. The metabolic phase begins with anabolic processes, including the formation of bone and blood vessel tissue [3]. During the transition from anabolism to catabolism, the secondary bone structures begin to form. Eventually, catabolic activity dominates as the bone structure returns to its pre-injury state. This whole process is supported by three main biological stages, from the inflammatory stage, to the intrachondral bone formation stage, to the bone remodeling stage [4]. Physical stimulation therapies [5], intermittent pneumatic compression, lncRNA [6] and miRNA indicator [7, 8] are effective treatments of fracture nonunion [9]. While some drugs (such as calcitonin) may have a potentially positive effect on fracture healing, most of the drugs studied (such as bisphosphonates, monoclonal antibodies, statins, and vitamin D) have not shown a significant boost to fracture healing [10]. Therefore, there are still deficiencies in the positive progression of drugs for the treatment of fracture nonunion, and more research is needed.

Exosomes are important participants in cellular communication under normal physiological and pathological conditions [11, 12]. A novel cell-free stem cell-derived extract containing exosomes can enhance tissue repair [13]. For bone regeneration, specific mesenchymal stem cells (MSC) must be recruitment, proliferated, and differentiated into osteoblasts [14]. Bone marrow mesenchymal stem cells (BMSC) served as pivotal progenitors for the osteogenic lineage [15]. The transplantation of BMSCs has been implied to be efficient in achieving regenerative capacity in fracture healing [16, 17]. The report shows the expression of SDF-1 is increased at the fracture site, especially in the periosteum at the fracture edge, and using anti-SDF-1 antagonists can damage fracture healing [18]. BMSCs are used in the repair process through the autocrine and paracrine pathways [17]. BMSC-exosome is a spherical vesicle that has been verified to mediate tissue regeneration in a variety of diseases [19–21]. Extracellular vesicles derived from MSCs showed potential as a treatment for COVID-19 due to their immunosuppressive and tissue regeneration properties [22]. It has been reported that BMSC-exosome enhanced osteogenesis and angiogenesis after transplanting to the fracture site of a rat femur fracture model accelerated the bone healing process [23], BMSC-exosome promotes muscle formation [24], endogenous angiogenesis [25] and bone formation [26]. However, it is not clear whether BMSC-exosome can improve the fracture healing process through metabolic reprogramming.

Current studies have shown that the gene-metabolic regulatory network is closely related to non-fracture healing [27]. However, it's unclear how BMSC-exosome improves fracture nonunion through the rat gene-metabolic network. We aim to elucidate the role of BMSC-exosome in remodeling metabolism to improve fracture nonunion in rats and provide a new basis for clinical treatment of fracture nonunion. Hence, we constructed a rat model of fracture nonunion and injected isolated exosomes from rat BMSCs into the fracture site of rats. The effects of exosomes on the treatment of fracture nonunion were detected by CT scanning and the effects of exosomes on metabolite changes were detected by LC-MS/MS. It provides a new perspective for the treatment and drug development of fracture nonunion, and brings light to the improvement of patients with fracture nonunion.

Materials and methods

BMSCs culture

BMSCs were cultivated in DMEM/F12 (1:1) medium (CORNING, 10-092-CVR) containing 10% fetal bovine serum (FBS, Gibco, USA) and 1% double antibiotics (penicillin/streptomycin mix, Sango, E607011). The BMSCs were conserved in an incubator at 37 °C and 5% CO2. The culture medium was replaced every 2 days. When the cells were 80% confluent, BMSCs were dissolved with trypsin (Sango, E607002) for passaging, and the 2–5 passages were used.

Isolation and identification of exosomes

To isolate exosomes from the supernatant of BMSCs. Initially, the BMSCs with 80% confluent were cultured for growth to logarithmic phase (about 10^7 cells/dish) in the complete medium, and the medium was moved to centrifuge for 10 min (300 g, 4 °C). Then, the supernatant was then centrifuged for 30 min (10000 g, 4 °C) to eliminate cellular debris. Next, the supernatant filtration was obtained by using a 200 μL filter to remove whole cells and excess cellular debris. Afterward, the supernatant was moved to new tubes for ultracentrifugation for 2 h (100000 g, 4 °C). Then the precipitate was collected, subsequently the ultracentrifugation was operated again. Ultimately, the supernatant without exosomes was collected for follow-up experiments.

NTA analysis

The size and concentration of exosomes were measured using NTA at VivaCell Shanghai with ZetaView PMX 110 (Particle Metrix, Meerbusch, Germany) and corresponding software ZetaView 8.04.02. The exosome samples

were suitably diluted by 1× PBS buffer (Biological Industries, Israel) to be detected. The ZetaView system was adjusted to standard using 110 nm polystyrene particles. Temperature was controlled around 23 °C and 30 °C. Finally, 11 positions were recorded and analyzed.

Western blotting

BMSCs were lysed with RIPA buffer (Thermo, USA), allowing for the extraction of proteins from the lysed samples. The concentration of protein was assessed using a BCA assay kit (Thermo, USA). The 10%SDS-PAGE was used to resolve the proteins. Gel electrophoresis was conducted to generate the gel plate, which was subsequently transferred onto a PVDF membrane. The PVDF membrane underwent incubation with primary antibodies Alix (Proteintech; 12422-1-AP), TSG101 (Proteintech; 67381-1-lg), CD9 (Proteintech; 20597-1-AP), CD63 (Proteintech; 25682-1-AP) and for either 3 h or overnight at 4 °C. Following this, Goat Anti-Mouse IgG H&L (abcam; ab205719) and Goat Anti-Rabbit IgG H&L (abcam; ab6721) serving as the secondary antibody, were applied to the PVDF membrane for 12 h at room temperature. Ultimately, color development was performed using a high-sensitivity ECL luminescence kit (Thermo, USA), followed by exposure to a chemiluminescence imaging system to capture images.

Construction of non-union fracture rat model

Twelve-week-old Wistar rats were purchased from SPF (Beijing) Biotechnology Co., Ltd (Beijing, China). The rats were randomly divided into model and BMSC-exo groups, $N = 5$. Before the experiment, the rats were fed for 7 days (temperature 24–26 °C, humidity 55–60%), alternating between light and dark at 12 h/12 h, and drank freely. Surgery was performed on rats anesthetized with 3% pentobarbital sodium [28, 29]. Concisely, a 10 mm lateral skin incision was taken and the right femur was exposed after blunt dissection to expose the muscle. The bone is then transverse with a bone knife and a hammer. Burn the periosteum with a hot needle. A 1.5 mm diameter needle (length 45 mm; Material, medical stainless steel 317 L) was interpolated through the distal femur medullary cavity. Next, the other end of the needle passes through the top of the greater trochanter of the femur. During the reduction and fixation of femoral fractures, a 0.4 mm blade was placed at an assistant of the fracture site to hold consistency in the length of the unhealed area. The penicillin was injected intraperitoneally with 100,000 IU/mL, 1mL /kg for continuous 3 days. The two groups were injected with PBS (100 μL) and exosomes (100 μL, 10^{10} particles) at the fracture site, respectively. After the successful completion of the nonunion model, the above measures were taken once a week. The time of model was constructed for 14 weeks.

Micro-CT

After 14 weeks, the rats were injected with heparinized normal saline (100 U/mL) and polyformaldehyde solution to flush the vascular system. MICROFIL (Flow Tech, Carver) was then injected intravenously. Subsequently, the rats were placed at 4 °C for 24 h. The fracture site of the lower right limbs was scanned by Skyscan 1176. The images were reconstructed with Hiscan Reconstruct software (Version 3.0, Suzhou Hiscan Information Technology Co.,Ltd) and analyzed with Hiscan Analyzer software (Version 3.0, Suzhou Hiscan Information Technology Co.,Ltd) and the bone parameters of rat femur were exported including bone volume, bone surface, percent bone volume, bone surface density, porosity, Euler number, connectivity, connectivity density, bone mineral content.

LC-MS/MS analysis

In this project, the metabolomics of the serum samples ($n = 5$) was studied using LC-MS/MS analysis platform [27]. The samples were first pre-treated to get rid of proteins and impurities, and the extract metabolites were obtained. Then the samples were sent to Major-Bio Technology Co., Ltd. (Shanghai, China) for detection and collection under positive and negative LC-MS modes. The MS and MS/MS information of metabolites was obtained. The metabolite annotation and data pretreatment were performed by Progenesis QI (Waters Corporation, Milford, USA) software. Finally, the list of metabolites and data of matrix were obtained. The differential abundance metabolites (DAMs) were screened by T-test and VIP (OPLS-DA), and the biological information of DAMs was further mined by personalized analysis such as pathway analysis, cluster analysis and association analysis. The DAMs were screened by T-test and VIP (OPLS-DA), Pvalue < 0.05 and VIP 1.

Statistics and analysis

The data management was performed in Excel 2019, then GraphPad Prism 8 was used to perform variance analysis by one-way ANOVA and the testing for difference by T-test. The values in the chart or table were regarded as mean ± standard deviation, the Pvalue < 0.05 indicated that there was a significant difference in groups.

Results

1. Isolation and identification of exosomes from bone marrow mesenchymal stem cells

BMSC was cultured in an exosome-free medium and then the exosomes were collected. First, the isolated exosomes were about 30–150 nm diameters and a saucer-like or concave hemisphere-like structure was observed by scanning electron microscopy (Fig. 1A), consistent with what has been reported [30]. Then, the particle size

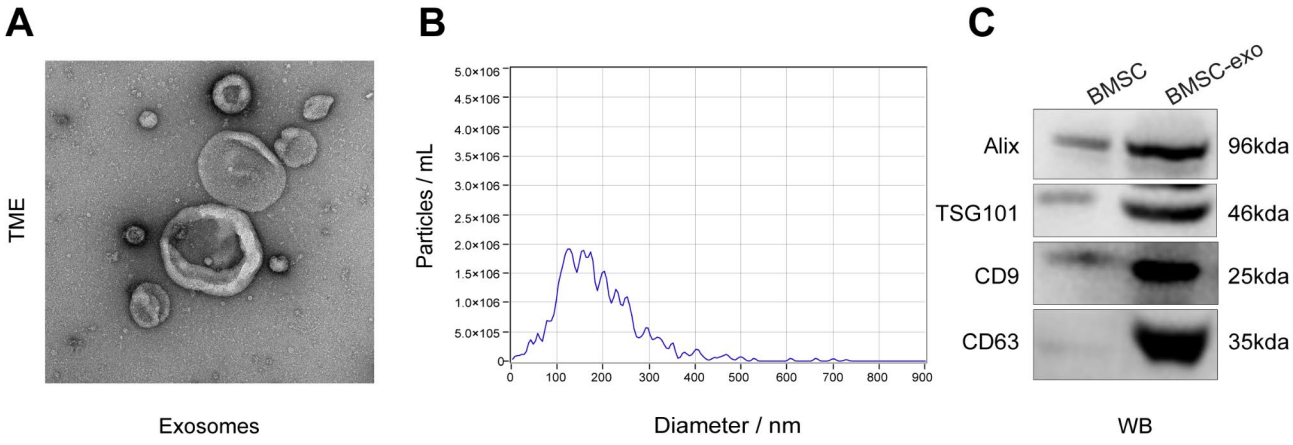

Fig. 1 Isolation and identification of exosomes from rat bone marrow mesenchymal stem cells. (**A**) The exosomes structure photographed by TEM; (**B**) The diameter peaks of exosomes detected by NTA; (**C**) The expression of Alix, TSG101, CD9 and CD63 detected by Western blot, scale bar = 200 nm

distribution of the particles in the samples was detected by NTA technology, which showed a peak value around 100 nm (Fig. 1B). We detected markers of exosomes by WB (Western blot) and found that Alix, TSG101, CD9 and CD63 were significantly expressed in BMSC exosomes (Fig. 1C). These results indicate that we successfully isolated exosomes from BMSC for further validation.

2. Micro-CT shows that BMSC exosomes promote bone repair in fracture nonunion rats

To explore whether BMSC exosomes can improve fracture non-healing, the midsection of femur bone was surgically removed to construct a rat fracture nonunion model with BMSC exosomes injected for treatment, and the formation of bone nonunion was observed by micro-CT after surgery (Fig. 2A). The CT results revealed distinct differences between the BMSC-exo group and the PBS group. In the BMSC-exo group, the bone tissue morphology appears more complete, with better fracture-end connections. There is a significant amount of bone tissue filling the gap between the fracture ends, indicating a certain healing trend. In contrast, in the PBS group, the fracture ends are markedly separated with poor continuity. The fracture-end gap is large, and there is minimal bone tissue formation (Fig. 2B). Through a comprehensive analysis of multiple indices related to the femur, it was conclusively demonstrated that the BMSC-exo group manifested significantly higher levels of bone volume, bone surface, bone volume percentage, and bone surface density compared to the PBS group. Similarly, in terms of microstructural and compositional characteristics, the connectivity, connectivity density and bone mineral content in the BMSC-exo group were also remarkably elevated when contrasted with those of the PBS group. These findings further highlighted the potential beneficial effects of BMSC-derived exosomes on bone-related properties. Conversely, when compared to the PBS group, the BMSC-exo group exhibited a significant decrease in porosity and Euler number (Fig. 2C). Such a reduction in these indices suggests an improvement in the structural integrity and organization of the bone tissue in the BMSC-exo group. These results revealed that BMSC-derived exosome treatment improved the femur phenotype in rats with nonunion fracture models.

3. The quality of the metabolomics analysis data is reliable by analysis of quality control

To explore the effects of BMSC exosomes on the metabolism of rats with nonunion fractures, we performed metabolomics analysis in the serum of rats ($N = 5$). To evaluate the effect of data preprocessing, we analyzed the evaluation map of QC samples. Over 70% of the QC sample peaks were exhibited when the RSD was no more than 30%, indicating satisfactory stability of the instrumental analysis system and validating the data for subsequent analysis (Fig. 3A). The results showed the correlation coefficient of QC samples were higher than 0.9, indicating the experimental repeatability is good (Fig. 3B). The PLS-DA model showed that the QC samples were aggregated and experimental samples were clearly distinguished, suggesting the good sample repeatability (Fig. 3C, D). Venn diagram showed that 1279 common metabolites were detected between the BMSC-exo group and the PBS group, indicating that the two groups had high similarity and stability in basic metabolic processes (Fig. 3E). It turns out that the quality of the metabolomics analysis data is reliable.

4. Differential abundance metabolites mediated by BMSC exosomes

To elaborate on the metabolic effects of BMSC-derived exosomes on fractured rats, DAMs were identified by the OPLS-DA model with the criteria of $P < 0.05$ and VIP > 1.

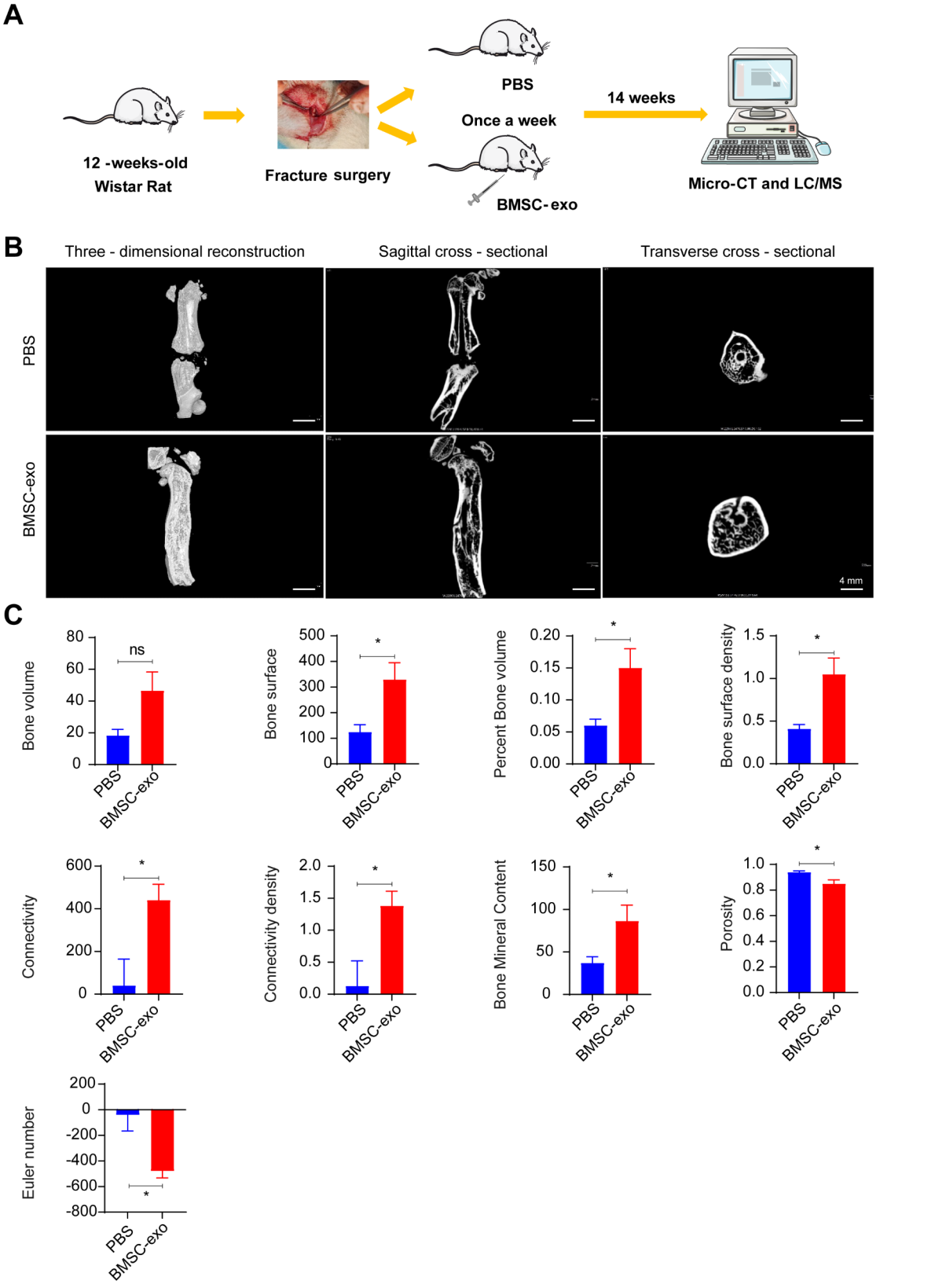

Fig. 2 Micro-CT shows that BMSC exosomes promote bone repair in fracture nonunion rats. (A) The construction of fracture nonunion rat model; (B) Micro-CT image features of PBS group and BMSC-exo group. (C) Statistical analysis of indexes of femur in fractured rats. * $P < 0.05$, *ns*, no significance, $N = 3$, scale bar = 4 mm

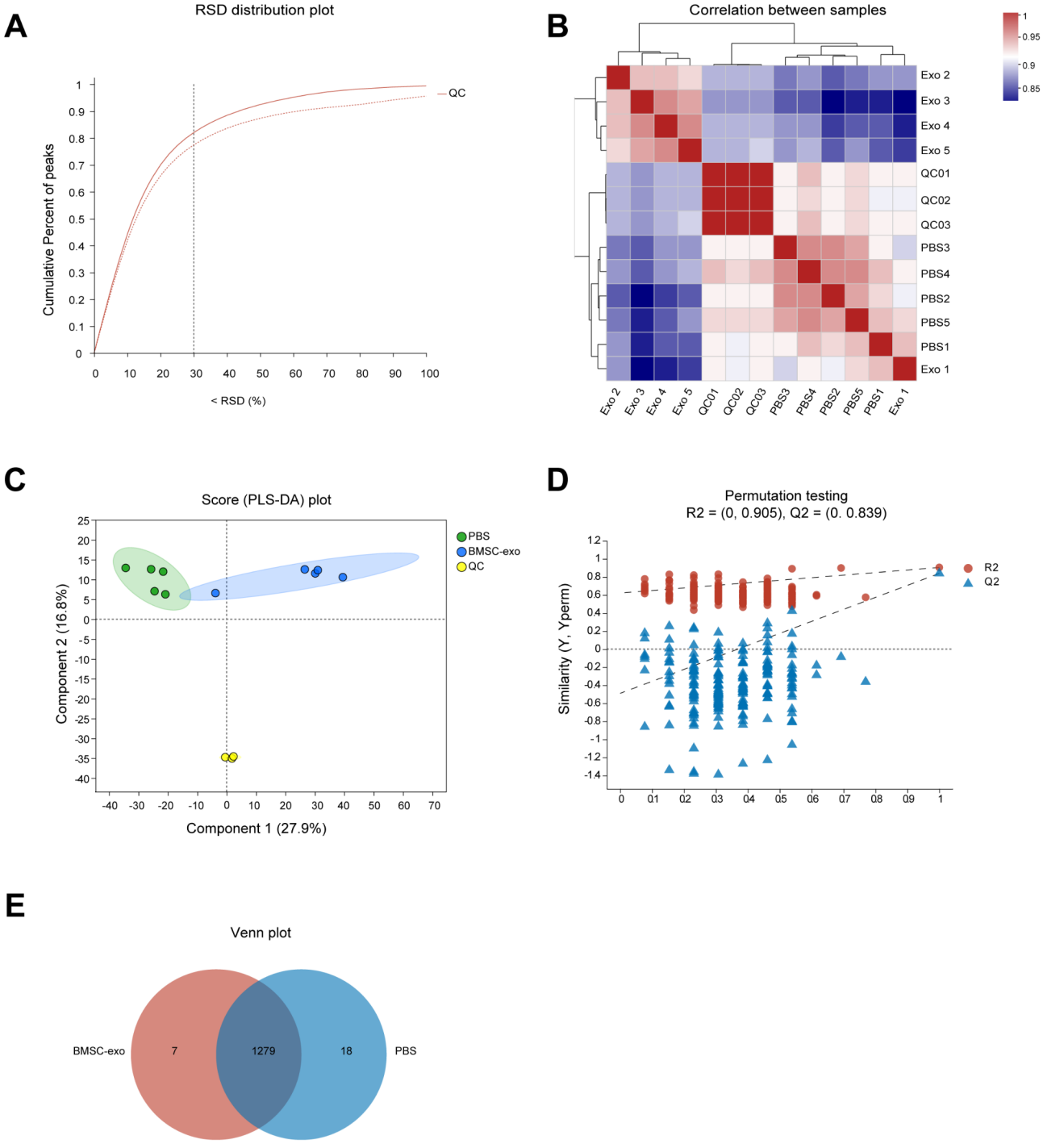

Fig. 3 The quality of the metabolomics analysis data is reliable by analysis of quality control. (A) RSD distribution plot examined by QC; (B) The correlation between samples; (C) PLS-DA score between samples and QC samples. (D) The permutation testing of samples. (E) The Venn plot between BMSC-exo group and PBS group

Compared with the PBS group, a total of 237 metabolites were differential abundance in fracture rats, including 158 up-regulated and 79 down-regulated DAMs were found in the BMSC-exo group in the volcano map (Fig. 4B). The cluster heat map disclosed distinct clustering patterns between the samples. Notably, samples from the BMSC-exo group and the PBS group segregated into distinct major clusters, highlighting significant differences in their metabolite expression profiles (Fig. 4C). The expression profile & VIP of metabolites showed that there were differences in the expression of different metabolites in the two groups, and the VIP values of

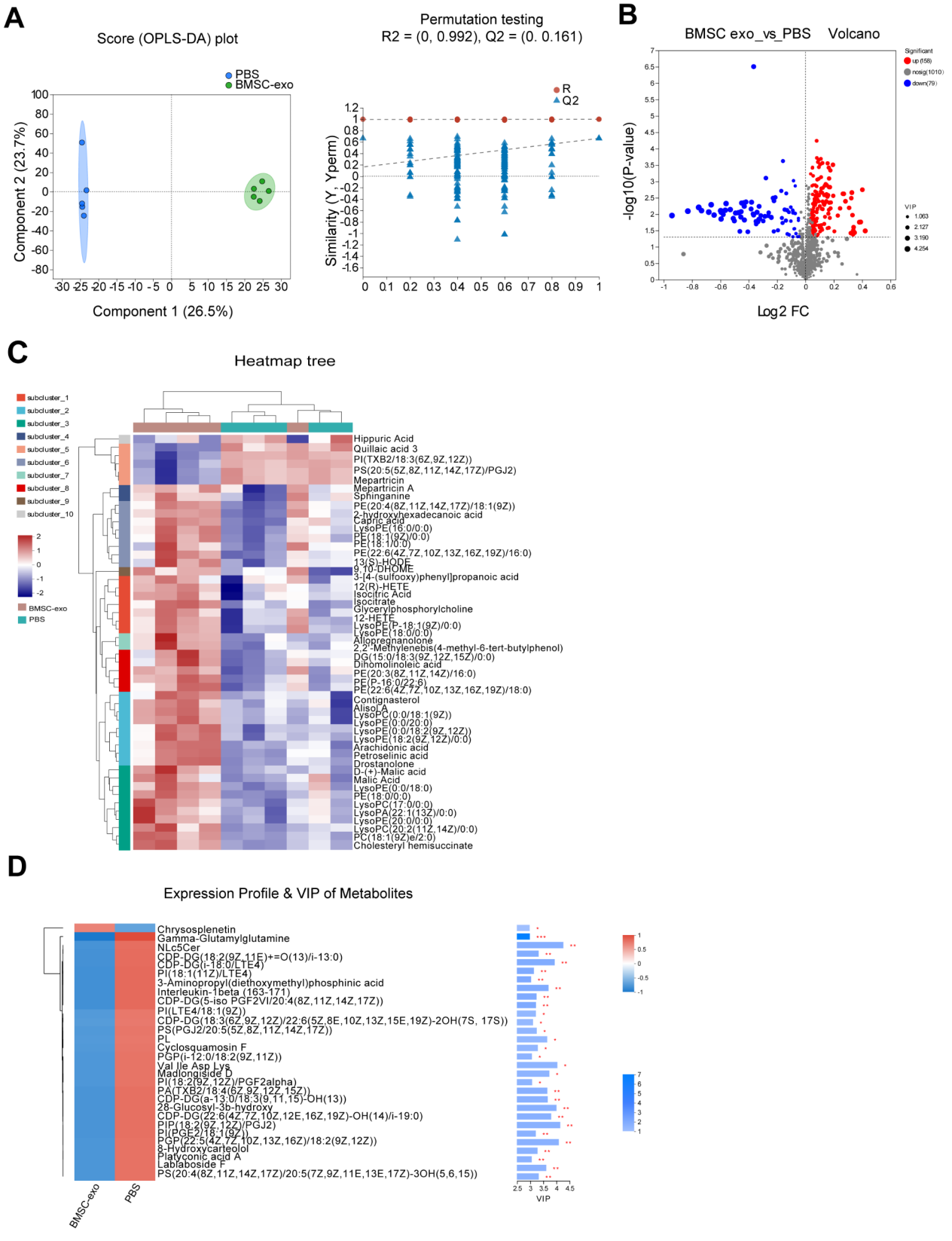

Fig. 4 The differential abundance metabolites mediated by BMSC exosomes. (A) The OPLS-DA score and permutation testing of BMSC-exo group and PBS samples. (B) Volcano map of metabolites. (C) The heat map tree of metabolites in BMSC-exo group and PBS group. (D) The expression profile & VIP metabolites in BMSC-exo group and PBS group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and VIP > 1

chrysosplenetin, γ-glutamylglutamine and NLc5Cer were higher. These reflected that they play a key role in distinguishing between the two groups of samples and were likely to be potential biomarkers (Fig. 4D). These results strongly demonstrated that BMSC exosomes can regulate nonunion fracture by altering serum metabolites in rats.

5. Key metabolic pathways mediated by BMSC exosomes

The 237 metabolites included 21 from secondary metabolism, along with 22 amino acids, peptides, and analogues, 22 glycerophosphoethanolamines, and 21 fatty acids and conjugates (Fig. 5A).To elucidate the biological processes associated with DAMs, we employed KEGG pathway analysis to identify pathways in which DAMs were significantly enriched, thereby establishing a foundation for further identification of these metabolites as potential biomarkers. Notably, pathways such as 'ErbB signaling pathway', 'NF-kappa B signaling pathway', 'Autophagy-other', 'Th1 and Th2 cell differentiation', 'EGFR tyrosine kinase inhibitor resistance', 'Fc-γ-R-mediated phagocytosis', 'PPAR signaling pathway', 'HIF-1 signaling pathway' show relatively high gene ratios and low FDR values, suggesting a significant enrichment of DAMs (Fig. 5B). The aforesaid 'Th1 and Th2 cell differentiation', 'PPAR signaling pathway', 'ErbB signaling pathway', and 'HIF-1 signaling pathway' were related to the function of cell proliferation and differentiation. These pathways may participate in the process of treating nonunion fractures with exosomes. These findings implied that the exosomes might exert their effects by modulating these metabolic and signaling pathways in comparison to the PBS group and provided valuable insights into the potential molecular mechanisms underlying the differences between the two groups.

Discussion

Fracture nonunion is one of the serious complications in the clinical treatment of fracture, and it is also the focus of current research. The increase in traffic accidents, diabetes and senile osteoporosis can lead to the destruction of bone microstructure [31, 32] and also inhibit the process of fracture healing. Therefore, effectively promoting fracture healing is the focus of current research. Stem cell transplantation has become a new insight for the treatment of bone nonunion, and MSCs are favored for their ability to regenerate bone [1, 33]. BMSC transplantation promotes bone and angiogenesis, and its osteogenic mechanism involves the PI3K/Akt pathway [34]. The combination of autologous stem cells and HA granules in the treatment of bone nonunion is limited by immune rejection and malignant transformation [35]. In recent years, a great deal of research on the cure of fracture nonunion has pointed to the field of exosomes with multiple physiological functions.

Exosomes effectively stimulate organ and tissue regeneration by mediating communication between different cells [36, 37]. For example, umbilical cord-derived MSC exosomes improve wound healing and skin regeneration in chronic diabetes via Pluronic F127 [38]. MSC-derived exosomes promote fracture healing in mice model [39], a cocktail therapy containing engineered endothelial cell-derived exosomes promotes fracture repair by mediating the balance of osteoblasts/osteoclasts and M1/M2 macrophages. In our study, we constructed a rat fracture model and injected MSC-derived exosomes into the rat model. Various bone features of rats show that exosome treatment is effective for fracture healing, these results are similar to Jintiange promote osteoporotic fracture healing in aged rats by enhancing bone microstructure [40]. Our findings implied that exosomes derived from BMSCs can promote fracture healing, indicating that they might constitute an efficacious drug for the treatment of non-healing fractures in the clinical setting.

Multiple studies utilizing LC-MS/MS have elucidated the regulatory mechanisms of bone-targeting drugs in modulating fracture healing metabolism. When the fracture area is exposed to inflammatory stimuli, macrophages undergo metabolic reprogramming to promote inflammatory responses by enhancing phagocytosis and cytokine release, and regulating itaconate concentrations in macrophages may be an effective way to mitigate early inflammatory responses in fracture healing [41, 42]. However, we didn't find itaconate in our results which implied that the pathway of exosomes regulating fracture healing may be estranged from the regulation of inflammation, there is a complex interaction between inflammatory response and fracture healing outcome [43]. In our study, we found that differential metabolites are significantly enriched in HIF-1 signaling pathway, HIF-1α exerts regulatory influence on both bone homeostasis and angiogenesis, thereby contributing to the development of bone metabolic disorders [44]. Phosphatidylethanolamine (PE) is a class of Glycerophospholipid (PL), PLs are involved in many important human activities, including cell integrity, proliferation and differentiation [45, 46]. Therefore, BMSC-exosomes are thought to influence the HIF-1 signaling pathway by modulating changes in DAMs-PE, thereby promoting bone regeneration.

Conclusion

Our study suggests that exosomes derived from BMSCs can effectively alleviate fracture-nonunion in rats. BMSC-exo changes the metabolic components of serum in rats without fracture healing, especially BMSC-exo-mediated the regulation of HIF-1 metabolic pathway. These data lay a foundation for further clinical application and drug research of fracture nonunion.

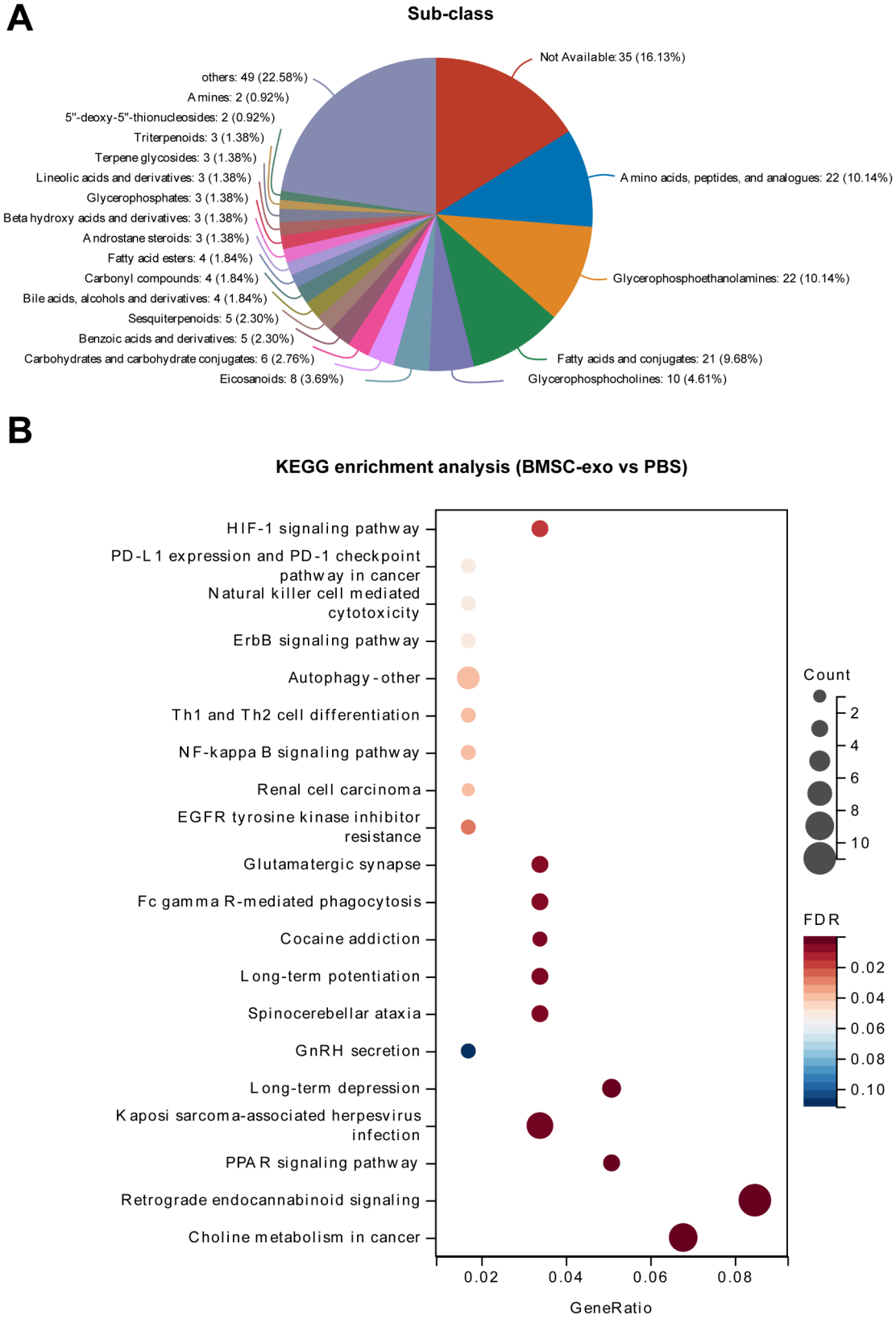

Fig. 5 Key metabolic pathways mediated by BMSC exosomes. (A) The sub-class of differential abundance metabolites in BMSC-exo group and PBS group. (B) KEGG enrichment analysis in BMSC-exo group and PBS group

Abbreviations

BMSC	Bone marrow mesenchymal stem cell
DAMs	Differential abundance metabolites
MSC	Mesenchymal stem cells
FBS	Fetal bovine serum
PE	Phosphatidylethanolamine
PL	Glycerophospholipid

Supplementary information

The online version contains supplementary material available at https://doi.org/10.1186/s13018-025-05721-3.

Supplementary Material 1

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Not applicable.

Author contributions
Cheng Li: Conceptualization, Methodology, Software, Writing- Original draft preparation; Ming Chen: Conceptualization, Methodology, Software, Writing-Original draft preparation; Lijun Guo: Data curation, Writing- Original draft preparation; Dadong Yu: Data curation, Writing- Original draft preparation; Zhonghai Xu: Investigation, and Writing- Reviewing and Editing; Bin Chen: Supervision, Validation, and Writing- Reviewing and Editing; Zhijian Xiao: Conceptualization, and Writing- Reviewing and Editing. All authors reviewed the manuscript.

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Data availability
Data is available from the corresponding author.

Declarations

Ethical approval
All rats' experimental operations were approved by the Medical Ethics Committee of Hangzhou Fuyang Hospital of TCM Orthopedics and Traumatology.

Competing interests
The authors have no relevant financial or non-financial interests to disclose.

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