Untargeted Metabolomics Reveal the Protective Effect of Bone Marrow Mesenchymal Stem Cell Transplantation Against Ovariectomy-Induced Osteoporosis in Mice

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Abstract
Bone marrow mesenchymal stem cell transplantation (BMSCT) is a potential treatment for osteoporosis, capable of contributing to bone tissue repair. BMSCT has demonstrated osteoinductive effects and the ability to regulate microenvironmental metabolism; however, its role and mechanisms in bone loss due to reduced estrogen levels remain unclear. In this study, the effect of BMSCT on ovariectomy (OVX)-induced osteoporosis in mice was assessed, and liquid chromatography–mass spectrometry (LC-MS) metabolomic studies of bone tissue were conducted to identify potential metabolic molecular markers. The results revealed that BMSCT reduces OVX-induced bone loss in mice while improving the mechanical properties of mouse femurs and increasing the expression of osteogenic markers in peripheral blood. In a metabolomic study, 18 metabolites were screened as potential biomarkers of the anti-osteoporotic effect of BMSCT. These metabolites are mainly involved in arachidonic acid metabolism, taurine and hypotaurine metabolism, and pentose and glucuronate interconversions. Collectively, these results illustrate the correlation between metabolites and the underlying mechanisms of osteoporosis development and are important for understanding the role and mechanisms of exogenous bone marrow mesenchymal stem cells (BMSCs) in osteoporosis management. This study lays the foundation for research on BMSCs as a treatment strategy for osteoporosis.

Keywords
bone marrow mesenchymal stem cells, ovariectomy-induced osteoporosis, cell therapy, metabolomics, biomarkers

Introduction
Osteoporosis is a metabolic systemic disease characterized by a decrease in bone mass and degeneration of the bone tissue microarchitecture1. With the increasing aging of the population in some regions, fractures caused by osteoporosis are extremely common and seriously affect the quality of life of the elderly2,3. The occurrence of osteoporosis was significantly correlated with sex. Women’s bones are affected by postmenopausal decline in estrogen and loss of bone mass at a faster rate, putting women at five times the risk of osteoporosis than men4. Therefore, exploring better methods for early prevention of bone loss in osteoporosis can have a positive effect on reducing complications such as fragility fractures, bone pain, and skeletal degeneration.

Recently, stem cell–based infusion therapy is becoming increasingly important in chronic and systemic diseases. Transplanted cells can respond to environmental signals from the lesion by secreting biologic factors and pro-regenerative pathways, even overcoming the deleterious effects of inflammation5,6. Mesenchymal stem cell therapy...
has been used to manage several diseases, including cardiac ischemia, osteoarthritis, cerebral infarction, and ischemic brain. It has been demonstrated that bone marrow mesenchymal stem cells (BMSCs) have marked osteogenic and immunomodulatory properties and are efficacious in the treatment of osteoporosis. BMSCs may be directly involved in tissue repair. They can also directly release factors or proteins, biologically active lipids, microRNA exosomes, and other substances through a paracrine mechanism to regulate several signaling pathways. However, the mechanisms underlying the biological effects of BMSCs on osteogenesis and osteolysis during bone repair in osteoporosis remain unclear.

Metabolomics allows the establishment of the relationship between changes in metabolite levels and biological phenotypes, thus enabling the exploration of the changes in all metabolites in living organisms during pathophysiological processes. Metabolomics studies have extensively used multivariate analysis methods such as data processing and analytical techniques such as principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), and orthogonal partial least squares discriminant analysis (OPLS-DA). In this study, we focused on the phenotypic alterations resulting from exogenous BMSC infusion in mice with osteoporosis and used liquid chromatography–mass spectrometry (LC-MS) techniques to analyze all metabolites within the bone. This study reveals specific metabolic pathways in the stem cell treatment of postmenopausal osteoporosis and explores potential molecular markers.

**Materials and Methods**

**Isolation and Identification of Primary BMSCs in Mice**

Bilateral femurs, tibiae, and fibulae of C57BL/6 female mice (Department of Experimental Animal Science, Kunming Medical University, 12-week-old, weight 24 ± 3 g) were obtained after cervical dislocation and immersed in 75% alcohol for 5 min. The bone marrow cavity was repetitively flushed with sterile phosphate-buffered saline (PBS), filtered using a cell filter and centrifuged (1,000 × g, 10 min). We added low-glucose GlutaMAX™ Supplement to Dulbecco’s modified Eagle medium’s (Gibco, Grand Island, NY, USA) containing 15% fetal bovine serum, 100 U/ml penicillin, and 100 g/ml streptomycin to the obtained cell precipitates and cultured them at 37°C in a 5% CO2 incubator (Thermo Fisher, Waltham, MA, USA). Cells were cultured by changing the culture medium every 2–3 days. The cells were digested with TrypLE™ Express Enzyme (12604021; Gibco) for passaging when they reached more than 90% of wall attachment. Differentiation induction was performed using BMSCs with stable passaged P3–P5 in osteogenic induction medium (Cyagen, Guangzhou, China), lipogenic induction medium (Cyagen), and chondrogenic induction medium (Cyagen), according to the induction medium. The cell differentiation ability was evaluated according to the time and steps described in the induction medium.

We used flow cytometry to characterize the BMSCs. Cells within five generations were digested and blown into individual cells, and resuspended cells were aspirated and added to CD16/CD32 (553142, 1 μg per 10⁶ cells in 100 μl volume; BD, San Jose, CA, USA) for containment and then centrifuged. For 10⁶ cells in 100 μl volume, they were incubated with anti-CD29 APC (Biolegend, San Diego, CA, USA), anti-CD44 Pacific Blue (10320; BioLegend), anti-CD45 PE/Cyanine5 (Biolegend), and anti-CD117 Alexa Fluor 700 (Biolegend) for 30 min, washed with PBS, resuspended, and detected using flow cytometry (BD) within 1 h. The above operations were performed under light-proof conditions. Data were analyzed using the FlowJo software (version V10.0).

**Ovariectomy (OVX) in Mice**

All experiments were approved by the Animal Experimentation Ethics Review Committee of Kunming Medical University (kmmu202111287) and conducted in accordance with the guidelines of the Animal Experimentation Ethics Review Committee of Kunming Medical University. The mouse ovariectomized model was referenced to the previous studies. C57BL/6 mice were purchased from the Experimental Animal Center of Kunming Medical University. Inclusion criteria: 12-week-old C57BL/6 female mice (weight 24 ± 3 g, well-proportioned and viable individuals). Exclusion criteria: male, non-12-week-old, 12-week-old substandard weight, or pregnant female mice. Withdrawal criteria: poor condition after OVX or bone marrow mesenchymal stem cell transplantation (BMSCT), dead mice. Mice were housed under standard conditions (12 h of light and 12 h of darkness, temperature 18–22°C, humidity 55% ± 5%). They were fed a standard mouse diet and drank water ad libitum.

We numbered the mice in order of their weight and randomly assigned them into three groups using a randomization tool: sham, OVX, and BMSCT groups, 12 mice in each group. Mice were anesthetized intraperitoneally with 3% pentobarbital sodium (Merck, Darmstadt, Germany). In a model with general anesthesia, the mice were removed from the dorsal hair and fixed in prone position on a sterile animal operating table. Through the bilateral dorsal entrances, the bilateral ovaries, the ovarian capsule, and part of the fallopian tubes including the tissue were removed, sutured, and re-sterilized. The surgical procedure and treatment of the sham-operated group were the same as those of the OVX group, but only the ovaries were exposed and a small amount of fat mass near the ovaries was removed. The mice were placed on a small animal warming blanket during and after surgery until they awoke. After the operation, the animals received intraperitoneal injection of carprofen (5 mg/kg, Merck, Germany) for 3 days as a postoperative pain reliever.

On postoperative days 7 and 21, the mice in the BMSCT...
group were infused with $1 \times 10^6$ BMSCs per mouse via the tail vein, whereas the sham and OVX group mice were infused with 200 μl of PBS. On day 35 after surgery, all mice were sacrificed by cervical dislocation to draw the heart blood and obtain serum. Bilateral femurs were collected from mice.

Immunosuppressants were not used in this experiment, mainly for the following reasons. First, it has been reported that BMSCs are low immunogenic stem cells and also that they are immune evasive18. Second, in studies of allogeneic BMSCs in osteoporosis16,17, immunosuppression was not used. Immunosuppression was also not used in some clinical trials with allogeneic BMSCs19,20. Third, immunosuppressants (eg, cyclosporine and cyclophosphamide) can lead to an impact on this study and the role of BMSCs could not be determined. Fourth, the use of immunosuppressants increases the group setting and also leads to changes in metabolite production. This does not facilitate our discovery of specific metabolites.

**Micro-CT for Bone Mass Detection**

The NEMO Small Animal High-Resolution Imaging Computed Tomography (CT) System (Pingseng Medical, Kunshan, Jiangsu Province, China) was used to scan and analyze the mouse femurs. The CT reconstruction algorithm was FDK, the CT field of view was 15 mm, the pixel size was 0.0146 mm, and the slice thickness was 0.025 mm. After reconstruction of the scanned data, regions of interest were selected for analysis of bone trabeculae and bone cortex. The analysis was performed using the Avatar3 software. The proximal femoral growth plate was used as a reference point, and the metaphysis region was selected. Bone volumetric density (BV/TV), number (N), thickness (Th), and bone mineral density (BMD) of the trabeculae were measured. The middle femur was then selected, and BMD, Th, bone volume (BV), and bone area to total area ratio (Ar/Tt.Ar) of the femoral cortical bone (Ct) were measured.

**Mechanical Testing of the Femur**

Femurs of mice were placed on a universal mechanical testing machine (HY-0230; Hengyi, Shanghai, China) to measure the biomechanical properties of femurs. The parameters were set as follows: the diameter of the indenter was 5 mm, the loading speed was 2 mm/min, and the span was 10 mm. The acquisition computer records the elastic load, maximum displacement, breaking load, and stiffness.

**Serological ELISA**

The concentrations of estradiol, alkaline phosphatase (ALP), osteoprotegerin (OPG), pyridinoline (PYD), and cross-linked N-telopeptide of type I collagen (NTXI) in mouse serum were measured using commercial ELISA kits (Jiancheng, Nanjing, Jiangsu Province, China). All assays and data processing were performed according to the manufacturer’s instructions.

**Pathological Examination Staining of Femurs**

Mouse femurs were fixed with 4% paraformaldehyde and decalcified by shaking with 0.5 M EDTA (Solarbio, Beijing, China). All sections were performed in the sagittal position. A portion of the decalcified femur was gradient dehydrated, transparent in xylene, and sealed with a neutral resin. An additional portion of the decalcified femur was immersed in 20% sucrose for 24 h. The bone tissue was embedded in optimal cutting temperature (OCT) (Sakura, Columbus, OH, USA) and 20 μm sections were prepared using a frozen section machine (Leica, Wetzlar, Germany). The sections were sealed after staining using Oil Red O staining kit (Solarbio, Beijing, China). The staining results were observed and photographed under a light microscope (Olympus, Tokyo, Japan). New bone surface area (Nb.S), fat surface area (Fat.S), and osteoclast surface (Oc.S) in bone surface (BS) were analyzed using ImageJ software (version 2.0).

**Untargeted Metabolomic Assay of LC-MS in Mouse Femur**

The mouse femoral tissue was added to the tissue extract (75% 9:1 methanol:chloroform, 25% H₂O) for grinding (55 Hz, 60 s). After centrifuging (12,000 × g, 4°C, 10 min) and drying, 50% acetonitrile solution was added to reconstitute the sample and filter it through a 0.22-μm membrane. Liquid chromatography detection was performed using an ACQUITY UPLC® HSS T3 column (2.1 × 150 mm, 1.8 μm; Thermo Ultimate 3000, Waltham, MA, USA). The autosampler temperature was set at 8°C, the column temperature was 40°C, the flow rate was 0.25 ml/min, and the injection volume was 2 μl. The mobile phase was 0.1% formic acid water–0.1% formic acid acetonitrile and negative ion was 5 mM ammonium formate water–acetonitrile.

Mass spectrometry was performed using a Q Exactive Plus (Thermo Fisher, Waltham, MA, USA) with an electrospray ionization source, positive ion spray voltage of 3.50 kV and negative ion spray voltage of 2.50 kV, sheath gas of 30 arb, auxiliary gas of 10 arb, and capillary temperature of 325°C. Based on the mass spectrometer performance specifications, a full scan with a resolution of 70,000 was chosen to be able to separate two adjacent mass components. A scan range of 81–1,000 and a secondary cleavage using high-energy collisional dissociation with a collision voltage of 30 eV were used. Unnecessary MS/MS information was removed using dynamic exclusion, where duplicate data are automatically removed.
Data Processing and Statistical Analyses

The GraphPad Prism software v9.0 (La Jolla, CA, USA) was used for data analysis and visualization. The normality of the data was judged using the Shapiro–Wilk test and P-P plots and Q-Q plots, and data that conformed to a normal distribution were expressed as mean ± standard deviation (SD). For data conforming to normal distribution and homogeneity, one-way analysis of variance (ANOVA) was used to compare the overall mean difference between multiple groups of data, and statistically different data were then tested with least-significant difference (LSD) test for differences between the two groups. For data that did not conform to a normal distribution, we used the Kruskal–Wallis test to compare the statistical significance of the groups. If P < 0.05 indicated the difference to be statistically significant.

Metabolomics raw data were converted into mzXML format using the Proteowizard software v3.0.8789 (Palo Alto, CA, USA). The XCMS package of the R programming language v3.3.2 was used for peak identification, filtration, and alignment. The main parameters are bw = 2, ppm = 15, peakwidth = c (5, 30), mzwid = 0.015, mzdiff = 0.01, and method = centWave. A data matrix, including the mass to charge ratio, retention time, and intensity, was obtained. A total of 7,821 precursor molecules were obtained in positive ion mode and 9,229 in negative ion mode. Batch normalization of peak areas was performed to compare data of different magnitudes. PCA, PLS-DA, and OPLS-DA were performed on the data. The PLS-DA model was evaluated based on the goodness of fit (R²) and cumulative goodness of prediction (Q²) values and cross-validation of permutation tests. Pathway analysis of potential biomarkers was performed using MetaboAnalyst (http://www.metaboanalyst.ca/) and the KEGG pathway database (http://www.genome.jp/kegg/).

Results

Characterization and Identification of Mouse BMSCs

Primary BMSCs were acquired from the femoral bone marrow of C57BL/6 mice (Fig. 1A). The obtained primary BMSCs exhibited stemness with multidirectional differentiation (Fig. 1B). Flow cytometry analysis indicated that the extracted cells highly expressed CD29 and CD44 and marginally expressed CD45 and CD117 (Fig. 1C). These features conformed to the features of the BMSCs.

Effect of BMSCT on Serum Biochemical Parameters and Mechanical Property of Bone

Serum estrogen, osteogenic, and osteolytic activity markers were measured on day 35 (Table 1). The serum markers of osteogenic activity, namely, alkaline phosphatase (ALP) (P = 0.0004) and OPG (P = 0.0126), were significantly decreased after OVX and significantly increased after BMSCT (P = 0.0165, P = 0.0173). Serum markers of osteolytic activity—NTXI and PYD—were significantly elevated after OVX (P = 0.0001, P = 0.0013) and significantly decreased after BMSCT (P = 0.0018, P = 0.0319). Serum estrogen levels decreased significantly after OVX (P = 0.0022). Moreover, compared with the OVX group, BMSCT increased the elastic load, breaking load, and stiffness of the femur (P = 0.0005, P = 0.0424, P = 0.0212) and decreased the maximum displacement (P = 0.0004).

BMSCT Promoted Bone Structural Remodeling and Reduced Marrow Cavity Steatosis

Masson and Oil Red O staining of bone tissue allowed us to evaluate changes in bone morphology and lipid metabolism after BMSCT (Fig. 2A, B). OVX resulted in reduced new bone formation near the femoral growth plate (P < 0.0001), whereas the BMSCT group demonstrated increased bone and collagen maturation compared with that in the OVX group (P = 0.0325). Oil Red O staining showed that the OVX group had a large amount of red-stained fat particles deposited in the femoral marrow cavity compared with the sham group (P < 0.0001); however, fat deposition in the femoral bone was significantly reduced after BMSCT (P = 0.0097). Trap staining of femur sections was performed to calculate the osteoclast surface per bone surface (Oc. S/BS) (Fig. 2A, B). Oc. S/BS demonstrated that OVX could increase bone resorption (P = 0.0011), but the process was inhibited by BMSCT (P = 0.0039).

BMSCT Improved BMD in Mice

Distal femoral trabeculae and cortical bone were reconstructed in three dimensions (3D) and quantitatively analyzed (Fig. 2C, D). The results of the quantitative analysis showed that the Tb.BV/TV (P = 0.0152), Tb.N (P = 0.0089), Tb.BMD (P = 0.0086), Ct.BM (P = 0.0297), Ct.BV (P = 0.0096), Ct.Th (P = 0.0451), and Ct.Ar/Tt.Ar of femoral tissue were significantly higher in the BMSCT group than in the OVX group (BV/TV (P = 0.026), Tb.N (P = 0.0205), Tb.BMD (P = 0.0298), Ct.BM (P = 0.043), Ct.BV (P = 0.0413), and Ct.Th (P = 0.0243)).

Metabolomic Analysis of Mouse Femurs

Twelve independent femur tissue samples from each group were fully scanned for positive and negative ions using LC-MS under optimal conditions. One QC sample was interspersed with every 10 samples. Good reproducibility of the LC-MS was observed from the typical base peak intensity chromatograms of the sham, OVX, and BMSCT groups (Fig. 3A). The PCA results showed significant differences in the variation between the groups (Fig. 3B). The effect of metabolite patterns on the femur after BMSCT was investigated using PLS-DA and OPLS-DA models. The results showed that R²X, R²Y, and
Figure 1. Culture and phenotype identification of bone marrow mesenchymal stem cells. (A) Morphology of C57BL/6 mouse primary bone marrow mesenchymal stem cells on days 1, 4, and 10 under an inverted microscope. (B) The staining results of osteogenic, adipogenic, and chondrogenic induction of bone marrow mesenchymal stem cells. (C) Flow cytometry analysis of the expression of primary mouse bone marrow mesenchymal stem cells (CD29, CD44, CD117, CD45, and CD117).

Table 1. Serum Biochemical Marker Parameters and Bone Mechanical Properties.

| Parameters        | Unit  | Sham         | OVX          | BMSCT         |
|-------------------|-------|--------------|--------------|---------------|
| Serum             |       |              |              |               |
| ALP               | µg/ml | 17.69 ± 3.22 | 12.03 ± 3.51*** | 16.61 ± 4.53# |
| OPG               | nmol/ml | 58.76 ± 14.25 | 44.97 ± 9.73** | 58.12 ± 14.35# |
| NTXI              | nmol/ml | 56.42 ± 26.15 | 109.8 ± 28.06*** | 71.16 ± 23.94### |
| PYD               | nmol/ml | 10.43 ± 4.57 | 20.45 ± 9.99** | 12.97 ± 5.19# |
| Estradiol E2      | nmol/ml | 50.32 ± 15.63 | 33.22 ± 10.12** | 34.15 ± 11.96 |
| Biomechanical properties |       |              |              |               |
| Elastic load      | N     | 12.88 ± 3.01 | 7.57 ± 2.49*** | 11.94 ± 2.66### |
| Maximum displacement | mm   | 0.19 ± 0.02 | 0.25 ± 0.03*** | 0.2 ± 0.02### |
| Breaking load     | N     | 16.92 ± 3.23 | 11.92 ± 3.06*** | 14.92 ± 3.37# |
| Stiffness         | N/mm  | 77.94 ± 12.44 | 52.17 ± 12.61### | 66.67 ± 12.20# |

Results are expressed as mean ± SD. ALP: alkaline phosphatase; BMSCT: bone marrow mesenchymal stem cell transplantation; NTXI: N-telopeptide of type I collagen; OPG: osteoprotegerin; OVX: ovariectomy; PYD: pyridinoline.

*P < 0.05, **P < 0.01, ***P < 0.001 vs Sham; #P < 0.05, ##P < 0.01, ###P < 0.001 vs OVX.
had positive modes of 0.397, 0.984, and 0.841 and negative modes of 0.294, 0.925, and 0.787, respectively, in the PLS-DA model score plot (Fig. 3C). In the OPLS-DA model, $R^2$, $Q^2$, and $R^2$ were 0.312, 0.988, and 0.864 in the positive mode and 0.281, 0.989, and 0.865 in the negative mode, respectively (Fig. 3D). In the PLS-DA replacement test positive mode, $R^2 = (0.0, 0.93)$, $Q^2 = (0.0, -0.14)$; in the negative mode, $R^2 = (0.0, 0.69)$, $Q^2 = (0.0, -0.51)$ (Fig. 3E). These results implied that the PLS-DA and OPLS-DA models were of high quality.

Identification of Potential Intraosseous Biomarkers in Mice After BMSCT

In the OPLS-DA model, variables with Variable Importance in Projection (VIP) values higher than 1.0 and $P$ values < 0.05 were selected, and the independent Student $t$ test between the two groups was used. Metabolites were initially identified based on the accurate quality provided by the HMDB (https://hmdb.ca/), METLIN (https://metlin.scripps.edu), LIPID MAPS (https://lipidmaps.org), and KEGG databases and validated using MS/MS fragment ion information. Finally, 18 metabolites with different abundances were identified. Basic information on the screened potential biomarkers, the fold change (FC), and $P$ value of the biologically corresponding pathways are presented in Table 2.

Box plots were used for differences in the levels of 18 potential biomarkers screened between the sham, OVX, and BMSCT groups (Fig. 4A). The results showed that the ability to promote 3-hydroxyanthranilic acid, l-dopa, d-xylitol, 5-l-glutamyl-taurine, citric acid, melphalan, phenylpropanoate, and prostaglandin after BMSCT. H2, lithocholic acid, prephenate, uridine, thioguanine, d-alanyl-d-serine, uracil 5-carboxylate, and diaminopimelic acid were increased in bone tissue, leading to reduced levels of acetylcholine chloride, lipoxin B4, and prostaglandin I2. Potential biomarkers of the anti-osteoporotic effect of BMSCT were mapped using an OPLS-DA-based ROC curve (Fig. 4B). The 18 biomarkers shown in the figure had high-sensitivity area under the curve (AUC) values (> 0.80), indicating that they can be used as potential bioactive targets and biomarkers in bone for the anti-osteoporotic effect of BMSCT.
Biological Pathway and Functional Analysis of Metabolites

The biomarkers of the BMSCT group were similar to those of the sham-operated group in the heat map (Fig. 5A). By contrast, there was a significant difference between the OVX and BMSCT groups, indicating that BMSCT significantly improved the metabolic status in the bone tissue of osteoporotic mice. Pathway and enrichment analyses of 18 important biomarkers were performed using the MetaboAnalyst 5.0. Arachidonic acid metabolism, taurine and hypotaurine metabolism, and pentose and glucuronate interconversions were identified based on pathway topology analysis calculations as the three most important metabolic pathways affected by BMSCT after treatment (Fig. 5B, results are shown in Supplemental Table 1). Enrichment analysis of metabolites showed that taurine and hypotaurine metabolism, catecholamine biosynthesis, transfer of acetyl groups into mitochondria, arachidonic acid metabolism, and phospholipid biosynthesis were the top five metabolite concentration sets altered by BMSCT (Fig. 5C). The above results suggest that the metabolism of amino acids and lipids are key factors involved in bone protection and prevention of osteoporosis using BMSCs.

Discussion

Postmenopausal osteoporosis is one of the most common types of primary osteoporosis. BMSCs have multiple differentiation potentials, such as osteogenesis, chondrogenesis, and vascularization; thus, they are widely used in bone repair and bone regeneration. Although exogenous BMSC infusion can promote osteogenesis in vivo and has potential therapeutic effects in osteoporosis, the pathway and mechanism of action remain unknown. Our results showed that BMSCT prevented osteoporosis by decreasing estrogen...
Table 2. Identified Potential Biomarkers, FC, and \( P \) Values Among Sham, OVX, and BMSCT Groups.

| No. | Metabolite                  | Retention time (min) | Adducts         | Theoretical \( m/z \) | Mass error (ppm) | OVX vs Sham FC | \( P \) values | BMSCT vs OVX FC | \( P \) values | BMSCT vs Sham FC | \( P \) values |
|-----|-----------------------------|----------------------|-----------------|------------------------|------------------|----------------|---------------|----------------|---------------|----------------|---------------|
| 1   | 3-Hydroxyanthranilic acid   | 183.30               | \([M+H]^+\)     | 154.05                 | 9.05             | 0.09           | 0.000         | 0.25           | 0.000         | 0.37           | 0.000         |
| 2   | L-Dopa                     | 93.52                | \([M]^-\)       | 197.07                 | 11.21            | 0.30           | 0.000         | 0.56           | 0.000         | 0.53           | 0.000         |
| 3   | D-Xylitol                  | 93.36                | \([M-H]^-\)     | 151.06                 | 3.44             | 0.25           | 0.000         | 0.51           | 0.000         | 0.50           | 0.000         |
| 4   | 5-O-Glutamyl-taurine       | 96.61                | \([M+H]^+\)     | 255.06                 | 1.82             | 0.41           | 0.000         | 0.60           | 0.000         | 0.69           | 0.000         |
| 5   | Citric acid                | 196.91               | \([M+H-H_2O]^+\) | 175.02                 | 17.78            | 0.70           | 0.017         | 0.60           | 0.000         | 1.17           | 0.012         |
| 6   | Melphalan                  | 327.43               | \([M+H]^+\)     | 305.09                 | 0.23             | 0.05           | 0.000         | 0.13           | 0.000         | 0.36           | 0.001         |
| 7   | Phenylpropanoate           | 478.96               | \([M-H]^-\)     | 149.06                 | 6.39             | 0.11           | 0.000         | 0.14           | 0.000         | 0.77           | 0.002         |
| 8   | Prostaglandin H2           | 586.68               | \([M-H]^-\)     | 351.22                 | 0.45             | 0.04           | 0.000         | 0.12           | 0.002         | 0.36           | 0.010         |
| 9   | Acetylcholine chloride     | 126.09               | \([M-H]^-\)     | 179.99                 | 1.01             | 2.70           | 0.000         | 1.85           | 0.010         | 1.46           | 0.026         |
| 10  | Lithocholic acid           | 824.31               | \([M]^+\)       | 376.31                 | 2.31             | 0.08           | 0.000         | 0.15           | 0.000         | 0.50           | 0.019         |
| 11  | Prephenate                 | 177.83               | \([M]^+\)       | 226.07                 | 0.49             | 0.04           | 0.000         | 0.15           | 0.000         | 0.29           | 0.002         |
| 12  | Lipoxin B4                 | 666.41               | \([M-H]^-\)     | 351.22                 | 0.56             | 3.78           | 0.000         | 1.52           | 0.004         | 2.49           | 0.000         |
| 13  | Prostaglandin I2           | 618.69               | \([M+H-H_2O]^+\) | 335.22                 | 1.10             | 0.00           | 0.000         | 0.41           | 0.017         | 0.50           | 0.045         |
| 14  | Uridine                    | 127.56               | \([M-H]^-\)     | 243.06                 | 1.40             | 0.49           | 0.004         | 0.47           | 0.000         | 1.27           | 0.035         |
| 15  | Thioguanine                | 98.08                | \([M-H]^-\)     | 166.02                 | 9.45             | 0.39           | 0.000         | 0.59           | 0.005         | 0.67           | 0.002         |
| 16  | D-Alanyl-d-serine          | 370.85               | \([M]^+\)       | 176.07                 | 2.76             | 0.21           | 0.000         | 0.42           | 0.002         | 0.50           | 0.002         |
| 17  | Uracil 5-carboxylate       | 71.90                | \([M+H]^+\)     | 156.97                 | 0.38             | 0.20           | 0.000         | 0.37           | 0.006         | 0.54           | 0.002         |
| 18  | Diaminopimelic acid        | 189.09               | \([M-H]^-\)     | 189.09                 | 1.07             | 0.51           | 0.000         | 0.73           | 0.005         | 0.72           | 0.001         |

FC represents the change in multiplicity between the two groups. FC with a value greater than zero indicates a high intensity of the bone metabolite, whereas an FC value less than zero indicates a low intensity of the bone metabolite. \( P \) values were calculated using one-way ANOVA. BMSCT: bone marrow mesenchymal stem cell transplantation; FC: fold changes; OVX: ovariectomy.
content and increasing Tb.BV/TV, Tb.N, Tb.BMD, Ct.BMD, Ct.BV, and Ct.Th in the femur of mice, which is consistent with previous reports. Although the systemic distribution after exogenous BMSCT and the mechanisms of action on target organs remain poorly understood, the regulatory effects of BMSC
on bone tissue have been more well established. Studies have shown that after intravenous infusion, BMSCs are rapidly cleared from the circulation and most of the infused cells are sequestered in the lungs within minutes. A few minutes after infusion, BMSCs are released back into the circulation and can be found in the liver, spleen, kidney, and heart minutes to days after infusion. BMSC then leaves the bloodstream, crosses the endothelial barrier, and reaches the site of the lesion where it plays an important role. It has been shown that homozygous BMSC can home into the bone to participate in repair. The regulation of the intraosseous microenvironment by exogenous BMSCs may be mediated through the inhibition of inflammation and induction of T cell apoptosis. Differences in energy metabolism and antioxidant defense systems of BMSCs from different sources have also been reported to influence the efficacy of cell therapy in osteoporosis treatment. BMSCs can also be involved in systemic or intraosseous immune regulation, angiogenesis, and inflammatory responses through the secretion of soluble paracrine factors, such as transforming growth factor-β (TGF-β), prostaglandin E2 (PGE2), and vascular endothelial growth factor (VEGF). We showed the ability of BMSC to elevate osteogenic marker levels and decrease osteoclastic marker levels in serum and to increase

**Figure 5.** Heat map of biomarkers and MetaboAnalyst pathway enrichment analysis. (A) Heat map analysis of biomarkers with increased and decreased levels indicated in red and green, respectively. (B) Metabolic pathway impact analysis. (C) Overview of the metabolite enrichment. BMSCT: bone marrow mesenchymal stem cell transplantation; OVX: ovariectomy; TCA: tricarboxylic acid.
the elastic load, breaking load, and stiffness of the femur and decrease maximum displacement. These results demonstrate the significant osteogenic and potential anti-fracture effects of BMSCT infusion. BMSCT promotes bone and collagen maturation while preventing intramedullary steatosis in the bone due to decreased estrogen levels. It has been shown that increased adipose tissue in the marrow cavity is accompanied by a significant decrease in BMD and an increased risk of osteoporotic fractures in both humans and rodents. This suggests that exogenous BMSC infusion may mediate the metabolism of lipogenesis and osteogenesis in bone, further acting as an anti-osteoporotic agent.

Existing metabolomics studies on osteoporosis are more often using urine or plasma samples to screen for specific metabolic biomarkers. Zhao et al screened 46 metabolites in the serum of the O VX rat model, involving various pathways such as linoleic acid metabolism, arachidonic acid metabolism, and glycerophospholipid metabolism. Luo et al identified serum sphingosine 1-phosphate, LPA (16:0) and arachidonic acid as key biomarkers in a mouse model of aging-induced primary osteoporosis. However, urine or blood is non-organ-specific and reflects many biochemical processes that occur in various tissues in the body. Metabolic analysis of tissue specimens, better reflects the organ dysfunctional processes of a specific disease. Therefore, the use of bone tissue to study osteoporosis provides a more realistic response to biological processes within the bone. Here, we performed LC-MS untargeted metabolomic assays against femoral tissue in a mouse osteoporosis model and screened 18 key metabolites. Moreover, the effect of BMSCT on metabolic pathways within osteoporotic bone was mainly associated with arachidonic acid metabolism, taurine and hypotaurine metabolism, and pentose and glucuronate inter-conversions, a result that was partially consistent with the metabolites found in serum in previous studies.

Metabolic disorders in the bone are an important predisposing mechanism for osteoporosis. 3-hydroxyanthranilic acid is a key product of tryptophan metabolism and its reduced concentrations may contribute to osteoporosis. 3-dopa has been shown to be closely associated with osteoporotic fractures in Parkinson’s patients. D-xylitol and prostaglandin are suggested to possess anti-osteoporotic effects. Age-related, ovariectomy-induced, or retinoic acid-induced osteoporosis in mice or rats has significantly reduced plasma and bone citric acid levels. Acetylcholine can be widely expressed by intraosseous mesenchymal stem cells, osteoblasts, and osteoclasts derived from macrophages and is regulated by acetylcholinesterase, which plays an important role in maintaining bone development and homeostasis. Lithocholic, a metabolite of arachidonic acid, blocks the production of reactive oxygen species, inhibits osteoclast formation and activation in the osteoporosis model of OVX, and is an important inflammatory regulatory product.

Intraosseous biomarkers are biochemical indicators that respond to a wide range of biological changes in bone, including physiological, biochemical, immunological, and metabolic, that occur in response to certain stimulating factors. Intraosseous metabolic biomarkers avoid potential confounding factors in blood and urine and provide a more complete view of the metabolic alterations that occur in osteoporosis and after treatment. This may provide a new way to diagnose osteoporosis, that is, bone tissue obtained by internal fracture fixation surgery, iliac bone harvesting, or bone marrow aspiration can be used to sensitively determine disease progression and treatment outcome of osteoporosis. And for the screened intraosseous biomarkers, future work will also carry out more mechanistic studies to discover possible biotherapeutic targets.

In this study, LC-MS was used to analyze changes in metabolites in the femurs of mice treated with BMSC tail vein infusion after OVX-induced osteoporosis to better understand the potential mechanisms and biomarkers of exogenous BMSC treatment. We believe that additional validation is necessary to elucidate all differentially expressed metabolites in the femur to comprehensively explain the mechanisms involved and indicate potential therapeutic targets. Furthermore, future work could identify metabolic biomarkers with better specificity and sensitivity from patients with osteoporosis.

Conclusion

This study investigated the protective effect of BMSCT against OVX-induced osteoporosis based on untargeted metabolomics of LC-MS and a multivariate data analysis approach. Moreover, iconographic, serological, and biomechanical analyses validated the protective effect of BMSCT against osteoporosis. A total of 18 potential biomarkers and 10 associated metabolic pathways were screened from the metabolomic studies of mouse femoral tissues. Our study shows that the method based on metabolomics LC-MS and multivariate analysis can reflect the regulatory mechanisms of stem cell transplantation therapy, making this approach a promising tool for assessing the efficacy of osteoporosis treatment.

Authors’ Contributions

W.W.Z. and W.YH. designed the research, wrote papers, and conducted the experiments. H.J., D.H., and W.ZH. assisted in data collection, and analysis and evaluation of raw data. H.F. provided technical support for the analysis and critical revision of the manuscript. All authors reviewed and approved the final manuscript.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical Approval

This study was approved by the Animal Experimentation Ethics Review Committee of Kunming Medical University (kmmu202111287).
Statement of Human and Animal Rights

All procedures in this study were conducted in accordance with the Animal Experimentation Ethics Review Committee of Kunming Medical University (kmmu20211287) approved protocol.

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

Declaration of Conflicting Interests

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Supplemental Material

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References

1. Choi KH, Lee JH, Lee DG. Sex-related differences in bone metabolism in osteoporosis observational study. Medicine (Baltimore). 2021;100(21):e26153.
2. Coughlan T, Dockery F. Osteoporosis and fracture risk in older people. Clin Med (Lond). 2014;14(2):187–91.
3. Goode SC, Beshears JL, Goode RD, Wright TF, King A, Crist BD. Putting the brakes on breaks: osteoporosis screening and fracture prevention. Geriatr Orthop Surg Rehabil. 2017;8(4):238–43.
4. Adler RA. Osteoporosis in men: a review. Bone Res. 2014;2:14001.
5. Hong H, Chen X, Li K, Wang N, Li M, Yang B, Yu X, Wei X. Dental follicle stem cells rescue the regenerative capacity of inflamed rat dental pulp through a paracrine pathway. Stem Cell Res Ther. 2020;11(1):333.
6. Ragni E, Perucca Orfei C, De Luca P, Mondadori C, Viganò M, Colombini A, de Girolamo L. Inflammatory priming enhances mesenchymal stromal cell secretopeut potential as a clinical product for regenerative medicine approaches through secreted factors and EV-miRNAs: the example of joint disease. Stem Cell Res Ther. 2020;11(1):165.
7. Esmaeili R, Darbandi-Azar A, Sadeghpour A, Majidzadeh-A K, Eini L, Jafarbeik-Iravani N, Hoseinpour P, Vajhi A, Oghabi Bakhshaiesh T, Masoudkabir F, Sadeghizadeh M. Mesenchymal stem cells pretreatment with stromal-derived factor-1 alpha augments cardiac function and angiogenesis in infarcted myocardium. Am J Med Sci. 2021;361(6):765–75.
8. Maheshwer B, Polce EM, Paul K, Williams BT, Wolfson TS, Yanke A, Verma NN, Cole BJ, Chahla J. Regenerative potential of mesenchymal stem cells for the treatment of knee osteoarthritis and chondral defects: a systematic review and meta-analysis. Arthroscopy. 2021;37(1):362–78.
9. Wu J, Sun Z, Sun HS, Wu J, Weisel RD, Keating A, Li ZH, Feng ZP, Li RK. Intravenously administered bone marrow cells migrate to damaged brain tissue and improve neural function in ischemic brain of mice. Cell Transplant. 2007;16(10):993–1005.
10. Song M, Mohamad O, Gu X, Wei L, Yu SP. Restoration of intracortical and thalamocortical circuits after transplantation of bone marrow mesenchymal stem cells into the ischemic brain of mice. Cell Transplant. 2013;22(11):2001–15.
11. Arjmand B, Sarvari M, Alavi-Moghadam S, Payab M, Goodarzi P, Gilany K, Mehrdad N, Larijani B. Prospect of stem cell therapy and regenerative medicine in osteoporosis. Front Endocrinol (Lausanne). 2020;11:430.
12. Ganguly P, El-Jawhari JJ, Giannoudis PV, Burska AN, Ponchel F, Jones EA. Age-related changes in bone marrow mesenchymal stromal cells: a potential impact on osteoporosis and osteoarthritis development. Cell Transplant. 2017;26(9):1520–29.
13. Mazziotto C, Lanzillotti C, Iaquinta MR, Taraballi F, Torrreggiani E, Rotondo JC, Oton-Gonzalez L, Mazzoni E, Frontini F, Bononi I, De Mattei M, et al. MicroRNAs modulate signaling pathways in osteogenic differentiation of mesenchymal stem cells. Int J Mol Sci. 2021;22(5):2362.
14. Zhao JF, Xu JY, Xu YE, Chen SL, Guo YX, Gao QY, Sun GC. High-throughput metabolomics method for discovering metabolic biomarkers and pathways to reveal effects and molecular mechanism of ethanol extract from epimedium against osteoporosis. Front Pharmacol. 2020;11:1318.
15. Li B, He X, Jia W, Li H. Novel applications of metabolomics in personalized medicine: a mini-review. Molecules. 2017;22(7):1173.
16. Sui BD, Chen J, Zhang XY, He T, Zhao P, Zheng CX, Li M, Hu CH, Jin Y. Gender-independent efficacy of mesenchymal stem cell therapy in sex hormone-deficient bone loss via immunosuppression and resident stem cell recovery. Exp Mol Med. 2018;50(12):1–14.
17. Zheng CX, Sui BD, Liu N, Hu CH, He T, Zhang XY, Zhao P, Chen J, Xuan K, Jin Y. Adipose mesenchymal stem cells from osteoporotic donors preserve functionality and modulate systemic inflammatory microenvironment in osteoporotic cytokine-thrapy. Sci Rep. 2018;8(1):5215.
18. Ankrum JA, Ong JF, Karp JM. Mesenchymal stem cells: immune evasive, not immune privileged. Nat Biotechnol. 2014;32(3):252–60.
19. Wilson JG, Liu KD, Zhuo H, Caballero L, McMillan M, Fang X, Cosgrove K, Vojnik R, Calfee CS, Lee JW, Rogers AJ, et al. Mesenchymal stem (stromal) cells for treatment of ARDS: a phase 1 clinical trial. Lancet Respir Med. 2015;3(1):24–32.
20. Steinberg GK, Kondziolka D, Wechsler LR, Lunsford LD, Coburn ML, Billigen JB, Kim AS, Johnson JN, Bates D, King
B, Case C, et al. Clinical outcomes of transplanted modified bone marrow-derived mesenchymal stem cells in stroke: a phase 1/2a study. Stroke. 2016;47(7):1817–24.

21. Zou Z, Liu W, Cao L, Liu Y, He T, Peng S, Shuai C. Advances in the occurrence and biotherapy of osteoporosis. Biochem Soc Trans. 2020;48(4):1623–36.

22. Chandra A, Rajawat J. Skeletal aging and osteoporosis: mechanisms and therapeutics. Int J Mol Sci. 2021;22(7):3553.

23. Fischer UM, Harting MT, Jimenez F, Monzon-Posadas WO, Xue H, Savitz SI, Laine GA, Cox CS. Pulmonary passage is a major obstacle for intravenous stem cell delivery: the pulmonary first-pass effect. Stem Cells Dev. 2009;18(5):683–92.

24. Kidd S, Spaeth E, Dembinski JL, Dietrich M, Watson K, Klopp A, Battula VL, Weil M, Andreiff M, Marini FC. Direct evidence of mesenchymal stem cell tropism for tumor and wounding microenvironments using in vivo bioluminescent imaging. Stem Cells. 2009;27(10):2614–23.

25. Nitzsche F, Müller C, Lukomska B, Jolkkonen J, Deten A, Boltze J. Concise review: MSC adhesion cascade-insights into homing and transendothelial migration. Stem Cells. 2017;35(6):1446–60.

26. Huang S, Xu L, Sun Y, Zhang Y, Li G. The fate of systemically administrated allogeneic mesenchymal stem cells in mouse femoral fracture healing. Stem Cell Res Ther. 2015;6:206.

27. Hofer HR, Tuan RS. Secreted trophic factors of mesenchymal stem cells support neurovascualar and musculoskeletal therapies. Stem Cell Res Ther. 2016;7(1):131.

28. Larrick JW, Mendelsohn AR. Mesenchymal stem cells for frailty? Rejuvenation Res. 2017;20(6):525–29.

29. Yeo GEC, Ng MH, Nordin FB, Law JX. Potential of mesenchymal stem cells in the rejuvenation of the aging immune system. Int J Mol Sci. 2021;22(11):3749.

30. Wang W, Wang Y, Tang Z, Chen Y, Liu Z, Duan H, Zhong Z, He F. Mesenchymal stem cells prevent ovariectomy-induced osteoporosis formation in mice through intraosseous vascular remodeling. Biochem Biophys Res Commun. 2021;582:64–71.

31. Sebo ZL, Rendina-Ruedy E, Ables GP, Lindskog DM, Rodeheffer MS, Fazeli PK, Horowitz MC. Bone marrow adiposity: basic and clinical implications. Endocr Rev. 2019;40(5):1187–206.

32. Li Z, Hardij J, Bagchi DP, Scheller EL, MacDougald OA. Development, regulation, metabolism and function of bone marrow adipose tissues. Bone. 2018;110:134–40.

33. Fan J, Jahed V, Klavins K. Metabolomics in bone research. Metabolites. 2021;11(7):434.

34. Luo D, Li J, Chen K, Rong X, Guo J. Untargeted metabolomics reveals the protective effect of Fufang Zhenshu Tiaozhi (FTZ) on aging-induced osteoporosis in mice. Front Pharmacol. 2018;9:1483.

35. Saisi M, Britz-Mckibbin P. New advances in tissue metabolomics: a review. Metabolites. 2021;11(10):672.

36. Jiang YC, Li YF, Zhou L, Zhang DP. UPLC-MS metabolomics method provides valuable insights into the effect and underlying mechanisms of Rhizoma Drynariae protecting osteoporosis. J Chromatogr B Analyt Technol Biomed Life Sci. 2020;1122:262.

37. Michalowska M, Znorko B, Kaminski T, Oksztulska-Kolanek E, Pawlak D. New insights into tryptophan and its metabolites in the regulation of bone metabolism. J Physiol Pharmacol. 2015;66(6):779–91.

38. Malochet-Guinaudand S, Durif F, Thomas T. Parkinson’s disease: a risk factor for osteoporosis. Joint Bone Spine. 2015;82(6):406–10.

39. Mattila PT, Svanberg MJ, Jämsä T, Knuuttila ML. Improved bone biomechanical properties in xylitol-fed aged rats. Metabolism. 2002;51(1):92–96.

40. Tagliaferri C, Wittrant Y, Davicco MJ, Walrand S, Coxam V. Muscle and bone, two interconnected tissues. Ageing Res Rev. 2015;21:55–70.

41. Chen H, Wang Y, Dai H, Tian X, Cui ZK, Chen Z, Hu L, Song Q, Liu A, Zhang Z, Xiao G, et al. Bone and plasma citrate is reduced in osteoporosis. Bone. 2018;114:189–97.

42. Luo X, Lauwers M, Layer PG, Wen C. Non-neuronal role of acetylcholinesterase in bone development and degeneration. Front Cell Dev Biol. 2020;8:620543.

43. Liu C, Guan H, Cai C, Li F, Xiao J. Lipoxin A4 suppresses osteoclastogenesis in RAW264.7 cells and prevents ovariectomy-induced bone loss. Exp Cell Res. 2017;352(2):293–303.