Research Note
Bone Viscoelastic Properties in an Animal Model with Osteoporosis after BMSC-Alendronate Sodium Intervention

Chengdong Piao1, Zhengwei Li1, Jie Ding2 and Daliang Kong3

1 Department of Orthopaedics, Second Hospital of Jilin University, Changchun, China
2 Department of Stomatology, Affiliated Hospital of Changchun University of Chinese Medicine, Changchun, China
3 Department of Orthopaedics, China-Japan Union Hospital, Jilin University, Changchun, China

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Abstract: This study aimed to test stress relaxation and creep of the femur in osteoporotic rats after bone mesenchymal stem cell (BMSC) intervention and to provide a viscoelastic basis for the prevention and treatment of osteoporosis. The osteoporotic animal models prepared by ovariectomy were intervened with BMSCs and alendronate sodium tablets (ASTs), respectively, for 30 days, and then compared for serum calcium (Ca), phosphorus (P), alkaline phosphatase (ALP), estradiol (E2), estrogen receptor (ER), superoxide dismutase (SOD), and malondialdehyde (MDA) levels, as well as for stress relaxation, creep, and bone mineral density (BMD). BMD and Ca, ALP, E2, ER, and SOD levels, as well as 7200 s stress reduction and 7200 s strain increase, were statistically higher in BMSC group than in MOD and AST groups (p < 0.05). However, P and MDA levels in BMSC group were lower than those in MOD and AST groups. BMSC intervention can improve bone quality and restore bone viscoelasticity in osteoporotic rat models.

Key words: Osteoporosis animal model, Bone marrow mesenchymal stem cells, Alendronate sodium, Stress relaxation, Creep

Introduction
The pathophysiology of osteoporosis is due to an imbalance between osteoclastic bone resorption and osteoblastic bone formation1-3. Bone mesenchymal stem cells (BMSCs) produce osteogenic factors and angiogenic growth factors, which contribute to bone formation, indicating that BMSCs have direct osteogenic effects4. Several studies conducted extensive research on the intervention of BMSCs and other drugs to treat osteoporosis5-9. Takada et al.9 showed that BMSC transplantation balances the balance between osteoclasts and osteoblasts, improves the quality and quantity of bone tissue cells, and increases the bone density in the area, concluding that BMSC transplantation can be used to treat osteoporosis. Uejima et al.10 showed that BMSC transplantation significantly increases bone density in the region and enhances the biomechanical strength of bone bending. Wang et al.11 investigated re-construction of osteoporotic rat femur using cultured BMSCs and calcium alginate gel mixture intervention and showed increasing trabecular bone density and strength. Previous studies5-9, however, did not investigate the effect of BMSCs and alendronate sodium tablets (ASTs) on viscoelasticity properties of the bone in an osteoporotic animal model. The authors hypothesized that stress relaxation, creep mechanical properties, bone mineral density (BMD), and serum bone metabolic markers exhibit changes in an osteoporotic animal model. Restoration of bone stress relaxation, creep properties, and serum bone metabolic markers in osteoporotic animal models after intervention with BMSCs and AST, needs verification. For this purpose, the authors duplicated the animal model of osteoporosis and validated the above hypothesis with BMSC and AST intervention treatment. The effects of AST and BMSCs on osteoporotic animals were evaluated for stress relaxation and creep characteristics.

Materials and Methods
Animals
A total of 66 five-month-old female Sprague Dawley (SD) rats (body weight 240-250 g) were provided by Changchun High-tech Medical Animal Experimental Center (Changchun, Jilin, China, License No. SCXK (Kyrgyzstan) 2003-0004).

Animal rearing and grouping
The 66 SD rats were randomly divided into four groups as follows: the control group (CON, n = 22), the model group (MOD, n = 22), the BMSC intervention group (BMSC, n = 22), and the alendronate sodium tablet intervention group (AST, n = 22). All the rats had free access to food and water.

Animal ethics: The experimental scheme was approved by the Animal Experimental Ethics Committee of the Second Hospital of Jilin University (No. 20170031). All experiments were carried out as per the International Association of Veterinary Editors’ Consensus on Authors’ Guidelines on Animal Ethics and Welfare and local and national regulations.

Preparation of ovariectomized osteoporotic rat model
Each rat was injected intraperitoneally with 2% pentobarbital sodium anesthetic (2 ml/kg) under aseptic conditions, and made a longitudinal incision (2.0 to 2.5 cm in length) via midline of the abdomen, fol-
Blood and specimen sampling

After five weeks, 20 ml of blood was collected from the rats in the CON group, MOD group, BMSC group, and AST group by cutting the abdomen cavity to prevent bacterial infection. The osteoporotic animal models prepared by ovariectomy (MOD group) were intervened with BMSCs (BMSC group) and alendronate sodium tablets (AST group) for 30 days, and the controls had no treatment (CON group).

**BMSC and AST intervention method for the treatment of osteoporosis in the animal model**

After 12 weeks of feeding, each rat in BMSC group received $3 \times 10^6$ BMSCs obtained from ScienCell Research Laboratories (San Diego, California, USA), and each rat in AST group received orally administered AST (2.264 mg/kg body weight, dissolved in 4 ml of distilled water) obtained from Beijing Wansheng Pharmaceutical Co., Ltd. (Beijing, China), once a day for 30 days.

**Blood and specimen sampling**

After five weeks, 20 ml of blood was collected from the rats in the CON group, MOD group, BMSC group, and AST group by cutting the tail, and the collected blood was allowed to sit for 15 min, followed by 20-min low-speed centrifugation at 2,000 rpm (Type: 4-5 R, Hengnuo instrument equipment Co., Ltd., Changsha, Hunan province, China). The supernatant was cryopreserved at -20°C for future use. Rats were anesthetized by intraperitoneal injection of 2% pentobarbital sodium, killed by decapitation, and femur and tibia were removed for BMD, stress relaxation, and creep measurements.

**Measurement of BMD**

Ten right tibias were selected randomly from each group, removed muscles, and BMD determined using the 400-type single photon bone densitometer manufactured by China Atomic Energy Academy (Beijing, China).

**Detection of ALP, P, and Ca**

Ten serum samples from each group were used for automatic detection of ALP, P, and Ca using Hitachi Ltd., 7180 (Tokyo, Japan) automatic biochemical analyzer, with automatic data output.

**Detection of estradiol (E2)**

The ELISA kit for detection of estradiol and estradiol receptors was purchased from R&D Corporation (Maryland, USA). Serum concentrations were determined by a radioimmunoassay (RIA) kit using an HBS-1096epro enzyme-labeled analyzer manufactured by Nanjing Detie Experimental Equipment Co., Ltd. (Nanjing, Jiangsu, China). The instrumental sensitivity was 1.1 pg/ml, together with an intra-assay coefficient of variation (CV) ≤ 10%. The sample determination used the double-tube parallel method, and the corresponding concentrations were obtained through instrument readings and derived from the standard curve.

**Detection of MDA and SOD**

The serum MDA kit and SOD kit were from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). MDA was detected according to the MDA kit operating instructions using the thiobarbituric acid colorimetric method. Measured serum MDA content (nmol/ml) = (absorbance of test - absorbance of blank) / (absorbance of standard - absorbance of blank) × standard concentration (10 nmol/ml) × sample dilution. SOD was detected according to the SOD kit operating instructions using the Astragalus oxidase method.

**Geometric analysis of femurs**

After the specimens were stored for three days, the femurs were taken out and the length and diameter measured using one reading CGA-5-microscope (Changchun Third Optical Instrument Factory, Changchun, Jilin, China). The samples ranged from 31.0-32.3 mm in length and 1.96-2.08 mm in diameter. The two ends of each sample were placed in a mold with an outer diameter of 10 mm, an inner diameter of 6 mm, and a depth of 5 mm, and the model was prepared using diluted tray powder.

**Stress relaxation test**

The experimental instrument was MODEL-55100 electronic universal testing machine manufactured by Changchun Test Machine Research Institute (Changchun, Jilin, China) according to the pre-conditioning and testing methods in reference$^{12-14}$. Each femur was placed on a tester bench and tested stress relaxation at a rate of 0.8 GPa/min. The experimental time was set to 7200 s. After reaching a preset time, the computer automatically outputs the results.

**Creep test**

The experimental equipment, experimental time, sample conditioning, and testing methods were the same as the stress relaxation test, with the strain increase rate of 0.05%/s.

**Statistical analysis**

The data were expressed as mean ± SD and processed using the SPSS16.0 software package (SPSS, Chicago, IL, USA). The intergroup differences were compared using the one-way analysis of variance and the Scheffe’s method, with $p < 0.05$ considered as statistically significant.

**Results**

**Comparison of BMD, Ca, P, and ALP in the serum of rats in each group (Table 1)**

The tibial BMD measurements showed the following results: BMD value of CON group was higher than that of BMSC group ($p < 0.05$), whereas BMD value of BMSC group was higher than that of AST intervention group ($p < 0.05$). BMD value of AST intervention group was higher than that of MOD group ($p < 0.05$). These results suggest that BMD is lower in the osteoporotic animal model, and BMSC intervention can restore BMD in osteoporotic rats. The results of serum Ca and ALP levels were similar to those of BMD measurements; Ca and ALP levels were higher in the CON group than in BMSC intervention group ($p < 0.05$), whereas Ca and ALP levels were higher in BMSC group > than in AST intervention group ($p < 0.05$); Ca and ALP levels were higher in AST intervention group than in MOD group ($p < 0.05$). The results of serum P levels in rats from each group showed that serum P levels were lower in CON group than in BMSC group ($p < 0.05$). The P levels were lower in BMSC group than in AST intervention group ($p < 0.05$), whereas they were lower in AST intervention group than in MOD group ($p < 0.05$). These results suggest that serum Ca and ALP levels in the osteoporotic animal models are decreased, while serum P levels are increased. BMSCs can increase Ca and ALP levels in osteoporotic animal models and decrease P levels.
Comparison of E2, ER, MDA, and SOD test results of rats in each group (Table 2)

The values of serum E2, ER, MDA, and SOD levels in all the groups showed the following order: CON group > BMSC intervention group (p < 0.05) > AST group (p < 0.05) > MOD group (p < 0.05). The MDA levels observed in the groups were as follows: CON group < BMSC group (p < 0.05) < the AST group (p < 0.05) < MOD group (p < 0.05). These results suggest that serum E2, ER, and SOD in osteoporotic models decreased, while MDA increased, and that BMSCs improve serum E2, ER, and SOD in osteoporotic animal models but reduce MDA.

Comparison of bone creep in rats of each group

The creep test showed that the strain of 7200 s in the CON group in-creased by 0.24%, which was 0.15% in the MOD group, 0.23% in the BMSCs group, and 0.19% in the AST group. The increase of 7200 s strain in the BMSCs group was not significantly different from that in CON group (p > 0.05). These results suggest that BMSCs can restore the creep properties of bone in osteoporotic models.

Comparison of bone stress relaxation in each group

The stress relaxation test showed that stress of 7200 s decreased in the following order: 1.52 MPa in the CON group, 1.51 MPa in the BMSC group, 1.29 MPa in the AST group and 1.01 MPa in the MOD group. The decrease in 7200 s stress in the CON group was higher than in the BMSCs group (p < 0.05), which was higher than in the AST group (p < 0.05), which was higher than in MOD group (p < 0.05). There was no significant difference between BMSCs group and the CON group (p > 0.05). These results suggest that AMSCs can restore the stress relaxation characteristics of osteoporotic animal models.

Discussion

The results of tibial BMD showed significantly lower BMD in the MOD group than the CON group, BMSCs group and AST group (p < 0.05), showing osteoporosis reduces bone formation and causes bone loss. BMD value of BMSCs group was higher than that of MOD group and AST group, and the P level of BMSCs group was lower than that of MOD group and AST intervention group, the difference was significant (p < 0.05). These results suggest that BMSCs can improve BMD in osteoporotic rats.

The results of serum Ca, ALP, and P levels showed that the serum Ca, and ALP of the model group decreased significantly, and the serum P increased significantly. The Ca and ALP in the BMSCs group were higher than those in the model group and AST group (p < 0.05). It indicated that BMSCs could increase Ca and ALP in the osteoporotic animal model and reduce the level of P.

Binding of estrogen and ER can affect the cell cycle, thus inducing apoptosis of osteoclasts, inhibiting the recruitment and differentiation of osteoclast precursor-forming cells, inhibiting the activity of osteoclasts, and promoting the proliferation of osteoblasts. The serum levels of E2...
and ER showed that the serum E2 and ER levels in the MOD group were significantly lower than CON, BMSCs, and AST groups (p < 0.05). The above results suggest that osteoporosis causes a decrease in serum E2 and ER in the osteoporotic animal models. The values of E2 and ER in BMSCs group were higher than those in MOD and AST groups (p < 0.05), indicating that BMSCs can restore serum E2 and ER in an osteoporotic animal model. Both SOD activity and AST activity in CON, BMSCs, and AST groups, with significant difference (p < 0.05). These results show that free radical release from osteoporotic model results in a decrease of SOD and an increase of MDA. The serum SOD value of BMSCs group was higher than CON and AST groups (p < 0.05). It showed that BMSCs and AST could improve the serum SOD and reduce MDA in the osteoporotic model. BMSCs and AST improve the free radical metabolism in animals to enhance the antioxidant ability and inhibit the formation of lipid peroxidation products to alleviate the damage by oxygen free radicals and lipid peroxidation products.

The stress relaxation test showed that the decrease of 7200 s stress in the CON group was more significant than in the BMSCs, AST and MOD groups (p < 0.05). There was no significant difference among BMSCs group and the CON group (p > 0.05).

The creep test showed an increase of 7200 s strain in the CON group was higher than in the BMSCs, AST and MOD groups (p < 0.05). BMSCs intervention group was not significantly different from that in the CON group (p > 0.05).

Earlier studies have shown that the decrease of bone volume fraction (BVF) and trabecular bone loss is accompanied by a decrease in osteoblasts and hematopoietic cells, and an increase of adipocytes and osteoclasts in ovariectomized rats due to the lack of estrogen. We conclude that the decrease in 7200 s stress and the increase in 7200 s strain in the femur of osteoporotic rat in the MOD group is lower than CON, BMSCs, and AST groups. Moreover, due to a decrease in BVF and loss of trabeculae after ovariectomy, was accompanied by a decrease in osteoblasts and hematopoietic cells, and an increase in adipocytes and osteoclasts as shown earlier, causing changes in the stress relaxation and creep characteristics. The stress relaxation, creep characteristics, BMD, and serum bone metabolic markers of osteoporotic model animals have been restored after intervention with BMSCs, consistent with the expected results.

BMSC dysfunction, such as decreased proliferative activity and decreased osteoblastic differentiation, is one of the critical factors leading to the onset of postmenopausal osteoporosis. Results from this study showed that after BMSC intervention, the osteoporotic animal model could repair the functional defect in BMSCs. BMSCs injected through the tail vein into the osteoporotic animal model plays a role in regeneration of bone tissue and bone formation within bone tissue, so that the bone quality of osteoporotic bones is improved. This study supports results from reference, in which BMSC transplantation into animals with sciatic nerve injury significantly increases bone density and enhances bone bending biomechanical strength. The difference between this study and reference is, this study investigated the mechanical properties, such as stress relaxation and creep viscoelasticity of bone after BMSC intervention, and this is the innovative part of this study.

Due to the limitations of experimental animals and individual differences among experimental animals, the experimental data had a certain degree of dispersion, but it still has significance for intervention in treatment against osteoporosis.

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Conflicts of Interest
The authors declare no conflict of interest.

References
1. Rivadeneira F and Mäkitie O. Osteoporosis and bone mass disorders: From gene pathways to treatments. Trends Endocrinol Metab 27: 262-281, 2016
2. Mafi Golchin M, Heidari L, Ghaderian SM and Akhavan-Niaki H. Osteoporosis: A silent disease with complex genetic contribution. J Genet Genomics 43: 49-61, 2016
3. Kumar S, Wan C, Ramaswamy G, Clemons TL and Ponnazhagan S. Mesenchymal stem cells expressing osteogenic and angiogenic factors synergistically enhance bone formation in a mouse model of segmental bone defect. Mol Ther 18: 1026-1034, 2010
4. Lazar A, Pacurar M and Campian RS. Bisphosphonates in bone diseases treatment. Revista de Chimie -Bucharest- Original Edition, 2017
5. Abdelazim IA, Faza MA and Ayash HM. Effects of raloxifene hydrochloride on bone mineral density and serum lipids in Kuwaiti postmenopausal women with osteoporosis. Archives of Osteoporosis 4: 1-5, 2015
6. Bhandari KH, Asghar W, Newa M, Jamali F and Doschak MR. Evaluation of bone targeting salmon calcitonin analogues in rats developing osteoporosis and adjuvant arthritis. Curr Drug Deliv 12: 98-107, 2015
7. Shiraki M, Ueda S, Sugimoto T, Kuroda T and Nakamura T. Treatment responses with once-weekly teriparatide therapy for osteoporosis. Osteoporos Int 27: 3057-3062, 2016
8. Ummarino D. Osteoporosis: Romosozumab versus teriparatide. Nat Rev Rheumatol 13: 512, 2017
9. Takada K, Inaba M, Ichioka N, Ueda Y, Taira M, Baba S, Mizokami T, Wang X, Hisha H, Iida H and Ikehara S. Treatment of senile osteoporosis in SAMP6 mice by intra-bone marrow injection of allogeneic bone marrow cells. Stem Cells 24: 399-405, 2010
10. Uejima S, Okada K, Kagami H, Taguchi A and Ueda M. Bone marrow stromal cell therapy improves femoral bone mineral density and mechanical strength in ovariectomized rats. Cytotherapy 10: 479-489, 2008
11. Wang Z, Goh J, Das De S, Ge Z, Ouyang H, Chong JS, Low SL and Lee EH. Efficacy of bone marrow-derived stem cells in strengthening osteoporotic bone in a rabbit model. Tissue Eng 12: 1753-1761, 2006
12. Zhang ZJ, Li YJ, Liu XG, Huang FX, Liu TJ, Jiang DM, Lv XM and Luo M. Human umbilical cord blood stem cells and brain-derived neurotrophic factor for optic nerve injury: a biomechanical evaluation. Neural Regen Res 10: 1134-1138, 2015
13. Wang Y, Li ZW, Luo M, Li YJ and Zhang KQ. Biological conduits combining bone marrow mesenchymal stem cells and extracellular matrix to treat long-segment sciatic nerve defects. Neural Regen Res 10: 965-971, 2015
14. Jin H, Yang Q, Ji F, Zhang YJ, Zhao Y and Luo M. Human amniotic
epithelial cell transplantation for the repair of injured brachial plexus nerve: evaluation of nerve viscoelastic properties. Neural Regen Res 10: 260-265, 2015

15. Griffith JF, Wang YX, Zhou H, Kwong WH, Wong WT, Sun YL, Huang Y, Yeung DK, Qin L and Ahuja AT. Reduced bone perfusion in osteoporosis: likely causes in an ovariectomy rat model. Radiology 254: 739-746, 2010

16. Griffith JF, Yeung DK, Antonio GE, Wong SY, Kwok TC, Woo J and Leung PC. Vertebral marrow fat content and diffusion and perfusion indexes in women with varying bone density: MR evaluation. Radiology 241: 831-838, 2006

17. Illing A, Liu P, Ostermay S, Schilling A, de Haan G, Krust A, Ameling M, Chambon P, Schinke T and Tuckermann JP. Estradiol increases hematopoietic stem and progenitor cells independent of its actions on bone. Haematologica 97: 1131-1135, 2012

18. Visnjic D, Kalajzic Z, Rowe DW, Katavic V, Lorenzo J and Aguila HL. Hematopoiesis is severely altered in mice with an induced osteoblast deficiency. Blood 103: 3258-3264, 2004

19. Bonnelly E, Saltel F, Chabadel A, Zirngibil RA, Aubin JE and Jurdic P. Involvement of the orphan nuclear estrogen receptor-related receptor α in osteoclast adhesion and transmigration. J Mol Endocrinol 45: 365-377, 2010

20. Gimble JM and Nuttall ME. The relationship between adipose tissue and bone metabolism. Clin Biochem 45: 874-879, 2012
