Comparative Analysis of Bone Mechanical Properties of Adipose-Derived Mesenchymal Stem Cells and Raloxifene in Treatment of Osteoporosis

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Abstract: This study aims to compare the bone mechanical properties of adipose-derived mesenchymal stem cells (ADMSCs) and raloxifene in treating osteoporosis and to provide related biomechanical basis. The serum of animal model of osteoporosis was sampled after ADMSCs or raloxifene intervention and measured BALP, TRACP-5b, MMP-9, and Cath-K. The femur was taken for compression test, tibia for shock test, vertebrae for bone mineral density test, humerus for histomorphometric index test. In Group AMSCs, BMD and BALP were higher than those in Group MOD, in Group RAL, TRACP-5b, MMP-9 mL-1 and Cath-K were lower than those in Group MOD with significant differences (p < 0.05). In Group AMSCs, BMD, BALP, and such mechanical performance indexes as maximum compressive load, maximum stress, maximum strain, elastic modulus, shock energy, and shock toughness were significantly larger than Group MOD and Group RAL (p < 0.05). After AMSCs intervention, the bone quality can be improved, and the compression and shock mechanical properties can be restored.

Key words: Osteoporotic animal model, Adipose-derived mesenchymal stem cells, Density bone, Histomorphometry index, Compression, Shock, Biomechanics

Introduction

The incidence and risk of osteoporosis are very high with pathological fracture as the most serious complication, the incidence of which is as high as 70%. WHO found that one osteoporotic pathological fracture happened every 30 seconds of the world, and the most common one was spinal fracture, while hip fracture often occurs many serious complications, including death. At present, the treatment of osteoporosis is mainly based on drug methods. Scholars have conducted a lot of research on the treatment effects of raloxifene, calcitonin, estrogen, vitamin D, or statins against osteoporosis. However, these treatments are expensive, require long treatment term, and have toxic side effects, but the efficacy is not ideal, so it is necessary to find a more ideal method. The main pathogenesis of osteoporosis is osteoblast deficiency. Adipose-derived mesenchymal stem cells (ADMSCs) can be isolated from adipose tissue and have the same proliferation and self-renewal ability as bone marrow-derived mesenchymal stem cells (BMSCs), which can differentiate into mesoderm and non-mesoderm cells in multiple directions. It has been confirmed that ADMSCs can be induced and differentiated into osteoblasts, promote new bone formation, increase bone density, improve bone microstructure, and repair bone defects, thus exhibiting certain therapeutic effects against osteoporosis. Tao et al. also injected ADSCs into the tail vein of osteoporotic rats for intervention and tested the serum Ca and alkaline phosphatase (ALP) levels 4 weeks later, as well as testing the three-point bending mechanics of the femur of rats. The results showed that the serum Ca and ALP levels were increased significantly, the serum P level was decreased significantly, and maximum femoral load and rigidity were increased significantly, indicating that ADSCs can promote bone mineralization, improve bone mass, strengthen bone strength, and improve the bending mechanical properties of bones. Park et al. used ADSCs to repair bone defects in rats and got good results, suggesting the feasibility of ADSCs as seed cells for bone tissue engineering. At present, stem cell transplantation has been paid more and more attention to the treatment of osteoporosis. More and more researchers hope to inhibit osteoclast proliferation and promote osteoblast differentiation by stem cell transplantation to achieve the purpose of treating osteoporosis. Previous studies on the mechanical properties of osteoporotic bone treated with ADSCs were mostly about three-point bending.

Comparison of treatment effects between ADMSCs and raloxifene (RAL) against osteoporosis have been reported from the view point of shock and compression mechanical properties. ADMSCs can be induced to differentiate into osteoblasts, promote new bone formation, increase bone density, improve bone microstructure, and repair bone defects, thus exhibiting certain therapeutic effects against osteoporosis. It has been confirmed that ADMSCs can be induced and differentiated into osteoblasts, promote new bone formation, increase bone density, improve bone microstructure, and repair bone defects, thus exhibiting certain therapeutic effects against osteoporosis. Tao et al. also injected ADSCs into the tail vein of osteoporotic rats for intervention and tested the serum Ca and alkaline phosphatase (ALP) levels 4 weeks later, as well as testing the three-point bending mechanics of the femur of rats. The results showed that the serum Ca and ALP levels were increased significantly, the serum P level was decreased significantly, and maximum femoral load and rigidity were increased significantly, indicating that ADSCs can promote bone mineralization, improve bone mass, strengthen bone strength, and improve the bending mechanical properties of bones. Park et al. used ADSCs to repair bone defects in rats and got good results, suggesting the feasibility of ADSCs as seed cells for bone tissue engineering. At present, stem cell transplantation has been paid more and more attention to the treatment of osteoporosis. More and more researchers hope to inhibit osteoclast proliferation and promote osteoblast differentiation by stem cell transplantation to achieve the purpose of treating osteoporosis. Previous studies on the mechanical properties of osteoporotic bone treated with ADSCs were mostly about three-point bending.
Materials and Methods

Animals

The experimental animals were 88 female SD (Sprague Dawley) rats (grade SPF (Specific pathogen free), 6 months old (body weight 240-250 g), which were provided by Changchun High-tech Medical Animal Experimental Center (Changchun, Jilin Province, China; license number: SCXK (Ji) 2003-0004). The feed was supplied by Shenyang Experimental Animal Feed Factory (Shenyang, Liaoning Province, China, Product Standard Code: DB-21741-93).

Animal feeding and grouping

The animals were of grade clean (CL) (Clean animal), bred at 25°C±1 °C with air circulation, relative humidity of 55-70%, and natural lighting. The safety level of the animal laboratory was ABSL-2 (Animal Biosafety Level-2). The 88 SD rats were randomly divided into the normal control group (CON, n=22), and the osteoporotic model group (MOD, n=22), the AMSCs intervention group (ADMSCC, n=22), and the raloxifene intervention group (RAL, n=22). All the animals were free to drinking water and diet in cages, and the feeding condition was level 2.

The experimental protocol was approved by the Animal Experimental Ethics Committee of the Second Hospital of Jilin University (20170039). The experimental process followed the ‘Consensus Guidelines on Animal Ethics and Welfare’ issued by the International Association of Veterinary Editors, as well as local/national regulations.

Modeling of osteoporotic rat model by ovariectomy

Each selected animal was intraperitoneally injected 2% pentobarbital Sodium Injection (2 ml/kg) for anesthesia under aseptic conditions. After successful anesthesia, a 2.0-2.5cm in length longitudinal incision was made through the median abdomen, through which the abdominal cavity was cut open layer by layer for searching the ovaries along the fallopian tubes. The bilateral ovaries were then ligated and removed, and 100,000 U penicillin was injected into the abdominal cavity after surgery to prevent infection.

In vivo ADMSCs intervention and drug treatment

After 12 weeks of feeding, the bone density of L5 vertebrae in 10 rats randomly selected in group CON and group MOD, respectively, were measured. The results showed that the bone density of the tibia in group MOD was significantly lower than group CON (p < 0.05), indicating the successful replicating of the osteoporotic animal model. The intervention of rats in group ADMSCs referred to the method of reference \(^{[9]}\): each SD rat was injected with 3 × 106 ADMSCs (Saive Biotechnology Co., Ltd., Suzhou, Jiangsu, China) via the tail vein. The intervention of rats in group RAL: each SD rat was orally administered with raloxifene suspension (1 mg/ml, Wuhan Jiangmin Huatai Pharmaceutical Chemical Co., Ltd., Wuhan City, Hubei Province, China, 60 mg per 1 tablet, which was ground and suspended in 60 ml of distilled water). According to the human/animal body surface area conversion formula, the dosage was calculated as 6.092 mg/(kg•d) (rat body weight: 200 g, and human body weight: 60 kg). The administration was performed once in the morning and evening, respectively, for 8 weeks.

Blood and specimen sampling

The rats in group CON were sampled blood by tail-cutting method, which was used as the basic control concentration. For group MOD and other intervention groups, each rat was intraperitoneally injected 10% chloral hydrate solution (3 ml/100 g – 1) after 8 weeks of administration for anesthesia and collected 10 mL of blood from the abdominal aorta, which was stored in anticoagulated tube containing 1% heparin sodium and centrifuged at low temperature for 15 min (centrifugal radius: 15 cm, rotation speed: 3000 r•min -1 ). The upper serum was taken and stored at -20 °C for detecting the serum bone metabolism markers. After blood collection, the L4 lumbar vertebrae of each rat was removed and fixed with 4% paraformaldehyde for detecting the bone mineral density (BMD) and bone histomorphometry. The humerus was taken for histomorphometric index test, femur was taken for compression test, tibia was taken for shock test.

Measurement of BMD of tibia

10 cases of L4 lumbar vertebrae were randomly selected from each group, removed muscles, and measured BMD using one 400 single photon bone densitometer (China Academy of Atomic Energy, Beijing, China).

Detection of bone metabolism markers (Serum BALP, TRACP-5b, MMP-9, and Cath-K)

The ELISA kits for these bone metabolism markers were produced by Shanghai Xitang Biotechnology Co., Ltd. (Shanghai, China). 10 serum samples of each group were tested using the microplate reader by LABsystems Co. (Helsinki, Finland) and double-antibody sandwich ELISA method. The procedure was in strict accordance with the ELISA kit instructions (Shanghai ELISA Biotechnology Co., Ltd., Shanghai, China).

Measurement method of bone histomorphometry

Dehydration of the tibia: the humerus specimen was dehydrated in 70% ethanol for 1 hr, 80% ethanol for 1 hr, 90% ethanol for 1 hr, and tert-butanol for 5 hr, followed by 5 hr direct waxing. The specimen was then embedded in wax, made into 4-μm paraffin slices, and baked at 67 °C for 3 hr. Staining steps: the slices were placed onto one slice holder and de-waxed for 10 min in xylene I and xylene II, respectively, followed by gradient dehydration using 100% ethanol I and 100% ethanol II (10 min each), 95% ethanol (10 min), and 80% ethanol (5 min). After HE staining, the slices were sealed in neutral gum and analyzed using the M550 microscopic image analysis system (Leica, Germany), which consisted of an optical microscope, a digital CCD (Charge-coupled Device) camera, a computer, a printer, etc. Its basic workflow was: taking microscope images through a digital camera, transferring the particle microscope images to the computer via USB (Universal Serial Bus) data transmission, and using QWinV2.3 multi-function color pathological image analysis software (Leica, Soarms, Germany) to analyze the bone-to-bone microstructure. The parameters were: Percent trabecular area (BV/TV), Trabecular thickness (Tb.Th), Trabecular number (Tb.N), Trabecular separation (Tb.Sp), and Osteoclast number (OC.N).

Methods for embedding and detecting geometrical dimensions of femur and tibia

After 3-day storage, the sample was taken out and measured the length and diameter using one reading microscope made by Changchun Third Optical Instrument Factory (Changchun, Jilin, China). The length of the femur sample was 31.2-32.4 mm, and the diameter was 1.95-2.06 mm. The length of the tibia was 30. 8-31. 2 mm, and the diameter was 2.19-2.26 mm. The two ends of each sample were placed in a mold having an outer diameter of 10 mm, an inner diameter of 6 mm, and a depth of 5 mm, which was filled with diluted denture powder and solidified for future use.
Experimental methods of femoral compression characteristic

One MODEL-55100 electronic universal testing machine produced by Changchun testing Changchun Testing Machine Research Institute (Changchun, Jilin, China) was used for the test according to the reference (11,12) after sample pre-conditioning. 12 femoral samples were randomly selected from group CON and other experimental groups, respectively, placed on the test machine table, and applied the load to the sample at a speed of 2 mm/min until the samples were destroyed. The data such as maximum load, maximum stress, maximum strain, and elastic modulus of each sample were printed automatically after the test.

Experimental methods of tibia shock characteristic

The experimental equipment was one small-type shock test machine produced by German Leipzig test machine factory (Leipzig, Saxony, Germany). 12 tibia samples were randomly selected from group CON and other experimental groups, respectively, placed on the test machine table, tested the sample, and calculated the shock toughness according to related formula.

Statistical analysis

The data were expressed as mean ± SD and analyzed by SPSS 20.0 SPSS16.0 (SPSS, Chicago, IL, USA) using the t test, Wilcoxon rank test, etc., according to data types, with p < 0.05 being considered as statistical significance.

Results

Measurement results of BMD, BALP, TRACP -5b, MMP -9 ml -1 and Cath – K

The measurement results of BMD and BALP in group MOD were lower than group CON and group RAL. In group AMSCs, the values of TRACP -5b, MMP -9 ml -1, and Cath-K were significantly greater than group CON and group RAL (p < 0.05). In group AMSCs, the values of BMD and BALP were greater than group MOD and group RAL, but the values of TRACP -5b, MMP -9 ml -1, and Cath-K were statistically smaller than group MOD and group RAL (p < 0.05).

Measurement results of bone histomorphometric parameters

The measurement results of bone histomorphometric parameters are shown in Table 2.
The experimental results showed that the values of BV/TV, Tb.Th, and TbN in group MOD were smaller than group CON and group AMSCs. The values of Tb.Sp and OC.N were greater than group CON, group AMSCs, and group RAL (p < 0.05). The values of BV/TV, Tb.Th, and TbN in group AMSCs were greater than group RAL, but the values of Tb.Sp and OC.N were significantly lower than group RAL (p < 0.05).

**Observation of humeral histomorphology (HE staining)**

The observation of humeral histomorphology of each group (HE staining) is shown in Fig. 1.

The humeral histomorphology of each group showed that the cortical bone of the rats in group CON was fine, the inner and outer surfaces were smooth, and the trabecular bone was arranged regularly and evenly (Fig. 1A). The trabecular area of the rats in group MOD became smaller, the distribution was sparse and scattered, the trabecular bone was sparse, smaller, and thinner, the trabecular spacing increased, and the trabecular bone was broken (Fig. 1B); In group AMSCs, the arrangement was denser, the arrangement was more regular, the area was increased, the thickness was increased, and the spacing was smaller (Fig. 1C). The arrangement in group RAL was more scattered, less dense, and more scattered, partial trabecular bone was thinner, the spacing was increased, partial trabecular bone was broken, and the area of the trabecular bone was reduced (Fig. 1D). The condition of osteoporosis in group AMSCs had a certain recovery.

**Results of femoral compression test**

The results of femoral compression test in each group are shown in Table 3.

The experimental results showed that the maximum load, maximum stress, maximum strain, and elastic modulus in group MOD were smaller than group CON, group AMSCs, and group RAL, and the differences were significant (p < 0.05); the maximum load, maximum stress, maximum strain, and elastic modulus in group AMSCs were significantly greater than group MOD and group RAL (p < 0.05).

**Results of bone shock test**

The results of bone shock test in each group are shown in Table 4.

The experimental results showed that the shock energy and shock toughness in group MOD were lower than group CON, group AMSCs, and group RAL, and the differences were significant (p < 0.05). The shock energy and shock toughness in group AMSCs were greater group RAL (p < 0.05).
Discussion

Bone mass and BMD are the key factors that determine the bone strength. The bone mass is determined by the microstructure, metabolic conversion, mineralization, and bone matrix characteristics of bones. BMD is expressed in terms of bone mass per unit area or per unit volume\(^1\). BMD measurements of tibia in each group showed that the BMD value in group MOD was significantly lower than group CON, group AMSCs, and group RAL, indicating that osteoporosis causes bone mass lost and bone formation decline. AMSCs/RAL intervention has the effect of improving the bone density, and AMSCs intervention shows the best effect.

BALP reflects the function of osteoblasts, derives from the osteoblasts, and is a commonly used metabolic marker for bone formation and bone turnover\(^2\). TRACP-5b released by osteoclasts during bone resorption is an effective marker reflecting the number and functional activity of osteoclasts\(^3\). During bone resorption, the osteoclasts release a large amount of TRACP-5b, and serum TRACP-5b content indirectly reflects osteoclast activity and bone resorption status. MMP-9 is a member of the MMPs gene family, is mainly expressed in the osteoclasts and bone marrow mononuclear cells, and is involved in the development and recruitment of osteoclasts\(^4\). MMP-9 is specifically expressed in the osteoclasts and has the function of degrading the extracellular matrix, thus playing an important regulatory role in the migration, erosion, and anchoring of osteoclasts\(^5\). The serum BALP test results of the rats in each group showed that the BALP value in group MOD was lower than group CON, group AMSCs, and group RAL. The BALP value in group AMSCs was greater than group RAL. The values of TRACP-5b, MMP-9 ml^{-1}, and Cath-K in group MOD were greater than group CON, group AMSCs, and group RAL. The BALP value in group MOD had changed values of serum BALP, TRACP-5b, MMP-9, and Cath-K, bone microstructure, bone density, bone compression and shock mechanical properties due to osteoporosis. The values of serum BALP, TRACP-5b, MMP-9, and Cath-K, bone mineral density, bone tissue microstructure, bone compression and shock mechanical properties were improved to a certain extent after AMSCs and RAL intervention, and AMSCs had better effect, consistent with our expectation\(^6\).

The biomechanical properties of bones are the main indicators for determining the quality of bone tissue. The “quantity” and “quality” of bones determine the strength of bones, that is, the ability of bones to resist fracture.

The core of bone formation reduction in osteoporosis is the reduction in the number of osteoblasts and the decline in function\(^7\). Therefore, inducing the differentiation of stem cells into osteoblasts can achieve the therapeutic purposes. A large number of studies have proved that\(^8-20\) ADSCs have multi-directional differentiation and self-proliferation ability, can be induced and differentiated into the osteoblasts, chondroblasts, neuroblasts, myoblasts, and lipoblasts; ADSCs also have low immunogenicity and immune regulation and can be used as seed cells for allogeneic transplantation; compared with the bone marrow mesenchymal stem cells, ADSCs have more wider sources, easier sampling methods, less painful for patients, and more isolated cell number. Therefore, transplantation of allogeneic ADSCs can promote bone formation by differentiating toward the osteoblasts and treat osteoporosis.

The differences between this study experiment and previous studies are in that not only the serum BALP, TRACP-5b, MMP-9, Cath-K, bone histomorphometry parameters, and bone mineral density were measured, but also the bone compression and shock mechanical properties were compared. In the past, the mechanical properties of osteoporotic animal models were mostly tested using the three-point bending test. This study compared the bone compression and shock mechanical properties, so it’s more practical.

This experiment is an in vitro biomechanical experiment and bone morphology measurement of osteoporosis animal model. There are certain differences between animals and human, also between in vitro and in vivo, that’s one of the limitations. With the development of biomedical engineering, in vitro biomechanical experiments of osteoporosis animal models will be used, which is an important direction for future research.

At present, clinical research of ADSCs is at an early stage, most of which are case reports, not randomized study. The clinical application of ASCs needs a lot of research. It is believed that through the great efforts of scientists and clinicians, stem cell technology will become a new medical technology to treat osteoporosis and other diseases beyond the reach of traditional medicine, which will play an important role in health care.

Due to the limitation of rat number and the differences among the
rats, the experimental data had a certain degree of dispersion, but it has certain value for the prevention and treatment of osteoporosis.

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Conflicts of Interest

The authors declare no conflict of interest.

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