

Beneficial effects of low-level laser irradiation on senile osteoporosis in rats

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Abstract. – **OBJECTIVE:** To investigate the effect of low-level laser irradiation (LLLI) on bone mineral density (BMD), bone structures, bone biomechanical properties and bone metabolism in senile osteoporosis, and to explore a relatively more secure and effective way to prevent and treat osteoporosis.

MATERIALS AND METHODS: Sprague-Dawley (SD) male rats at different age stages (4 months old, 12 months old and 20 months old) were selected and randomly divided into six groups. The rats in the treatment group were treated with LLLI for 12 weeks, and then the microstructure of bones was analyzed by micro-computed tomography (micro-CT) scanning. The biomechanical indexes of the femur were detected by the three-point bending test. Levels of the blood calcium (Ca)²⁺, blood phosphorus (P)³⁺, urine Ca, urine P and urine creatinine (CREA) were detected using an automatic biochemical analyzer. The contents of serum osteocalcin (OCN) and bone alkaline phosphatase (BAP) were measured by enzyme-linked immunosorbent assay (ELISA). The bone formation rate (BFR) was analyzed by double fluorescent labeling with calcein and tetracycline. Hematoxylin and eosin (HE) staining and toluidine blue staining were used to analyze the number of bone marrow osteoblasts and adipocytes.

RESULTS: Micro-CT results showed that compared with those in the young group, the bone mineral density (BMD) in the old group was significantly decreased, and the trabecular microstructure was seriously damaged. LLLI could significantly enhance the BMD and improve the damage to the trabecular microstructure; the three-point bending test revealed that LLLI could significantly improve the biomechanical properties and enhance the mechanical

strength of the femur in the old group; the biochemical analysis showed that LLLI could significantly reduce Ca and P losses and elevate the levels of serum BAP and OCN; the bone histomorphology analysis results indicated that LLLI could increase BFR and mineral apposition rate (MAR), increase the number of osteoblasts and decrease the number of adipocytes in the bone marrow in the old group.

CONCLUSIONS: LLLI can effectively improve osteoporosis, increase BMD, improve bone structure and improve bone biomechanical properties in old rats; at the same time, it increases the levels of serum BAP and OCN and the number of osteoblasts in the bone marrow, suggesting that the osteogenesis function of osteoblasts is enhanced.

Key Words:

Low-level laser irradiation, Senile osteoporosis, Bone mineral density, Bone structure, Bone biomechanical properties, Bone metabolism.

Introduction

Osteoporosis is a common and frequently occurring disease in the elderly. Among the global population aged over 50 years old, 1/3 of women and 1/5 of men suffer from osteoporosis^{1,2}. The most common and serious complications of osteoporosis are all types of fractures. The number of fractures in osteoporosis each year exceeds the sum of myocardial infarction and stroke in the United States, in which the mortality rate of hip fractures accounts for 24%, and its hazards can-

not be ignored^{3,4}. Senile osteoporosis is a physiological degenerative change that occurs with the increase in age. The onset age is about 70 years old. The main lesions of osteoporosis are cancellous bone and cortical bone^{5,6}, and its occurrence is related to the body's senility, hypogonadism, genetic disease, endocrine, nutrition and physical factors⁷. Bone loss and slow bone turnover in the elderly are performances of senile osteoporosis, and age and hypogonadism are the main determinants. Characteristics of senile osteoporosis: The activities of osteoclasts and osteoblasts are decreased; the bone formation rate (BFR) of osteoblasts is lower than the bone resorption rate of osteoclast, which is known as low conversion type of osteoporosis^{8,9}. Although a variety of drugs have been used in clinical treatment of osteoporosis, the desired effect is still not achieved due to the long course of treatment, the inconvenient administration route, poor patient compliance, great toxic and side effects, etc., so the prevention and treatment of senile osteoporosis have become urgent problems to be solved.

Low-level laser irradiation (LLLI) therapy is a physical therapy, in which the LLLI (usually lasers at the wavelength of 630-1000 μm from infrared to near-infrared) is applied to the biological response with the lossless and the non-thermal mechanism caused by lesions or monolayer cells, thus achieving the treatment goals^{10,11}. Studies^{12,13} have confirmed that LLLI therapy has a positive effect on anti-inflammatory analgesia, promotion of the repair of skeletal muscles and other tissues, reduction of free radical production through antioxidant, improvement of mitochondrial function and promotion of motor fatigue recovery. Recently, a large number of studies¹⁴⁻¹⁶ have shown that the biological effects of LLLI can stimulate bone formation, but its role in senile osteoporosis is not clear. Therefore, the rat model of senile osteoporosis was established in this study to investigate the effect of LLLI on bone mineral density (BMD), bone structures, bone biomechanical properties and bone metabolism in senile osteoporosis, and to explore a relatively more secure and effective way to prevent and treat osteoporosis.

Materials and Methods

Experimental Animals and Grouping

Sprague-Dawley (SD) male rats at different age stages (4 months old, 12 months old and 20 months old) were selected as the experimental

subjects, which were provided by the Animal Center of Kunming University of Technology. The rats were randomly divided into six groups: the young control group (Y-C), the young treatment group (Y-T), the middle-aged control group (M-C), the middle-aged treatment group (M-T), the old control group (O-C) and the old treatment group (O-T).

This study was approved by the Animal Ethics Committee of by the Animal Center of Kunming University of Technology.

Experimental Schemes and Specimen Collection

The rats in the treatment group were treated with LLLI therapy using the GaAlAs laser irradiator at the wavelength of 810 nm. The rats were irradiated on both sides of the femoral neck. Irradiation points were manually controlled, and the irradiation point of each site was 30 s (50 mW, 11.94 J/cm). Rats were subcutaneously injected with tetracycline (25 mg/kg) on the 14th and 13th day before sacrifice, and subcutaneously injected with calcein (5 mg/kg) on the 4th and 3rd day before sacrifice. 12 weeks later, the urine, blood, bone tissue specimens of rats were collected.

Micro-Computed Tomography (micro-CT) Analysis

The microstructure of bones was analyzed by micro-CT scanning. Scan parameters: the current was 400 μA ; the voltage was 60kV; 360° rotation scan; the angle gain was 2°; the scan time for each specimen was about 20-30 min. The entire femoral image was scanned for reconstructing a three-dimensional (3D) image with a resolution of 12 μm . The selected region of interest (ROI) was located at the metaphysis of the femur, and at least 100 layers were scanned. MicroView v.2.1 software was used to integrate these planar images into 3D images.

Analysis of Biomechanical Properties

Biomechanical indexes of the femur were tested by the three-point bending test with a universal testing machine. The femoral sample was placed on a bracket with the span of 10 mm. 0.5 N pressure was pre-posed to the femoral surface, followed by the action of 0.1 mm/min contact forces on the middle of the femur, and the pressure was increased by 1 mm/min after the test was started. The load-deformation curve was recorded using the instrument, and the vernier caliper was used to measure the b, B, h, H values of the

inside and outside diameters of the cross section after the test so as to calculate the elastic load parameters and the maximum load parameters.

Detection of Biochemical Markers of Bone Metabolism

Levels of the blood calcium (Ca), blood phosphorus (P)³⁺, urine Ca, urine P and urine creatinine (CREA) were detected using an automatic biochemical analyzer. Levels of serum osteocalcin (OCN) and bone alkaline phosphatase (BAP) were measured by enzyme-linked immunosorbent assay (ELISA).

Determination of Bone Histomorphometry Indexes

After undecalcified tissue sections were polished, the fluorescence of tetracycline and calcein was observed under a fluorescence microscope, and mineral apposition rate (MAR, $\mu\text{m}/\text{d}$) and BFR ($\mu\text{m}/\text{d}$) were measured and calculated by the automatic image analysis system, and the new bone formation speed was observed. HE staining and toluidine blue staining were conducted for decalcified tissue sections, and sections were observed under a microscope after mounting.

Statistical Analysis

Measurement data were expressed as $\bar{x} \pm s$, and all the data were analyzed by Statistical Product and Services Solution (SPSS) 19.0 software (Version X; IBM, Armonk, NY, USA). The *t*-test was used for intergroup comparisons. The analysis of variance was used for multi-sample comparisons. Student-Newman-Keuls (SNK)-*q* test was used for pairwise comparisons. $p < 0.05$ represented that the difference was statistically significant.

Results

LLLI Improved the Trabecular Microstructure of Old Rats

Bone histomorphometry analysis was performed using micro-CT. The results showed that in comparisons of the bone mass of rats in different age groups, the bone mass of young rats was significantly larger than that of middle-aged and old rats, indicating that the bone mass of rats decreases with the increase in age. Compared with that of young rats, the BMD of the femur was significantly decreased, while structure model index (SMI) and trabecular spacing (Tb.Sp) were significantly increased. The bone volume per total volume (BV/

TV), trabecular number (Tb.N) and trabecular thickness (Tb.Th) of old rats were significantly lower than those of young rats, indicating that the trabecular microstructure is seriously destroyed. Compared with those of rats in the control group, the LLLI had no significant effects on BMD, BV/TV, Tb.N, Tb.Th, SMI, Tb.Sp and other trabecular microstructure parameters of young rats, and it had no significant effects on BMD, BV/TV, Tb.N, and Tb.Th of middle-aged rats, but it could significantly decrease SMI and Tb.Sp of them; LLLI could significantly increase BMD, BV/TV, Tb.N and Tb.Th of old rats, and significantly decrease SMI and Tb.Sp of them. The above results suggested that LLLI does not increase the bone mass of normal bone tissues, but can significantly increase the bone mass of old rats with osteoporosis and improve the trabecular microstructure (Figure 1).

LLLI Enhanced the Mechanical Strength of the Femur

The bone biomechanical analysis was carried out using a universal material testing machine through the three-point bending test. The maximum load of the mechanical strength index of the femur, structural hardness, bone stress and elastic modulus in three groups of rats were compared. The results showed that the mechanical strength of the femur was gradually decreased with the increase in age. Compared with those of young rats, the maximum load, structural hardness, bone stress and elastic modulus of the femur of old rats were significantly decreased. Compared with those of rats in the control group, LLLI exerted no significant effects on the maximum load, structural hardness, bone stress and elastic modulus of young rats; it exerted no significant effects on the maximum load, structural hardness and bone stress of middle-aged rats, but increased the elastic modulus of them. LLLI could significantly increase the maximum load, structural hardness, bone stress and elastic modulus of old rats. The above results indicated that LLLI does not affect the mechanical strength of normal bone tissues, but can significantly improve the biomechanical properties of the femur, improve the internal and external characteristics of it and enhance its mechanical strength (Figure 2).

LLLI Reduced the Calcium and Phosphorus Losses and Elevated the Levels of Serum BAP and OCN

The results revealed that compared with those of young and middle-aged, the levels of urine

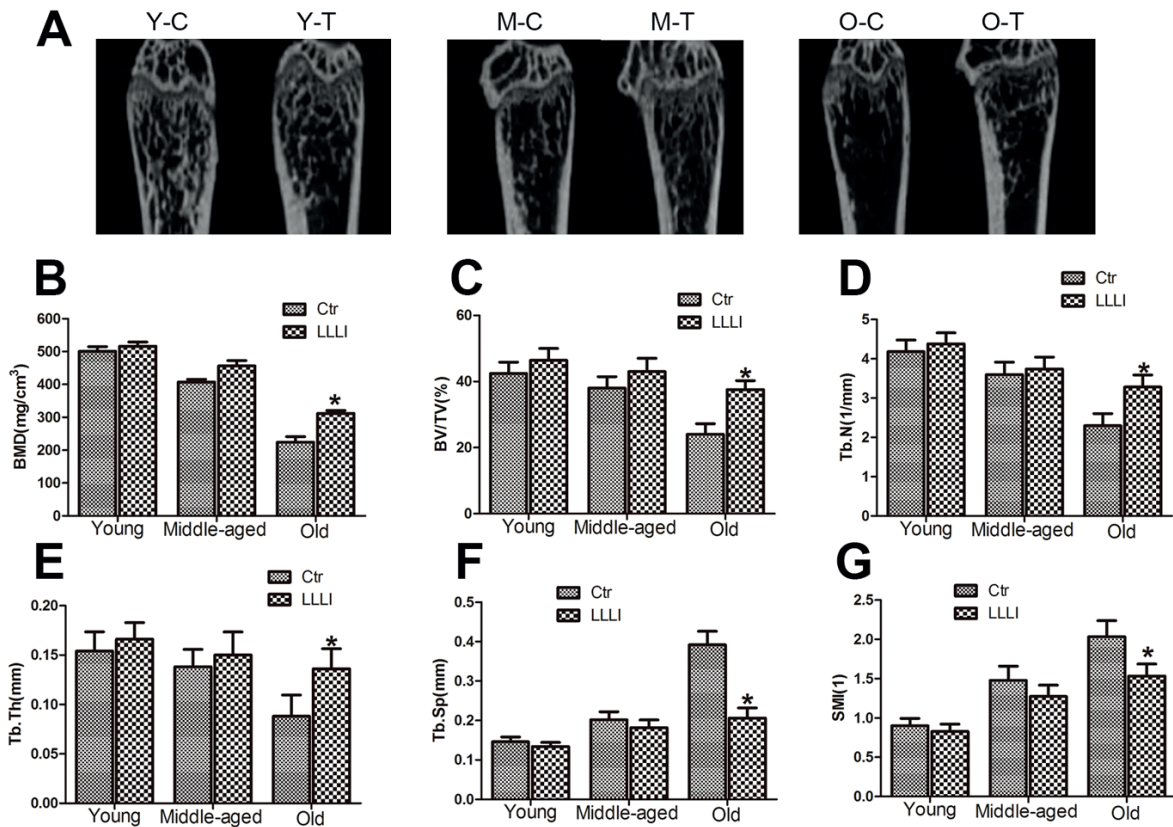


Figure 1. Micro-CT analysis shows that LLLI improved the trabecular microstructure of old rats. (A) Representative images from micro-CT of trabecular bone from the distal femur metaphyses. (B-G) Micro-CT analysis of BMD, BV/TV, Tb.N, Tb.Th, Tb.Sp, SMI show LLLI improved the trabecular microstructure of old rats.

Ca²⁺ and P³⁺ of the old rats were significantly increased, while the levels of serum BAP and OCN were significantly decreased, indicating that the bone resorption was significantly enhanced, bone formation was significantly weakened, and bone steady state was imbalanced in old rats. Compared with that in the control group, LLLI could significantly increase the concentration of serum Ca²⁺, but exerted no significant effects on serum concentration of P³⁺. LLLI can significantly decrease the ratios of Ca/chromium (Cr) and P/Cr and the levels of serum BAP and OCN. The above findings showed that LLLI could significantly reduce the calcium and phosphorus losses in the urine in the old group, increase the levels of serum BAP and OCN, suggesting that LLLI inhibits bone resorption while promoting bone formation, and bone resorption and bone formation represent osteoclast activity and osteoblast activity, respectively. Therefore, it was believed that LLLI could inhibit osteoclast activity while promoting osteoblast activity (Figure 3).

LLLI Increased BFR and MAR of old Rats

Double fluorescent labeling with calcein and tetracycline was used to investigate the bone formation activity by analyzing the deposition rate of bone. Data revealed that the BFR and MAR of the young rats were the highest, followed by middle-aged rats, and those of old rats were the lowest, indicating that BFR is gradually reduced with the increased in age. Compared with those in the control group, LLLI exerted no effects on young rats and middle-aged rats in the LLLI treatment group, but significantly increased MAR and BFR of old rats, suggesting that LLLI promotes the bone formation (Figure 4).

LLLI Increased the Number of Osteoblasts in the Bone Marrow and Decreased the Number of Adipocytes in Old Rats

As shown in bone tissue sections stained by HE and toluidine blue, the number of osteoblasts in bone tissues of old rats was significantly lower

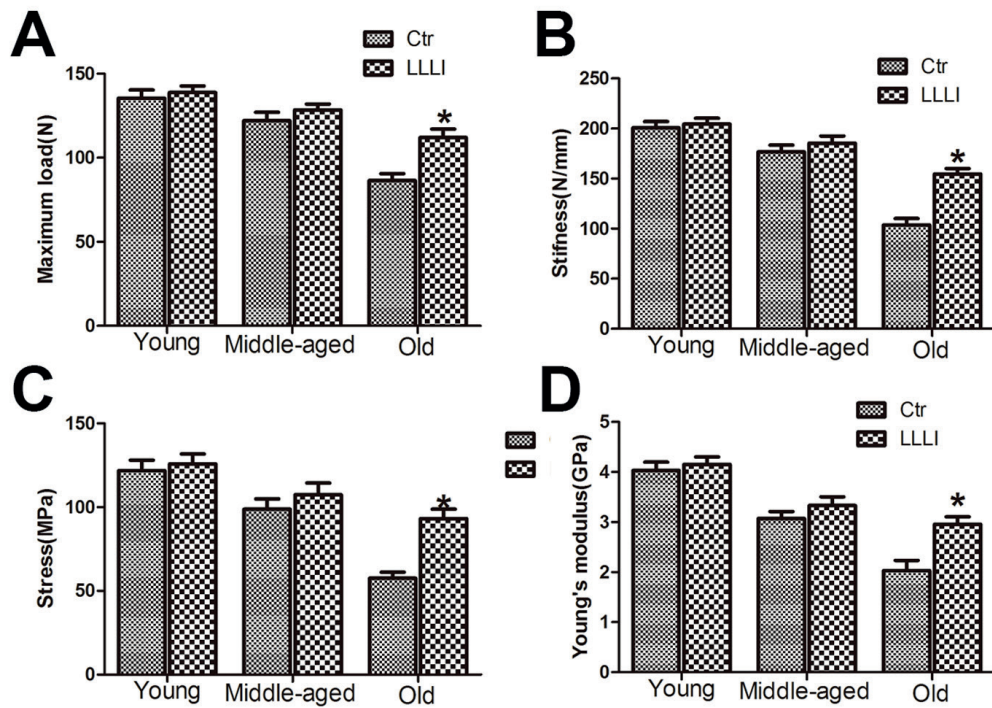


Figure 2. LLLI enhanced the mechanical strength of the femur by three-point bending test and axial compression test. (A-D) Analysis of maximum load, stiffness, maximum stress, young's modulus indicated LLLI enhanced the mechanical strength of the femur.

than that of the young rats, while the number of adipocytes was significantly higher than that of the young group, indicating that with the increase

in age, osteoblast formation in the bone marrow is significantly reduced, while the formation of adipocytes is significantly increased. After the

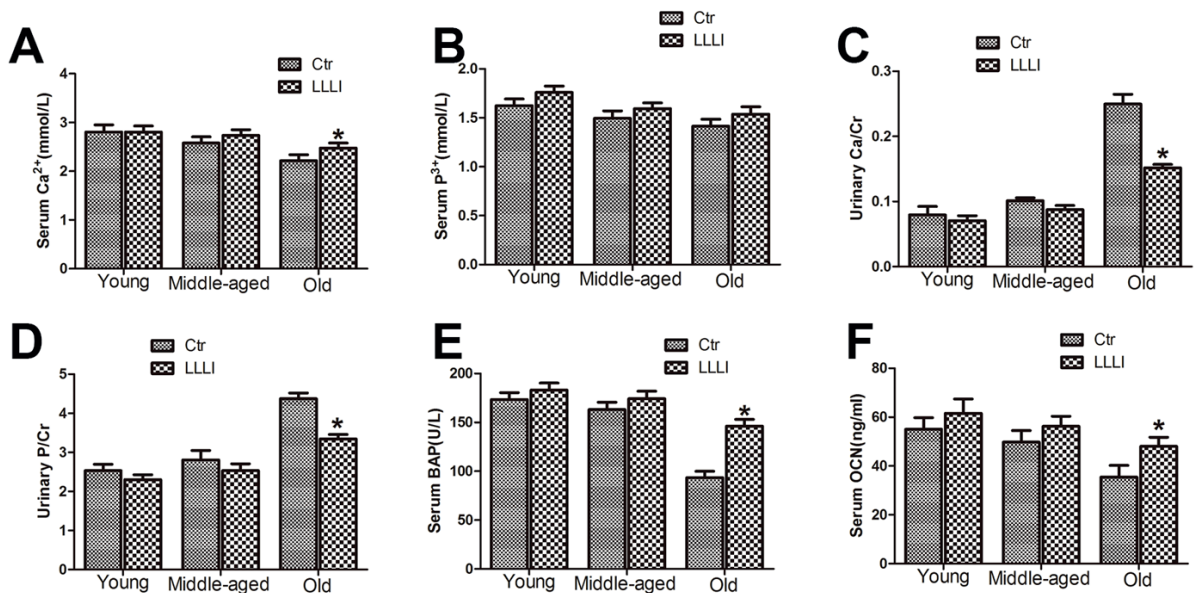


Figure 3. LLLI reduced the calcium and phosphorus losses and elevated the levels of serum BAP and OCN. (A-B) LLLI increased serum levels of Ca²⁺ and P³⁺. (C-D) LLLI reduced urinary levels of Ca²⁺ and P³⁺. (E-F) LLLI increased serum levels of BAP and OCN.

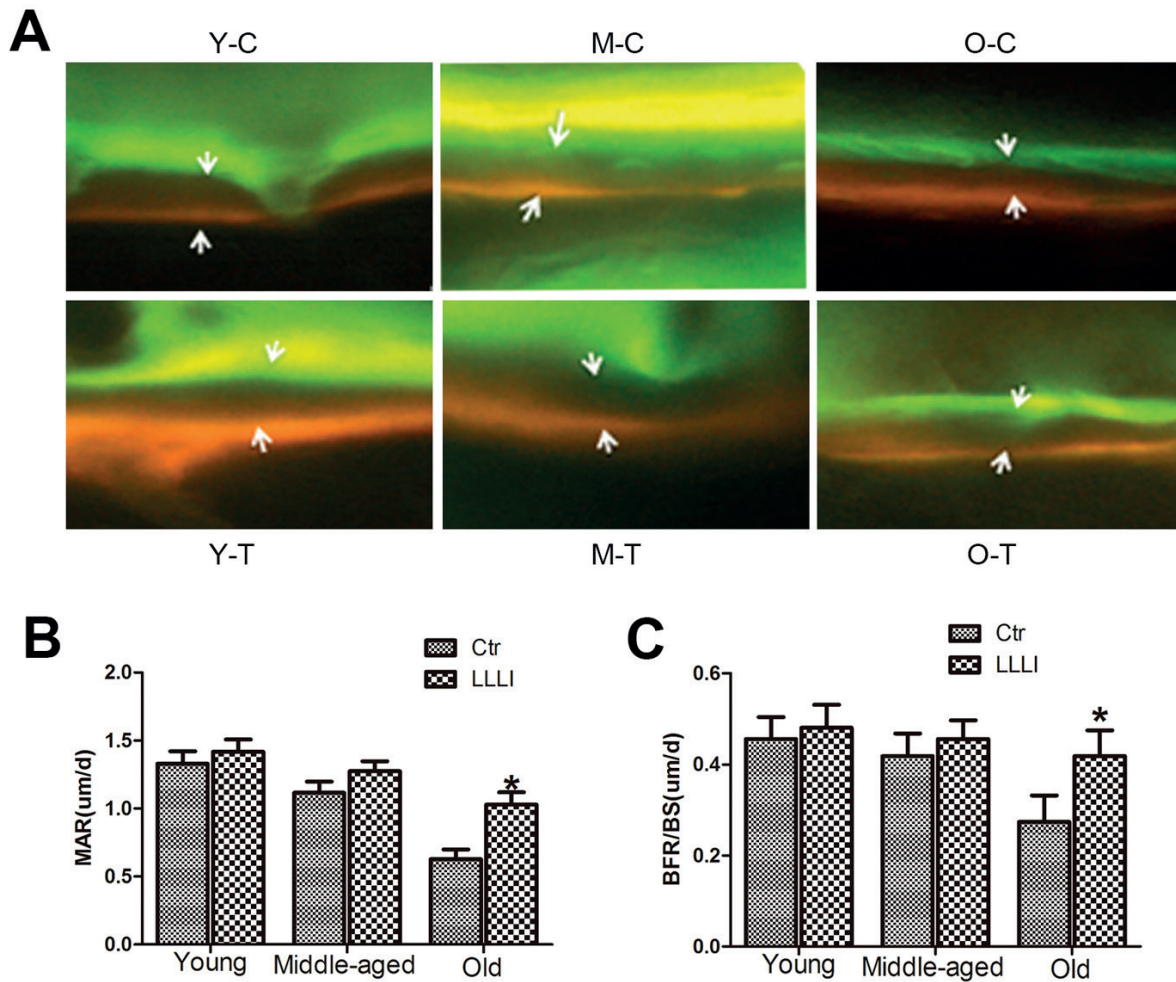


Figure 4. LLLI increased BFR and MAR of old rats by calcein and tetracycline labeling of the femur trabecular bone sections. (A) Representative images showing new bone formation examined by calcein and tetracycline labeling of the femur trabecular bone sections. (B-C) Analysis of BFR and MAR show LLLI increased bone formation.

treatment with LLLI, the number of adipocytes was significantly decreased while the number of osteoblasts was significantly increased in the old group compared with those in the control group, indicating that LLLI can inhibit the formation of adipocytes and promote the formation of osteoblasts, so it can promote the formation of bone (Figure 5).

Discussion

Primary osteoporosis is a systemic bone disease characterized by reduced bone mass and the diminution, fracture and reduced quantity of trabeculae, which ultimately increase bone fragility and risks of fracture¹⁷. Senile osteoporosis is the

most common type of osteoporosis, and pathological manifestations of the age-related osteoporosis in natural aging animals are mostly similar to those of senile osteoporosis in human. The life cycle of rats is generally 3 to 4 years. In rats at the age over 6 months old, changes in BMD and Ca in the femur have been quite small, and when they grow to 12 months old, the bone parameters have reached the platform level, followed by a relatively slowly decrease in BMD. It has been reported that the bone of male SD rats at the age of 9 months old has been fully mature, from which the age-related bone loss occurs. This process is similar to male osteoporosis. Therefore, it is recommended that male SD rats at the age of more than 9 months old can be used for establishing the age-related bone loss model. Therefore, male SD

rats at different age stages were selected as the experimental subjects.

Micro-CT is a non-destructive 3D imaging technique, through which we can clearly understand the internal microstructure of a sample without destroying it¹⁸. The biggest difference between it and ordinary clinical CT is that its resolution is extremely high and can even reach micron (μm) level¹⁹. Trabecular microstructure-related parameters include BMD, BV/TV, Tb.N, Tb.Th, Tb.Sp, and SMI. BMD refers to bone mineral density, and when osteoporosis occurs, the BMD value is decreased; BV/TV is the relative bone volume or bone volume fraction, and when osteoporosis occurs, the BV/TV value is decreased; Tb.N, Tb.Th, and Tb.Sp refer to trabecular number, trabecular thickness and trabecular spacing, respectively, and when osteoporosis occurs, Tb.N and Tb.Th are decreased, while Tb.Sp is increased; SMI is the structure model index, and it is the important index reflecting the material structure. The range of it is 0-3. In bone tissues, 0 represents plate trabeculae and 3 represents rod-shape trabeculae. When osteoporosis occurs, the SMI value is increased. The results of micro-CT showed that among rats in the old group, middle-aged group and young group, the BMD, BV/TV, Tb.N, and Tb.Th of rats in the old group were the lowest, while Tb.Sp and SMI were the highest, and the differences between the old group and the young group were statistically significant. All the indexes of rats in the mid-

le-aged group were between those of rats in the young group and the old group, indicating that the loss of bone mass is gradually increased with the increase in age and the elderly is extremely prone to senile osteoporosis. LLLI could significantly improve the BMD, BV/TV, Tb.N, Tb.Th, SMI, and Tb.Sp. related to the trabecular microstructure of old rats. The above results suggested that LLLI can significantly increase bone mass in old rats with osteoporosis and improve the trabecular microstructure.

The three-point bending test is suitable for the determination of the mechanical properties of the femur and long bones^{20,21}. The maximum load and structural hardness of bone samples are related to the size and geometry of them, and they are indexes reflecting the mechanical properties of the bone structure, which are also known as extrinsic properties. Bone stress is the load value per unit area of bone samples. Elastic modulus refers to the intrinsic hardness of bone samples. These two indexes are not affected by the size of bones, and they are indexes reflecting the mechanical properties of bone material, which are also known as intrinsic properties. The results of this study revealed that the maximum load, structural hardness, bone stress and elastic modulus of the old rats with osteoporosis were significantly reduced, and LLLI could significantly improve the above indexes, suggesting that LLLI can significantly improve the biomechanical properties of the femur, improve the internal and external properties

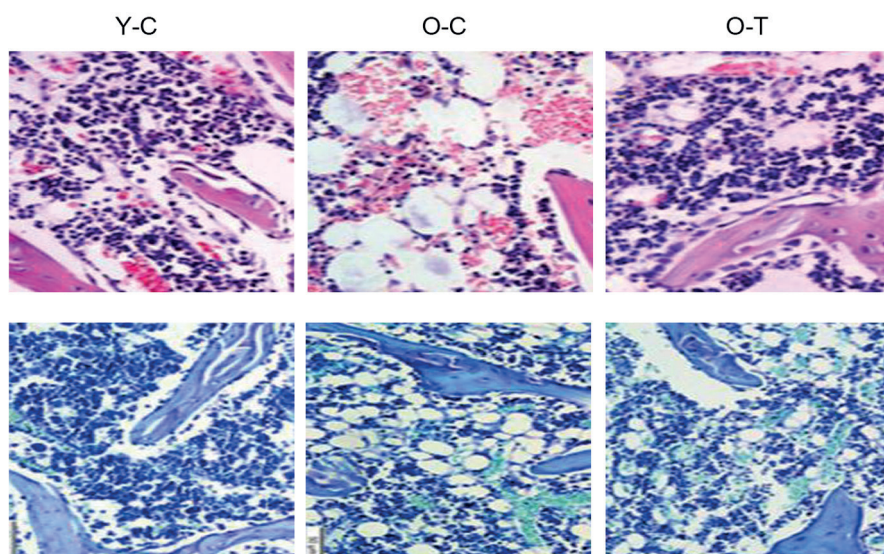


Figure 5. LLLI increased the number of osteoblasts in the bone marrow and decreased the number of adipocytes in old rats. Representative images of tibial sections from rats stained with HE and toluidine blue (magnification 200 \times).

of the femur, and enhance its mechanical strength. It was found that LLLI could significantly improve the osteoporosis and the biomechanical properties of the femur in old rats, but what mechanism does LLLI rely on to play its roles? The basic pathological mechanism of osteoporosis is the steady-state imbalance between bone formation and bone resorption. Bone resorption capacity greater than bone formation capacity leads to bone loss and increases bone fragility, but how does LLLI play a role in anti-osteoporosis, is it through bone formation or bone absorption? Based on this, the indexes reflecting bone steady balance, including urine Ca, urine P, serum alkaline phosphatase (ALP) and serum OCN were detected. When the bone resorption activity is increased, Ca and P in bones were resorbed by osteoclasts, thus increasing the concentrations of urine Ca and P; when the bone formation activity is increased, the concentrations of serum OCN and ALP are increased. The study results showed that the bone resorption in old rats was significantly enhanced, while bone formation was significantly reduced, and LLLI could significantly reduce the urine Ca and P losses in the old group, increase the levels of serum ALP and OCN, suggesting that LLLI inhibits bone resorption while promoting bone formation.

Double fluorescent labeling with tetracycline and calcein analysis is an important indicator for bone formation activity²². MAR = the average distance between tetracycline fluorescence band and calcein fluorescence band / the time interval between two times of tetracycline and calcein labeling; BFR = BMR × tetracycline and calcein labeling line edge length / bone-like surface. In this study, the detection of BMR and BFR revealed that BMR and BFR of middle-aged and young rats were significantly higher than those of the old rats, indicating that the middle-aged and young rats had stronger bone formation activity than the old rats, and LLLI can significantly increase MAR and BFR in older rats, suggesting that glucagon-like peptide 1 receptor (GLP-1R) agonists increase bone formation activity and promotes bone formation.

It was found from the detection of bone histomorphology that the number of osteoblasts in the bone marrow of rats was significantly decreased with the increase in age, while the number of adipocytes was increased significantly, and LLLI could significantly increase the number of osteoblasts and reduce the number of adipocytes in old rats. Osteoblasts and

adipocytes in the bone marrow are originated from bone marrow mesenchymal stem cells, which have the potential of multi-directional differentiation and can differentiate into osteoblasts, adipocytes, fibroblasts, etc. The results of this study indicated that LLLI may promote bone marrow mesenchymal stem cells to differentiate into osteoblasts and inhibit their differentiation into adipocytes so as to play a role in promoting bone formation.

Conclusions

LLLI can effectively improve osteoporosis, increase BMD, improve bone structure and improve bone biomechanical properties in old rats; at the same time, it increases the levels of serum BAP and OCN and the number of osteoblasts in the bone marrow, suggesting that the osteogenesis function of osteoblasts is enhanced.

Conflict of interest

The authors declare no conflicts of interest.

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