Effect of Senolytic to alleviating Osteoporosis in Mice induced by Retinoic Acid

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Abstract. Senolytic is a potential new anti-aging drug, and it is worth exploring whether it has an inhibitory effect on osteoporosis. Osteoporosis models in mice were established by gavage of retinoic acid(RA), and Senolytic drugs (dasatinib and quercetin) were used to treat for 8w. Serological indexes were measured, and bone mineral density was detected by dual-energy X-ray absorptiometry, then parameters of trabecular bone were quantified by μ CT, by which three-dimensional images of lumbar trabecular bone were rebuild for comprehensive analysis. In osteoporosis model mice the content of serum calcium and alkaline phosphatase in mice were increased, the bone mineral density decreased, bone volume fraction and trabecular number decreased significantly, and the trabecular separation increased significantly. After applying the Senolytic drug, the serum calcium of group A and B were significantly lower than that of the blank control group. The alkaline phosphatase content in the two groups was (272.5 ± 42.7) U/L and (258.4 ± 90.2) U/L, which was also significantly lower than that in the blank control group (389.0 ± 31.1) U/L. After the treatment of Senolytic, the bone mineral density of group A and group B were improved, the bone volume fraction were increased compared with the blank control group. The trabecular bone separation of the two groups was significantly reduced. Therefore, these reduced osteoporosis. From our study, it can be confirmed that the senolytic drug has a certain alleviating effect on osteoporosis.

1 introduction

China has entered an aging society, which has brought a series of problems, among which the great medical challenge is the increasing incidence of diseases related to aging[1]. Osteoporosis is a common disease characterized by decreased bone mass and increased bone fragility. Its incidence increases year by year as the age increases, and it is one of the main causes of fracture in the elderly and postmenopausal women. The two basic processes of bone metabolism are bone formation and bone resorption. Normal bone is a state of balance between the two, while osteoporosis is caused by the imbalance of bone metabolism and the amount of bone destruction is greater than the amount of bone formation, among which, excessive osteoclast function is the main cause[2]. By contrast, if it can inhibit the function of osteoclasts or reduce the number of osteoclasts in the population, it should be able to fight osteoporosis. Osteoclasts themselves are mature differentiated cells, not naive progenitor cells, and the rate of mature and even aging cells in the population is high. With this in mind, our team proposed that Senolytic drugs, specifically designed to eliminate senescent cells, might reduce the degree of osteoporosis by reducing the number of "mature active agents" in osteoclasts, thus designing the following experiment.

2 Materials and methods

2.1 Experiment materia

Experimental animals: 40 healthy and female SPF KM mice[weighing (30.1 ± 1.6) g] were provided by Hunan Slake Jingda Experimental Animal Co. LTD, production certificate No. SCXK(Xiang)2011-0003. All mice were raised in the standardized laboratory of Changsha Medical University. The mice were fed in different cages and ate freely.

Experimental supplies: Dasatinib and quercetin were donated by Dr. Liu Shuiping, School of Life Sciences, Central South University. Calcium content, phosphorus content, osteocalcin, alkaline phosphatase kit were provided by Nanjing JianCheng Institute of Biological Engineering.

2.2 Experimental procedure

2.2.1 Model making and intervention treatment

Forty female KM mice were randomly divided into four groups with 10 mice in each group. Experimental group A, B and blank control group were gavage with retinoic acid 70mg/kg for 60d, while normal control group was

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gavage with distilled water. After 4d, therapeutic gavage was started: Dasatinib and quercetin dissolved in 10% PEG400 were given doses of 5mg/kg and 50mg/kg respectively. Experimental group A was given gavage once A week, while experimental group B was given gavage twice A week. The blank control group and the normal control group were given 10% PEG400 by gavage once a week. The treatment time of each group lasted 8W. If the modeling gavage and the therapeutic gavage were performed on the same day, the interval between them was greater than 6 hours.

2.2.2 Detection of bone - related serological indicators

Mice were sacrificed, and their whole blood was collected through heart puncture, and serum was separated. The content of calcium and phosphorus in serum was determined by absorbance after chemical reaction. The levels of osteocalcin and alkaline phosphatase were detected by enzyme - linked immunosorbent assay (ELISA).

2.2.3 Determination of bone mineral density and bone trabecula

Lumbar L2-L3 was isolated and immobilized in formalin for bone mineral density and μ CT. The mineral density of lumbar vertebrae was measured with GE Prodigy Advance dual-energy X-ray absorptiometry. Bone volume/total volume (BV/TV), Trabecular number (Tb.N), trabecular thickness (Tb.Th), and trabecular separation (Tb.Sp) were quantitatively analyzed using a CT scanner (55KV,140mA) from Scanco Medical Co. LTD. Three-dimensional images of the lumbar spine segments were reconstructed using two-dimensional data from 200 scan sections.

2.2.4 data statistics

SPSS 10.0 statistical software was used to process experimental data, and measurement data were expressed in the form of mean $\pm$ standard deviation. One-way ANOVA was used for comparison, and the test level value was set at 0.05 on both sides.

3 Results

3.1 Daily status of mice

Weight gain, diet and exercise were normal in each group.

3.2 Serological index detection

The content of serum calcium, phosphorus and alkaline phosphatase in blank control group was higher than that in normal control group, indicating successful modeling. After treatment with Senolytic dasatinib and quercetin, serum calcium levels in group A and group B were

significantly lower than those in the blank control group and similar to those in the normal control group. There was no statistical difference between the two groups and those in the normal control group, experiment group A and group B. The content of alkaline phosphatase in group A and group B was (272.5 $\pm$ 42.7) U/L and (258.4 $\pm$ 90.2) U/L, which was also significantly lower than that in the blank control group (389.0 $\pm$ 31.1) U/L and close to that in the normal control group (180.6 $\pm$ 22.8) U/L. There was no significant difference between the groups in blood phosphorus concentration and osteocalcin content. (Table 1)

Table 1 Effect of Senolytic drug on calcium related indexes in serum of mice ($\bar{x}\pm s$, n=10)

group	Calcium (mmol/L)	Phosphorus (mmol/L)	Osteocalcin (μ g/L)	alkaline phosphatase (U/L)
group A	1.35 $\pm$ 0.21 ^a	1.71 $\pm$ 0.33	1.12 $\pm$ 0.30	272.5 $\pm$ 42.7 ^a
group B	1.44 $\pm$ 0.12 ^a	1.52 $\pm$ 0.70	0.78 $\pm$ 0.41	258.4 $\pm$ 90.2 ^a
blank control group	1.78 $\pm$ 0.17	1.81 $\pm$ 0.29	0.49 $\pm$ 0.35	389.0 $\pm$ 31.1
normal control group	1.42 $\pm$ 0.16 ^a	1.38 $\pm$ 0.14 ^a	1.22 $\pm$ 0.56	180.6 $\pm$ 22.8 ^b
F value	3.58	0.45	1.99	4.01
P value	0.034	12.92	1.303	0.019

a. compared with blank control group, 0.01<P<0.05; b. compared with blank control group, P<0.01

3.3 Bone mineral density

Dual-energy X-ray absorption is the "gold standard" for clinical diagnosis of osteoporosis [3], which can be used for quantitative analysis of bone mineral density. The results showed that the bone mineral density of the blank control group was lower than that of the normal control group after retinoic acid administration (P<0.05), indicating the successful preparation of the mouse model of osteoporosis. After Senolytic therapy, the bone mineral density of group A and Group B was (0.366 $\pm$ 0.012) g and (0.373 $\pm$ 0.021) g, which was higher than that of the blank control group (0.302 $\pm$ 0.049) g (P<0.05). There was no significant difference between the two experimental groups, between the experimental group and the normal control group. (Table 2)

Table 2 Effects of Senolytic drug on bone mineral density in mice ($\bar{x}\pm s$, n=10)

group	BMD(g)
group A	0.366 $\pm$ 0.012 ^a
group B	0.373 $\pm$ 0.021 ^a
blank control group	0.302 $\pm$ 0.049
normal control group	0.398 $\pm$ 0.023 ^a
F value	3.89
P value	0.030

a. compared with blank control group, 0.01<P<0.05

3.4 Trabecular bone condition

Quantitative analysis of bone trabeculae at microscopic level for each group can be done by μ CT. The results showed that the bone volume fraction(BV/TV) of the blank control group was significantly lower than that of the normal control group ($P<0.01$), while the number of trabecular bone(Tb.N) was significantly decreased ($P<0.01$), and the separation degree of trabecular bone(Tb.Sp) was significantly increased ($P<0.01$). After treatment, BV/TV and Tb.N in group A and group B increased and Tb.Sp significantly decreased compared with the control group ($P<0.01$). The changes of the above indexes were similar to that of the normal control group. There was no significant difference in bone trabecular thickness among the groups.(Table 3)

Table 3 Effects of Senolytic drug on trabecular structure parameters in mice ($\bar{x}\pm s$, n=10)

group	BV/TV(%)	Tb.N(1/m m)	Tb.Th(mm)	Tb.Sp(mm)
group A	12.84±2.12 _a	3.11±0.41 _a	0.044±0.003	0.36±0.05 ^b
group B	13.31±2.29 _a	2.92±0.72	0.042±0.002	0.39±0.04 ^b
blank control group	9.12±1.15	1.90±0.33	0.040±0.001	0.58±0.04
normal control group	16.60±2.34 _b	3.56±0.24 _b	0.049±0.003 _a	0.30±0.02 ^b
F value	4.38	2.97	0.25	18.82
P value	0.010	0.047	38.10	0.000

a. compared with blank control group, $0.01<P<0.05$; b. compared with blank control group, $P<0.01$

Fig.1. Effect of Senolytic drug on trabecular structure of lumbar vertebrae in mice

Based on μ CT plain scan, the reconstructed 3d images showed that the normal control group had normal shape, complete structure and dense trabecular bone (Fig.1.D). In the blank control group, the trabecular bone structure was damaged, with sparse overall structure and many fractures, and the joint cavity formed was large and obvious (Fig.1.C). After simultaneous treatment with Dasatinib and quercetin, more trabecular bones and significantly reduced large cavities were observed in the two groups (Fig.1.A and B).

4 Discuss

Biological senescence is mainly targeted at proliferative cells. DNA damage and/or accumulation of other cellular stressors (such as carcinogenesis, reactive metabolites, and protein-toxic stress) cause a decline in cell proliferation and enter terminal differentiation[4]. Age-related diseases have also been linked to senescent cells. Studies have shown that with the increase of age, various cell types in the bone microenvironment gradually senescence, which is closely related to senile osteoporosis[5]. In order to verify the hypothesis of senescent cells (especially mature and senescent osteoclasts) and bone loss, Senolytic drug was used to treat retinoid-induced osteoporosis mouse model, and the possibility of Senolytic drug for osteoporosis was explored.

Senolytic drug is a big breakthrough in the field of anti-aging research in 2015, with a wide range of applications and great medical potential[6], which has been reported by various countries[7]. A research team from the United States has developed a new kind of drug that can significantly slow the aging process in animal models -- reducing symptoms of weakness, improving

heart function, and extending healthy life. Scientists call the new drugs "Senolytic"[8], Dasatinib and quercetin [9-10].

The drugs extend the healthy lives of mice by clearing away senescent cells. The preliminary experiment of dasatinib and quercetin single drug intervention was carried out at the beginning of this project, and the experimental effect was found to be limited. Therefore, according to the suggestion of Weivoda research group, dasatinib 5mg/kg and quercetin 50mg/kg were combined for intermittent administration, and the significant effect was found to take up to 8 weeks at the earliest, so the experimental scheme was determined. In this study, compared with the pure osteoporosis model mice (blank control group), the blood calcium and alkaline phosphatase content of mice treated with Senolytic drug were significantly reduced, suggesting that Senolytic drug inhibited the osteoclast destruction of bone. In addition, the bone mineral density of the spine was increased, and the trabecular bone microstructure was significantly improved. This partly confirms our hypothesis that osteoclasts belong to a more "mature" or even "somewhat senescent" cell population, and therefore are more affected by Senolytics than osteoblasts, and may be cleared more, thus reducing the degree of bone decomposition and absorption and reducing the occurrence of osteoporosis.

In this study, the Senolytic drug were used for mice as a whole. In addition to the removal of senescent cells, studies have also confirmed that it can improve cardiovascular function[11-14] improve insulin sensitivity, whether because of improved the other physiological functions of mice model which reduces the degree of osteoporosis, still uncertain. So the team plan that Senolytic drug directly act on osteoclasts and osteoblasts, in order to make clear the mechanism of drug to osteoporosis. Whether or not Senolytic drugs are eventually used in osteoporosis and other diseases of aging, our trial provides an idea for the treatment of age-related bone loss.

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