Chin J Integr Med 2019 Mar;25(3):210-215

Chinese Journal of Integrative Medicine

Available online at link.springer.com/journal/11655
Journal homepage: www.cjim.cn/zxyjhen/zxyjhen/ch/index.aspx
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Original Article

Ursolic Acid Prevents Retinoic Acid-Induced Bone Loss in Rats*

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ABSTRACT **Objective:** To examine the effects of ursolic acid (UA) on mitigating retinoic acid (RA)-induced osteoporosis in rats. **Methods:** Fifty female Sprague-Dawley rats were randomly divided into the control group (n=10) and the osteoporosis group (n=40). The 40 osteoporosis rats were induced by 75 mg/(kg•d) RA once daily for 2 weeks, and then were randomly assigned to vehicle control (model), low-, middle-, and high-dose UA [(UA-L, UA-M, UA-H; 30, 60, 120 mg/(kg•d), respectively] groups (10 rats each). UA were administered once daily to the rats from the 3rd weeks for up to 4 weeks by gavage. Bone turnover markers [serum alkaline phosphatase (ALP), osteocalcin (OCN), urine deoxypyridinoline (DPD)] and other parameters, including serum calcium (S-Ca), serum phosphorus (S-P), urine calcium (U-Ca), urine phosphorus (U-P), and bone mineral density (BMD) of the femur, 4th lumbar vertebra and tibia, bone biomechanical properties and trabecular microarchitecture, were measured. **Results:** The osteoporosis in rats was successfully induced by RA. Compared with the model group, UA-M and UA-H significantly reversed the RA-induced changes in S-P, U-Ca, U-P, ALP, OCN and urine DPD ratio and markedly enhanced the BMD of right femur, 4th lumbar vertebra and tibia (P<0.05 or P<0.01). Further, biomechanical test and microcomputed tomography evaluation also showed that UA-H drastically improved biomechanical properties and trabecular microarchitecture (P<0.05 or P<0.01). **Conclusion:** UA could promote bone formation, increase osteoblastic activity and reduce osteoclastic activity in rats, indicating that UA might be a potential therapeutic of RA-induced acute osteoporosis.

KEYWORDS osteoporosis, bone turnover, ursolic acid, bone mineral density, biomechanics, microcomputed tomography

As a derivative of vitamin A, retinoic acid (RA) is one of the commonly used drugs for skin diseases and tumors. In the last century, it was noticed that large doses of vitamin A (RA) in a short period (1–3 weeks) could cause oxidative stress, and decrease bone mineral density (BMD), and cause histomorphometrical structural changes, manifestations of osteoporosis.[1-4] Therefore, RA is frequently used for inducing acute osteoporosis animal model due to its simple operation, short time period, low cost, high successful rate, and great similarity to typical clinical symptoms of osteoporosis.[5-7]

Many natural compounds from plants have been shown to prevent bone loss in osteoporotic rats.[8-11] Ursolic acid (UA) is one of ubiquitous triterpenoids in medicinal herbs, such as *Fructus Ligustri Lucidi*, *Cornus officinalis*,[12] etc. UA was detected throughout the plant kingdom and constituted an integral part of the human diet.[13] Pharmacological effects of UA include anticancer,[14] anti-liver damage,[15-17] pro-differentiation,[18] antiviral,[19] antileukemia,[20] antibacteria,[21] and antiinvasion activities.[22] UA rich herb *Fructus Ligustri Lucidi* has been shown to be protective against bone loss in rodents.[23,24] However, the pharmacological effect of UA on RA-induced osteoporosis remains unknown. This study was to evaluate whether UA had effects on osteoporosis induced by RA in rats.

METHODS

Animals

Fifty female Sprague-Dawley (SD) rats (specific-pathogen-free grade, National Grade A experimental

*Supported by Qinling-Bashan Mountains Bioresources Comprehensive Development Collaborative Innovation Center (No. QBXT-Z(Z)-15-4)

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DOI: https://doi.org/10.1007/s11655-018-3050-y

animal) 90 days old, weighing 160–200 g, were obtained from the Experimental Animal Center of the Fourth Military Medical University (approval No. SCXK2007-007). The rats were housed at 21±2 ℃ with 60%–70% humidity and a 12-h light/dark cycle and free access to food and water. The animal care and experimental procedures were conformed to the Guide for the Care and Use of Laboratory Animals and all protocols were approved by Institutional Animal Care and Usage Committee of Fourth Military Medical University.

Grouping and Administration

Fifty rats were randomly divided into the control group (*n*=10) and the osteoporosis group (*n*=40) by random number table method. The 40 osteoporosis rats were treated with 75 mg/(kg•d) RA (batch No. 201403, Xiya Chemical Technology Co., Ltd., China) once daily in the first 2 weeks,[5-7] and then were randomly assigned to 4 groups (10 rats each) by random number table method: RA with distilled water as vehicle (model); RA with UA ($C_{30}H_{48}O_3$, batch No. 201312, Nanjing Dilger Medical Techology Co., China) in low-, middle-, and high-doses [UA-L, UA-M, UA-H; 30, 60, 120 mg/(kg•d), respectively]. UA were administration to the rats once daily from the 3rd week for up to 4 weeks by gavage.

All test components were dissolved in ethanol and further dilutions were made in water before feeding the rats. The final concentration of ethanol ⩽ 0.5% (v/v), ethanol (0.5%) was used in the control and model groups. All rats were maintained under the same living conditions during the study.

Sample Collection and Preparation

Urine samples were collected in metabolic cages without feeding them for a day. Serums were prepared by centrifugation (2,000 r/min for 20 min) of the blood which collected via abdominal aorta puncture. The collected urine and serum samples were stored at –80 ℃ until use. The rats were sacrificed by CO_2 inhalation. The left femur, 4th lumbar vertebra and tibia were dissected and filled in physiological saline and stored at –20 ℃ for measuring BMD by dual-energy X-ray absorptiometry (DEXA), trabecular microarchitecture by microcomputed tomography (microCT), and bone biomechanical parameters by three-point bending test and crush test.

Serum and Urine Analyses

The serum and urine samples were used to measure bone metabolism-related biochemical indicators with a suitable commercial kit (Zhongsheng Beikong Bio-technology and Science, China), including serum calcium (S-Ca), serum phosphorus (S-P), urine calcium (U-Ca) and urine phosphorus (U-P). Enzyme-linked immuno sorbent assay (ELISA) kits (Quidel, San Diego, USA) were used to determine the levels of serum alkaline phosphatase (ALP), osteocalcin (OCN), and urine deoxypyridinoline (DPD).

Bone Analysis

Two-dimensional BMD of the right femur were measured using Lunar Prodigy Advance by DEXA (GE Healthcare, USA) equipped with appropriate software for bone density assessment in small laboratory animals as reported elsewhere.[25] The isolated right femurs were assessed for three-point bending test by using the 858 Mini Bionix material testing machine (MTS, Eden Prairie, Minnesota, USA).[26] The isolated left femurs were assessed for structural strength test (compression test) by using tensile strength testing apparatus (YLS-16A, Jinan Yiyan Technology Development Co. Ltd., Shandong, China).[27]

The morphometric parameters of right distal femur from each group were evaluated by Explore Locus SP Pre-Clinical Specimen microCT (GE Healthcare, USA).[28] This region included 350 images obtained from each femur using 1024×1024 matrix resulting in an isotropic voxel resolution of 22 μm^3.[29] The volume of interest (VOI) was selected as a region 25 slices away from the growth plate at the proximal end of the femur to 125 slices. Three-dimensional (3D) image data of the femur was acquired with a voxel size of 12 μm in all special directions.[30] The following densitometry and architectural parameters were directly determined from the binarized VOI: bone volume over total volume (BV/TV), trabecula number (Tb.N), trabecula separation (Tb.Sp), trabecula thickness (Tb.Th), connectivity density (Conn.D), and structure model index (SMI). The operator conducting the scan analysis was blinded to the treatments associated with the specimen.[31]

Statistical Analysis

All statistical analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Data were expressed as mean±standard deviation ($\bar{x}\pm s$). Inter-group differences were analyzed by one-way ANOVA, followed by the least-significant difference multiple-

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range test. Statistical significance was set at $P<0.05$.

RESULTS

BMD Analysis

The BMD of the femur, 4th lumbar vertebra and tibia of RA-induced osteoporosis rats in the model group was significantly reduced compared with the control group ($P<0.05$). The 28-day treatment with UA-H and UA-M markedly enhanced the BMD of femur, 4th lumbar vertebra and tibia compared with the model group ($P<0.05$ or $P<0.01$, Figure 1).

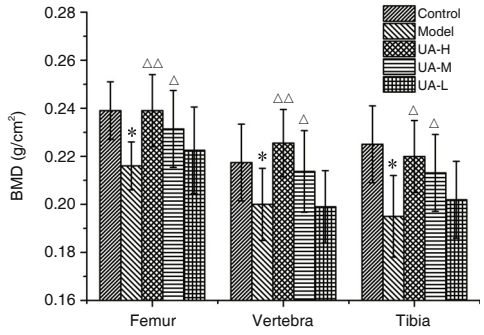

Figure 1. Effects of UA on Total BMD in Femur, 4th Lumbar Vertebra and Tibia of Rats by DEXA ($\bar{x}\pm s$, n=10)

Notes: UA: ursolic acid; RA: retinoic acid; BMD: bone mineral density; DEXA: dual-energy X-ray absorptiometry. $^{*}P<0.05$ *vs*. control group; $^{\triangle}P<0.05$ *vs*. model group

Biochemical Assay

The S-Ca and S-P levels were significantly decreased and U-Ca level was significantly increased in the model group compared with the control group ($P<0.05$ or $P<0.01$). UA-H, UA-M, and UA-L substantially reversed the RA-induced changes in S-P, U-Ca and U-P levels ($P<0.01$). Moreover, UA-H significantly increased the S-Ca level, compared with the model group ($P<0.05$, Table 1).

Table 1. Effects of UA on Biochemical Parameters in Serum and Urine of Rats (mmol/L, $\bar{x}\pm s$)

Group	n	S-Ca	S-P	U-Ca	U-P
Control	10	2.43±0.15	2.03±0.22	0.24±0.10	0.79±0.15
Model	10	2.21±0.07**	1.70±0.16*	0.27±0.33*	0.91±0.04
UA-H	10	2.33±0.06$^{\triangle}$	2.03±0.77$^{\triangle\triangle}$	0.23±0.01$^{\triangle\triangle}$	0.73±0.12$^{\triangle\triangle}$
UA-M	10	2.26±0.05	2.14±0.37$^{\triangle\triangle}$	0.24±0.01$^{\triangle\triangle}$	0.75±0.09$^{\triangle\triangle}$
UA-L	10	2.25±0.05	2.16±0.26$^{\triangle\triangle}$	0.24±0.01$^{\triangle\triangle}$	0.76±0.04$^{\triangle\triangle}$

Notes: S-Ca: serum calcium, S-P: serum phosphorus, U-Ca: urine calcium, U-P: urine phosphorus. $^{*}P<0.05$, $^{**}P<0.01$ *vs*. control group; $^{\triangle}P<0.05$, $^{\triangle\triangle}P<0.01$ *vs*. model group

RA caused significant increase in serum ALP, OCN and urine DPD ratio by 47%, 36%, and 40%, respectively compared with the control group ($P<0.01$). UA-H and UA-M significantly reversed the RA-induced changes in ALP, OCN and urine DPD ratio ($P<0.05$ or $P<0.01$). UA-L group had no significant effect in reducing bone turnover markers ($P>0.05$, Figure 2).

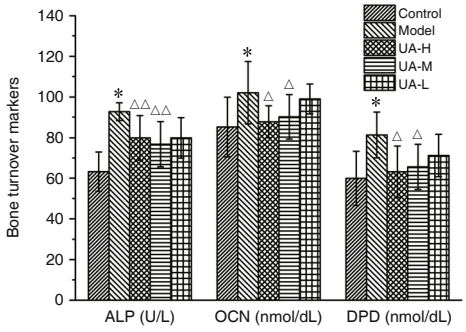

Figure 2. Effects of UA on Bone Turnover Markers in Rats ($\bar{x}\pm s$, n=10)

Notes: ALP: alkaline phosphatase, OCN: osteocalcin, DPD: deoxypyridinoline. $^{*}P<0.01$ *vs*. control group; $^{\triangle}P<0.05$, $^{\triangle\triangle}P<0.01$ *vs*. model group

Biomechanical Test

RA caused drastic reduction of the maximum load, stiffness, energy, maximum stress, elastic modulus, and compression strength ($P<0.01$). The 28-day treatment with UA-H resulted in marked increase of maximum load, stiffness, energy, maximum stress, elastic modulus, and compression strength compared with the model group ($P<0.05$ or $P<0.01$). Treatment of RA rats with UA-M increased the maximum load, energy, elastic modulus and compression strength ($P<0.05$ or $P<0.01$) but not the stiffness or maximum stress ($P=0.071$ and 0.062, respectively, Figure 3).

MicroCT Evaluation

Two weeks after oral large doses of RA, the model rats had lower values in BV/TV, Conn.D, Tb.N and Tb.Th, and higher values in Tb.Sp and SMI, when compared with the control group ($P<0.01$). Compared with the model group, treatment with UA-H and UA-M reversed the abovementioned findings ($P<0.05$ or $P<0.01$, Table 2). Figure 4 shows that UA-H treatment reduces the RA-induced damage to trabecula.

DISCUSSION

Previous study suggested that large doses of RA had outstanding short-term effects on producing the osteoporosis model and it led to increase of periosteal resorption in relation with periosteal extension or disposal of minerals,[32] which ultimately led to bone mineral loss. Plant compounds have pharmacological applications and therapeutic value for treating or preventing several bone diseases

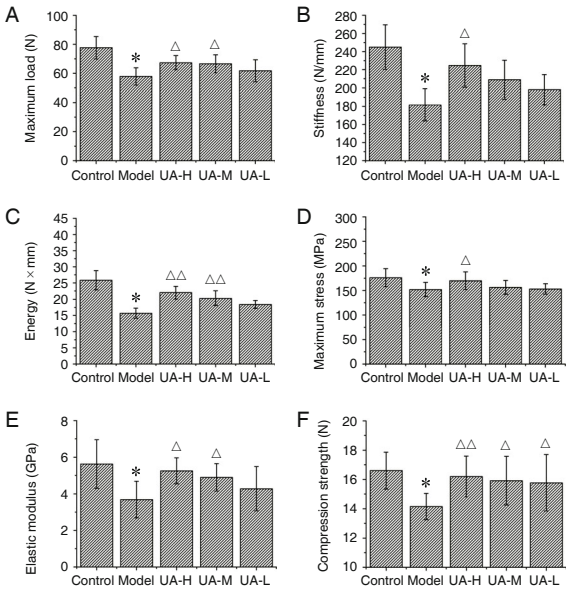

Figure 3. Effects of UA on Bone Biomechanical Parameters Measured by Three-Point Bending Test and Compression Test in Femoral of Rats ($\bar{x} \pm s$, n=10)

Notes: $^{*}P$<0.01 *vs*. control group; $^{\triangle}P$<0.05, $^{\triangle\triangle}P$<0.01 *vs*. model group

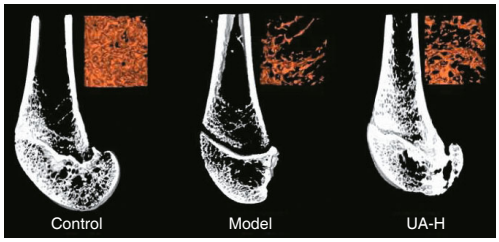

Figure 4. Representative Sample of 2D Mapping and 3D Architecture of Trabecula Bone in Distal Femur Region of Rats

characterized by excessive bone resorption.[33,34] Lee, et al[13] have shown that UA had certain preventive effect against osteoporosis in mice by promoting bone formation, inhibiting bone resorption, and regulating bone metabolism. It is speculated that UA exerted its anabolic effect on RA-induced osteoporosis in rats. This study demonstrated that UA stimulated bone formation based on both 2D and 3D bone histomorphometric analysis, suggesting that UA might be a potential therapeutic agent for osteoporosis.

The investigation showed that UA-H was able to prevent the decreased S-Ca and S-P and lower the increased rates of U-Ca and U-P excretion induced by RA, which might be resulted from increased intestinal absorption and kidney secretion of Ca and phosphor.[35] DPD served as an important role in monitoring bone resorption.[36] ALP,[37-39] OCN[40-42] were biomarkers of bone formation, released from osteoblasts into serum and reflected the activity of osteoblasts.[43] This study showed that treatments with UA-H and UA-M for 2-weeks significantly decreased the bone turnover markers ALP, OCN, and DPD in RA-induced osteoporosis rats.

As expected, large dose of RA resulted in significant decrease of BMD in the femur, 4th lumbar vertebra and tibia after 2 weeks. Those results were in accordance with the previous observation.[44] At the same time, large dose of RA significantly reduced the values of Tb.N, Tb.Th and Conn.D, while increased the value of Tb.Sp, which were the typical changes of cancellous bone loss. Furthermore, the findings were in agreement with other investigator.[45] Although UA-H treatment significantly reversed the above changes, it was not able to restore trabecular bone completely. This loss of bone mass was accompanied by a significantly increased in bone remodeling, as was evidenced by the enhanced levels of the bone turnover markers ALP, OCN and DPD. Treatment with UA-H and UA-M prevented the decline in BMD, which were reflected by the decreases in serum ALP, OCN and urine DPD ratio.

We utilized both the three-point bending test and compression test to determine biomechanical properties of bone.[46-49] Six indicators of the biomechanical properties of bone were assessed after the test of three points bending and crushing force of the femur in rats,

Table 2. MicroCT 3D Parameters of Trabecula Bone in Distal Femur Region of Rats ($\bar{x} \pm s$)

Group	n	BV/TV	Conn.D (1/mm^3)	SMI	Tb.N (1/mm)	Tb.Th (mm)	Tb.Sp (mm)
Control	10	0.307 ± 0.005	49.342 ± 1.300	1.480 ± 0.110	5.950 ± 0.170	0.070 ± 0.006	0.137 ± 0.002
Model	10	0.168 ± 0.019*	36.491 ± 2.260*	2.560 ± 0.120*	2.071 ± 0.121*	0.043 ± 0.004*	0.236 ± 0.002*
UA-H	10	0.273 ± 0.111$^{\triangle\triangle}$	43.353 ± 1.473$^{\triangle\triangle}$	1.400 ± 0.107$^{\triangle\triangle}$	4.280 ± 0.066$^{\triangle\triangle}$	0.063 ± 0.001$^{\triangle\triangle}$	0.183 ± 0.005$^{\triangle\triangle}$
UA-M	10	0.224 ± 0.062$^{\triangle}$	41.524 ± 1.109$^{\triangle\triangle}$	1.855 ± 0.119$^{\triangle}$	4.080 ± 0.085$^{\triangle\triangle}$	0.058 ± 0.001$^{\triangle\triangle}$	0.205 ± 0.001$^{\triangle\triangle}$
UA-L	10	0.180 ± 0.015$^{\triangle}$	38.881 ± 1.095	2.239 ± 0.131	3.080 ± 0.057$^{\triangle\triangle}$	0.053 ± 0.003$^{\triangle\triangle}$	0.210 ± 0.002$^{\triangle}$

Notes: BV/TV: bone volume over total volume, Tb.N: trabecula number, Tb.Sp: trabecula separation, Tb.Th: trabecula thickness, Conn.D: connectivity density, SMI: structure model index. $^{*}P$<0.01 *vs*. control group; $^{\triangle}P$<0.05, $^{\triangle\triangle}P$<0.01 *vs*. model group

including maximum load, stiffness, energy, maximum stress, elastic modulus and compression strength. Among them, maximum stress and elastic modulus were indicators of the intrinsic characteristics of bones, while maximum load, stiffness, energy and compression strength were indicators of the extrinsic characteristics of bones.[50] Large dose of RA caused the decrease of bone biomechanical properties compared with the control group. Treatment with UA-H greatly strengthened the biomechanical properties, which might be due to the maintenance of the trabecular bone structure compared with the model group.

In conclusion, this study clearly demonstrated that 4-week administration of UA for RA-induced osteoporosis adult rats could prevent the bone loss and deterioration of trabecular microarchitecture, thereby, and maintain biomechanical competence of bones. These results also suggested that UA might be a potential therapeutics of RA-induced acute osteoporosis. The molecular mechanism of the effect might be related to the rate of bone turnover down-regulated by UA, and further investigation was required to understand the mechanism.

Conflict of Interest

The authors reported no declarations of interest.

Author Contributions

Cheng M was responsible for conception and design of the study, carried out the literature search, performed data analysis, and drafted the manuscript. Liang XH performed data extraction and drew figures and tables. Wang QW and Deng YT participated in animal experiment. Zhao ZX participated in critically revised the manuscript. Liu XY participated in conception and design of the study. All authors read and approved the final manuscript.

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(Accepted November 29, 2016; First Online August 30, 2018)
Edited by YU Ming-zhu