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Rosmarinic acid alleviates diabetic osteoporosis by suppressing the activation of NLRP3 inflammasome in rats

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ABSTRACT

Background: Diabetic osteoporosis is a common metabolic bone disorder characterized by bone loss in diabetic patients, which causes an enormous social burden due to the unsatisfactory outcome of current therapeutic strategy. *Methods:* Based on the importance of inflammasome activation in diabetic osteoporosis, we evaluated the protective effect of an antioxidant, rosmarinic acid (RA) in diabetic osteoporosis. Bone marrow-derived monocytes isolated from rats were treated with receptor activator of nuclear factor kappa-B ligand (RANKL) and macrophage colony stimulating factor to differentiate into mature osteoclasts (OCs). Next OCs were stimulated with RA under high glucose condition to evaluate bone resorption. Next, streptozotocin (STZ)-injected rats were orally treated with 50 mg kg^{-1} RA to analyze its effect on diabetic osteoporosis. *Results:* RA inhibited high glucose-stimulated inflammation and inflammasome activation in OCs. Bone resorption was also reduced after RA treatment as shown by the resorption pits assay. Moreover, RA significantly reduced bone resorption, alleviated bone weight loss and increased bone mineral density by inhibiting the activation of NACHT-LRR-PYD domains–containing protein 3 (NLRP3) inflammasome in STZ-induced diabetic rats, leading to the improvement of diabetic osteoporosis. *Conclusion:* RA effectively ameliorates diabetic osteoporosis in STZ-induced rats by inhibiting the activation of NLRP3 inflammasome in OCs, which suggests that RA might serve as a potential candidate drug for treating diabetic osteoporosis.

KEYWORDS

rosmarinic acid, diabetic osteoporosis, inflammasome, osteoclast

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INTRODUCTION

Osteoporosis is a systemic bone disorder caused by a dysfunction in bone remodeling characterized by micro-architectural deterioration of bone tissue, low bone mass, reduced bone strength, decreased bone quality and induced bone fragility, leading to the increase in fracture risk [1]. Osteoporosis is also the most common metabolic bone disease in the United States.

Diabetic osteoporosis is one of the common complications of diabetes in the skeletal system. Although osteoporosis was not traditionally recognized as a diabetic complication, their important relationship has recently been proven by more evidence [2–4]. With the ageing trend of world population and the increased annual incidence of diabetes, the prevalence of diabetic osteoporosis is also significantly growing and the disease emerges as a major public health problem due to its association with fractures [5, 6]. Therefore, evaluating the pathogenesis and developing new drugs for diabetic osteoporosis is critical for preventing and managing this disease.

Several factors are implicated in diabetic osteoporosis, including reactive oxygen species (ROS) [7]. Hyperglycemia stimulates the excessive production of ROS, leading to the imbalance of oxidants and anti-oxidants, and consequently to inflammation-related diseases, including diabetic osteoporosis. ROS mediates diabetic osteoporosis through multiple mechanisms. For examples, ROS deteriorated osteoclastic bone resorption [8]. Numerous studies indicated that ROS-induced NACHT-LRR-PYD domains–containing protein 3 (NLRP3) inflammasome activation played a critical role in the development of diabetic osteoporosis. In addition, several natural compounds, mostly anti-oxidants, were studied as alternative options for treating diabetic osteoporosis, which could complementarily overcome the inherent side effects of the current therapies under long-term use [8]. Rosmarinic acid (RA) also is a natural compound extracted from natural plants. It has anti-inflammatory, anti-oxidative and insulin-sensitizing activities. A previous study revealed that RA inhibited high glucose-activated inflammasome activation in endothelial cells [9]. In this investigation, we hypothesized that RA as an anti-oxidant had an inhibitory effect on the activation of NLRP2 inflammasome and a protective role in diabetic osteoporosis.

MATERIALS AND METHODS

In vitro osteoclastogenesis

Osteoclasts were obtained as previously described [7]. Briefly, the long bones of Sprague-Dawley rats (8-week-old males) were flushed to get whole bone marrow cells, then cells were cultured in 100-mm dishes in α-minimum essential medium (MEM) containing fetal bovine serum (FBS, 10%, Gibco, Grand Island, NY) at 37 °C with 5% CO_2 overnight. Nonadherent cells were collected and cultured in the medium supplemented with recombinant rat macrophage-colony stimulating factor (M-CSF, 25 ng ml^{-1}, PeproTech, Bedford, MA) for 48 h to get purified bone marrow–derived monocytes (BMMs). BMMs were cultured in α-MEM with M-CSF and the recombinant rat receptor activator of nuclear factor kappa-B ligand (RANKL, 50 ng ml^{-1}). After 7 days, rat osteoclasts that were large, multinucleated and spread cells were observed. To confirm the differentiation of multinucleated osteoclasts, cells were stained with tartrate-resistant acid phosphatase (TRAP) (Leukocyte Acid Phosphatase Kit, Millipore, Billerica, MA). TRAP-positive

cells with 3 or more nuclei were observed under the microscope. All experiments were performed using BMM-differentiated mature osteoclasts (OCs).

OCs were seeded into 6-well plates at a density of 1×10^5 cells/ml in a total volume of 2 ml medium, and treated with 35 mM high glucose (HG) for 36 h to induce inflammation, followed by the treatment with 2 µl phosphate-buffered saline (PBS) or indicated doses of RA (Sigma, St. Louis, MO) for 96 h.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cell viability was measured using the MTT colorimetric assay as previously described [10]. In brief, treated OCs were stained with MTT reagent (0.5 mg ml^{-1}) for 4 h, followed by the addition of 10% sodium dodecyl sulfate in 0.01 M HCl at 37 °C overnight. Cell viability was next measured at 550 and 690 nm using a Tecan Infinite M200 micro-titre plate reader.

ELISA

The secretion of interleukin-1β (IL-1β) and cathepsin K in the medium and tartrate-resistant acid phosphatase 5b (TRACP-5b) and deoxypyridinoline in the urine was measured using the corresponding ELISA kits (Nanjing Jiancheng Biotech, Nanjing, China) according to the manufacturer's instructions.

Western blot

For *in vitro* study, protein was extracted from treated cells. For *in vivo* study, proximal tibia from treated rats were used to extract protein. Western blot was next performed as previously described [11]. GAPDH was used as loading control. Antibodies used were Apoptosis-associated speck-like protein containing a CARD (ASC) (1:1000, Abcam, Cambridge, MA), Caspase-1(1:1200, Abcam), NLRP3 (1:1000, Abcam), GAPDH (1:2000, Sigma) and IL-1β (1:1000, Sigma).

RT-PCR

Total mRNA was extracted from treated cells using TRIzol reagent (Invitrogen Life Technologies, Waltham, MA), then transcripted into cDNA. PCR was performed as previously described [12]. All the genes were normalized to *GAPDH*.

The sequences of primers were as below:

GAPDH, Sense: 5′-GTCGGTGTGAACGGATTTG-3′; antisense: 5′-TCCCATTCT CAGCCT TGAC-3′;

ASC, sense: 5′-TGCTGGATGCTCTGTATGG-3′; antisense: 5′-CAAGTAGGGC TGTGT TTGC-3′;

Caspase-1, sense: 5′-GACAAGCCCAAGGTTATC-3′; antisense: 5′-GGCCTTCTTA ATGCCATC-3′;

cathepsin K, sense: 5′-CCCAGACTCCATCGACTATCG-3′; antisense: 5′- CTGTACC CTCTGCACTTAGCTGCC-3′;

NLRP3, sense: 5′-CCAGGGCTCTGTTCA TTG-3′; antisense: 5′-CCTTGGCT TTCACTTC G-3′.

Bone resorption pit assay

Bone resorption pit assay was performed as previously described [10]. In brief, BMMs-differentiated osteoclasts were detached from the flask using 25 mM EDTA solution to seed on bovine bone slices at a density of 50,000 cells per well in 0.5 ml medium in a 96-well plate, then treated with 35 mM high glucose in the presence or absence of 0.5 µl RA for 72 h as mentioned above. Medium was collected for measuring the secretion of cathepsin K, cells were collected for RT-PCR, and slides were stained using 1% toluidine solution. Seven fields were randomly selected on each bone slide to observe the bone resorption pits formed by osteoclasts using a wide field microscope with side illumination. Image J software was used to analyze the area of bone resorption pits.

Animal procedure

After overnight fasting 8-week-old male Sprague-Dawley rats were intraperitoneally injected with 60 mg kg^{-1} streptozotocin (STZ, Sigma) to induce diabetes, which was confirmed by fasting blood glucose >11.1 mM. STZ was dissolved in 0.05 M citrate buffer (pH 4.5). 4 days after the STZ injection, 400 µl 50 mg kg^{-1} RA or the same volume of saline was orally administered to the rats daily for 12 weeks. There were 7 rats in each group. Rats were scanned by dual-energy X-ray absorptiometry (DXA; Hologic, USA) to analyze bone mineral density and bone mineral contents. We used resolution scan with line spacing set at 0.05 cm to measure the bone density of the whole body and measured with the pDEXA Sabre and Sabre Research software (both from Norland Medical Systems Inc., Fort Atkinson, Wisconsin) as previously described [13]. At the end of the experiments, femur, tibia and proximal tibia were collected and weighted after removing the muscle and tendons. Blood and urine were also collected. All the animal experiments were approved by the Ethical Committee of the Second People's Hospital of Anhui Province (2020/03/07.124).

Statistical analysis

Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test, and two-way ANOVA followed by Bonferroni's post hoc test. Post hoc analyses were only done with a significant overall ANOVA value. Data were presented as means ± SD. Experiments were repeated independently three times to confirm the results. $^{*}P < 0.05$ was considered as statistically significant.

RESULTS

RA reduced IL-1β secretion under HG conditions in OC

The structure of RA is shown in Fig. 1A. We first stimulated mature osteoclasts with 35 mM high glucose to activate the inflammatory responses in osteoclasts, then treated them with different doses of RA. After 96 h, high glucose-reduced cell viabilities were dose-dependently increased by RA treatment (Fig. 1B). Notably, cell viabilities were significantly improved by RA with a concentration >50 µM. Next, we observed that IL-1β secretion, which were induced by high glucose, were also reduced by RA in a dose-dependent way (Fig. 1C). Similarly to previous

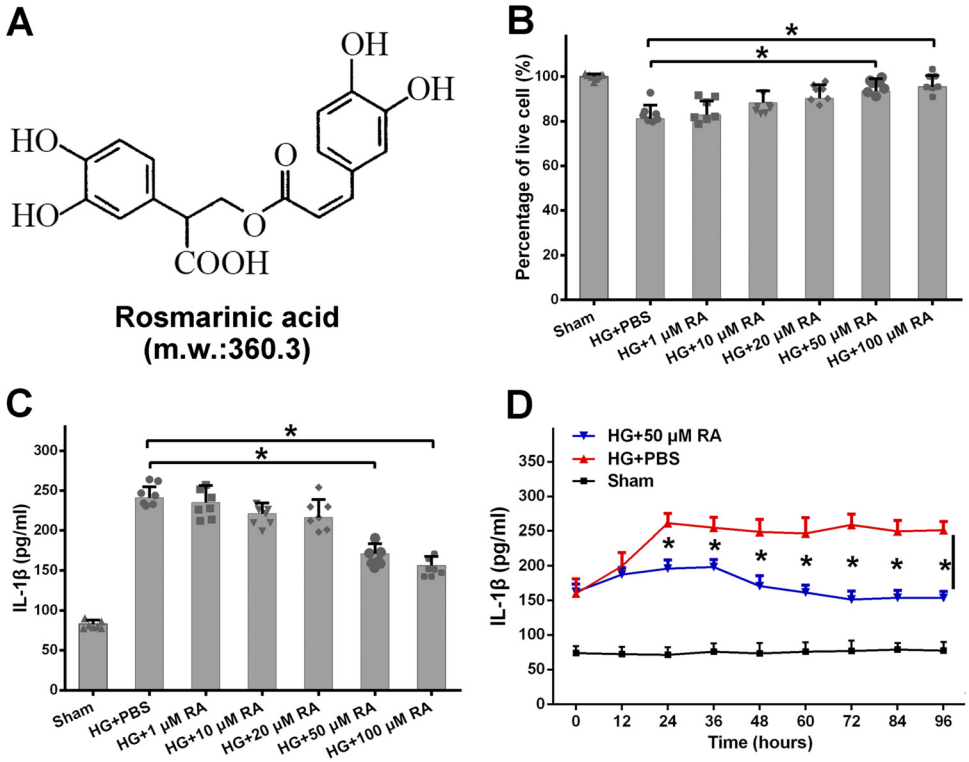

Fig. 1. RA reduced IL-1β secretion in OC under HG conditions. (A) Structure of rosmarinic acid. (B) Effect of RA on OC viability. OCs were treated with different doses of RA for 96 h, and then cell viability was measured by the MTT method. $^{*}P < 0.05$ between the indicated groups. One-way ANOVA with Tukey's post hoc test. (C) Concentrations of IL-1β released by OC treated with different doses of RA for 96 h and detected by ELISA. $^{*}P < 0.05$ between the indicated groups. One-way ANOVA with Tukey's post hoc test. (D) Concentrations of IL-1β released by OC treated with 50 μM RA for different times and detected by ELISA. $^{*}P < 0.05$ between the HG+PBS group (red) and HG+RA group (blue). Two-way ANOVA with Bonferroni's post hoc test. Results are expressed as means ± SD ($n = 7$)

data, RA with a concentration higher than 50 μM significantly decreased the secretion of IL-1β. Therefore, we chose 50 μM RA for the subsequent studies. Compared to PBS-treated ones, IL-1β secretion in the medium of RA-treated cells was significantly downregulated after 24 h and stayed stable after 72 h (Fig. 1D).

RA inhibited the inflammasome activation in OCs

High glucose activated the inflammasome in OC as judged by the increased protein expression of inflammasome–related proteins including NLRP3, caspase-1 and ASC, and the activated inflammasome was inhibited by RA treatment for 72 h (Fig. 2A and B). Moreover, RNA expression of these inflammasome-related proteins was also induced by high glucose and reduced by RA treatment (Fig. 2C–E).

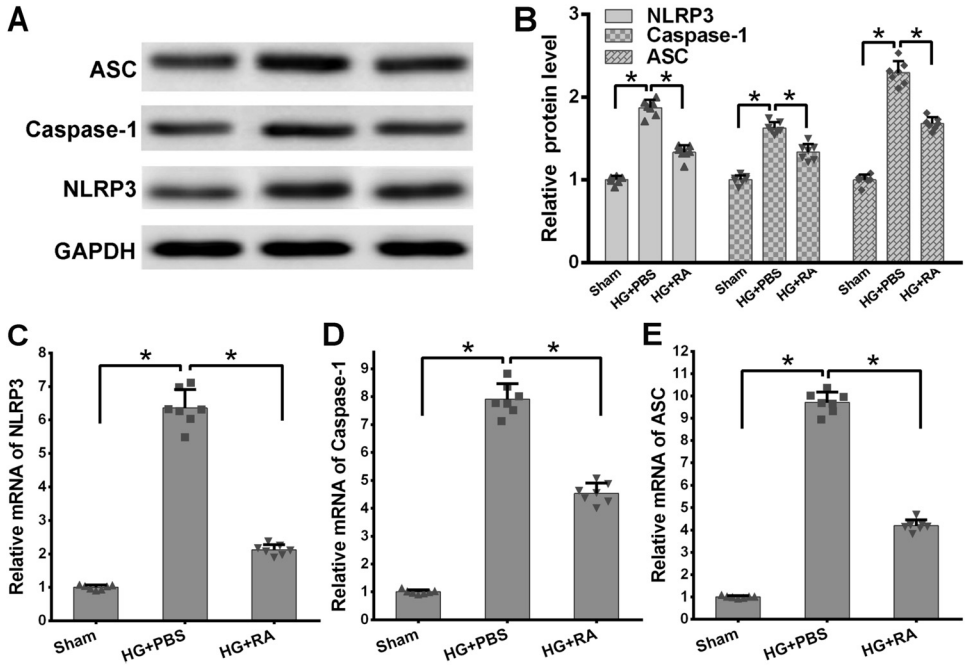

Fig. 2. RA inhibited the activation of NLRP3 inflammasome in OCs. (A) Western blot was used to detect the expression of NLRP3, Caspase-1 and ASC in OC. GAPDH was used as an internal reference. (B) The relative grayscale of WB results was analyzed by image J software. (C–E) qRT-PCR was used to analyze the mRNA levels of NLRP3 (C), Caspase-1 (D) and ASC (E) in OCs. One-way ANOVA with Tukey's post hoc test. Data are presented as means ± SD ($n = 7$). $^*P < 0.05$ between the indicated groups

RA reduced bone resorption on bone model

To further assess the role of RA in OC, we evaluated its effect on mature osteoclast resorbing bovine bone slices. Compared to sham, high glucose increased the bone resorption as reflected by the upregulated resorption area, which was significantly reduced by RA (Fig. 3A and B). Moreover, we evaluated the expression of cathepsin K to assess the activity of osteoclasts. The secretion and mRNA levels of cathepsin K were promoted by high glucose in comparison with the sham group, and RA significantly inhibited this increase in cathepsin K expression (Fig. 3C and D).

RA alleviated bone weight loss in STZ-induced rats

To further explore the role of RA in diabetic osteoporosis, we injected STZ into rats to induce diabetes, and orally administered 50 mg kg^{-1} RA or PBS daily to the diabetic rats. We monitored the bone mineral content and bone mineral density by DXA every week. Compared to the sham group, PBS-treated diabetic rats had gradually decreasing bone mineral content and bone mineral density, but RA treatment significantly inhibited these declines in bone mineral content

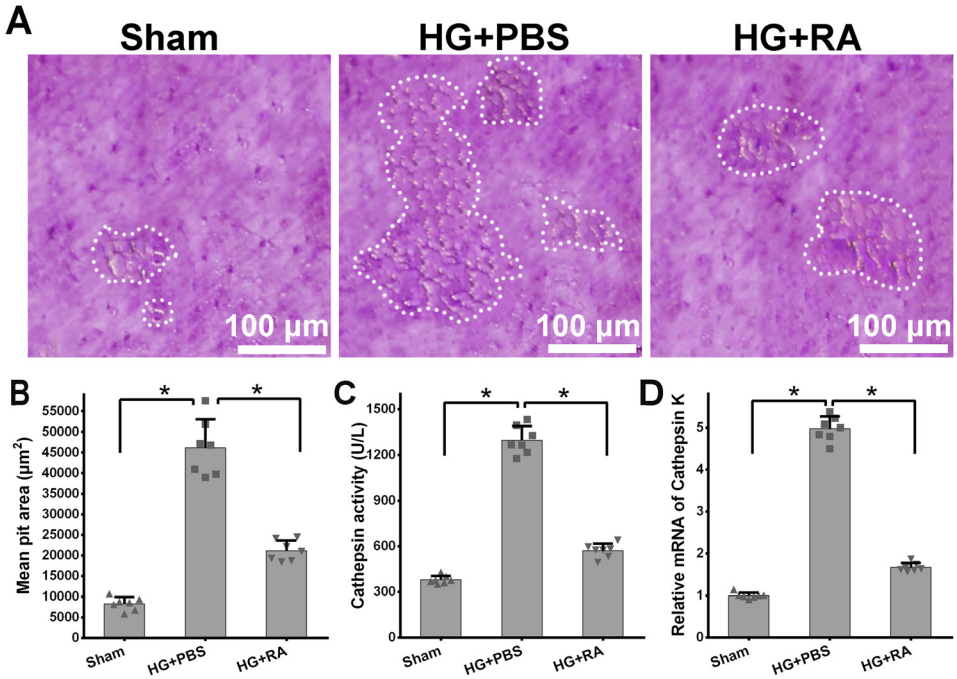

Fig. 3. RA reduced the bone-resorption capacity in OCs. (A) Resorption pits induced by OC with or without RA. Pits as indicated by white contours were formed by osteoclasts resorbing on bovine bone slices. (B) Quantitative analysis of average pit area (μm^2) per visual field on bovine bone slices. (C) The levels of cathepsin K in the serum were detected by ELISA. (D) qRT-PCR was used to analyze the mRNA levels of cathepsin K in the OC. One-way ANOVA with Tukey's post hoc test. Data are presented as mean ± SD ($n = 7$). $^*P < 0.05$ between the indicated groups

and bone mineral density from week 9 (Fig. 4A and B). However, RA treatment did not alter decreased body weight and increased blood glucose levels in STZ-induced diabetic rats (Fig. 4C and D). After 12 weeks, the femur, tibia and proximal tibia were collected from treated rats and weighted. Consistently with previous data, the femur, tibia and proximal tibia weights were all remarkably decreased in STZ-induced diabetic rats, and significantly protected from decline by treatment with RA (Fig. 4E–G). These data indicated that RA could effectively alleviate the symptoms of bone weight loss in rats with diabetic osteoporosis.

RA reduced bone resorption and inflammasome activation *in vivo*

Next, we assessed the effect of RA on bone resorption *in vivo* by measuring the expression of some markers of bone resorption, including deoxypyridinoline, TRACP-5b and cathepsin K. The secretion of deoxypyridinoline in the urine and that of TRACP-5b and cathepsin K in the serum were all induced in PBS-treated diabetic rats compared to sham, which could be significantly restored by RA treatment (Fig. 5A–C). In addition, the expression of inflammasome-related proteins such as NLRP3, ASC and Caspase-1 were all increased in PBS-treated

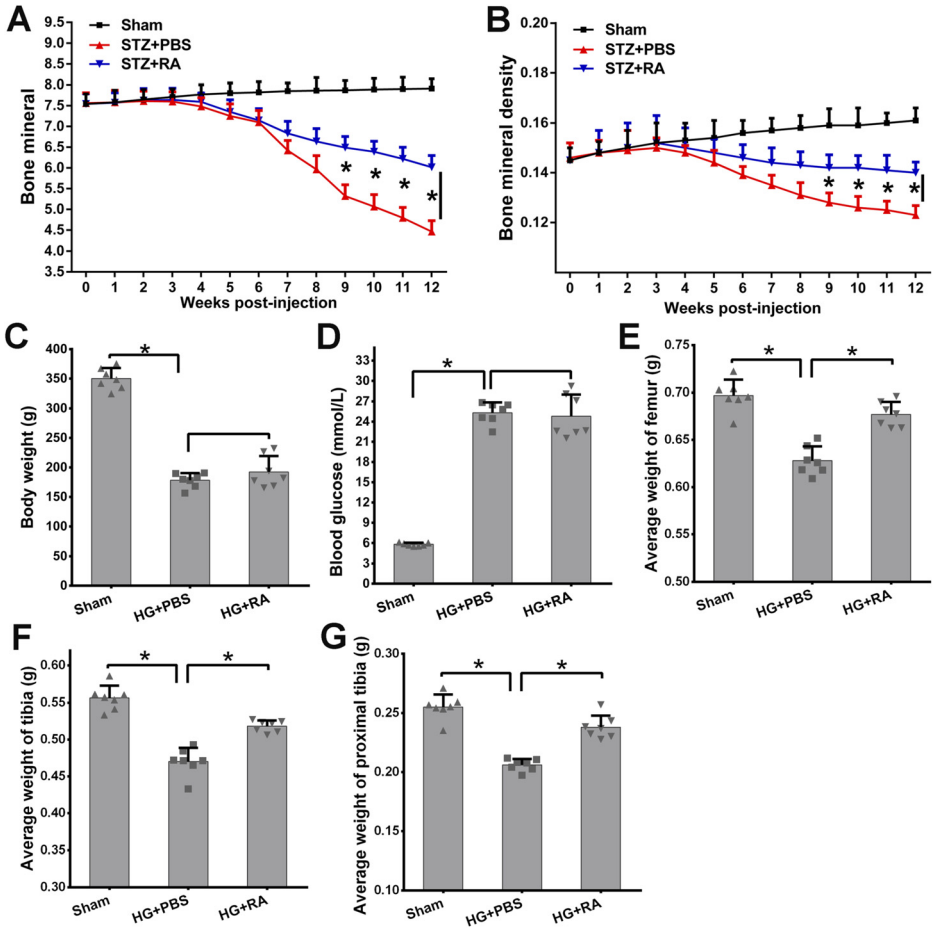

Fig. 4. RA improved bone mineral content, bone mineral density and bone weight in rats with diabetic osteoporosis. (A–B) DXA was used to assay the bone mineral content (A) and bone mineral density (B) in diabetic osteoporosis model rats. Two-way ANOVA with Bonferroni's post hoc test. $^{*}P < 0.05$ between the STZ+PBS group (red) and STZ+RA group (blue). The body weight (C) and blood glucose concentration (D) of rats were measured at sacrifice. One-way ANOVA with Tukey's post hoc test. The weights of femur (E), tibia (F) and proximal tibia (G) were measured. One-way ANOVA with Tukey's post hoc test. $^{*}P < 0.05$ between the indicated groups. Data are presented as mean ± SD ($n = 7$)

diabetic rats, and inflammasome was deactivated by RA treatment (Fig. 5D and E). Moreover, IL-1β expression, which was greatly increased in diabetic rats, was also significantly reversed after RA treatment (Fig. 5D and E). This indicated that RA remarkably reduced the inflammatory responses in OC. Taken together, RA administration in mice inhibited the activation of osteoclasts by suppressing NLRP3 inflammasome activation of osteoclasts under high glucose stimulation, thereby alleviating the symptoms of diabetic osteoporosis.

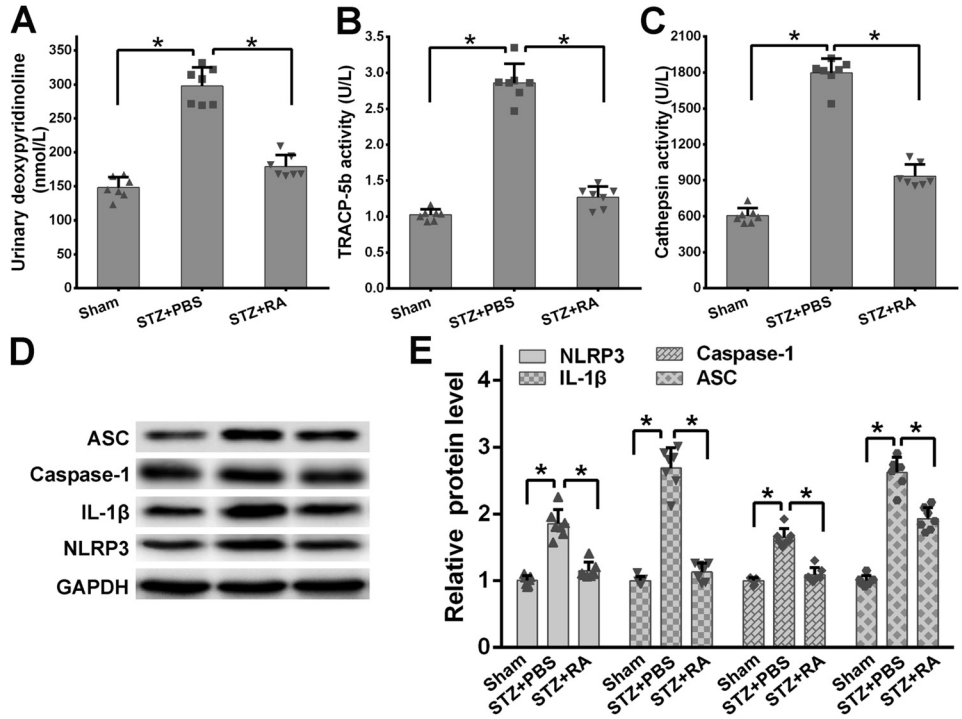

Fig. 5. RA improved osteoporosis in rats with diabetic osteoporosis. (A) The levels of deoxypyridinoline in the urine were detected by ELISA. (B–C) The levels of TRACP-5b (B) and cathepsin K (C) in the serum were detected by ELISA. (D) Western blot was used to detect the expression of NLRP3, IL-1β, Caspase-1 and ASC in bone tissues. GAPDH was used as an internal reference to ensure equal loading. (E) The relative grayscale analysis of WB results by image J software. One-way ANOVA with Tukey's post hoc test. Data are presented as mean ± SD ($n = 7$). $^{*}P < 0.05$ between the indicated groups

DISCUSSION

Diabetes is a pandemic metabolic and chronic disease with substantial mortality and morbidity. Patients with either type I or type II diabetes always have reduced bone mineral density and bone mass, leading to a decrease in bone strength and a high risk of fracture, i.e. osteoporosis. Osteoporosis is the most important and prevalent metabolic skeletal disease in patients with diabetes mellitus. Osteoporosis is a "silent" disease with no symptoms until fractures occur; and diabetes not only increases the risk of fracture, but also delays healing of the bone fracture [14], therefore osteoporosis exerts unfavorable effects on the health and life quality of patients with diabetes. Moreover, the social burden caused by osteoporosis is enormous. It was estimated that only the direct medical cost of osteoporosis was as high as $26 million in 2005 [15], and this burden was dramatically growing due to the increasing number of patients with diabetes mellitus. Therefore the awareness, prevention and treatment of osteoporosis are pivotal [2]. However, the therapeutic strategy of osteoporosis has mostly focused on anti-resorptive

therapies that inhibit bone turnover, which might not be effective based on the current outcome [16]. Therefore, novel therapeutic strategies need to be explored.

Osteoclasts are the major cells that mediate bone resorption; they originate from the monocyte-macrophage lineage in the bone marrow, called hematopoietic cells [17]. The differentiation of hematopoietic cells determines the resorptive efficacy of mature osteoclasts, in which is dependent on M-CSF and RANKL. Mature osteoclasts express TRAP with multinuclear form and bone-resorbing activity. Therefore in our study bone marrow-derived macrophages were supplemented with M-CSF and RANKL to induce osteoclasts, and the mature osteoclasts were confirmed by TRAP-staining as well as multinuclear images (data not shown) as well differentiated osteoclasts for the subsequent studies. Our mature osteoclasts had good activity of bone resorption, which was promoted by the stimulation of high glucose. These data were consistent with previous results [7], indicating that our mature osteoclasts were well differentiated.

Nowadays, a body of studies supports the theory that provoked oxidative stress and stress-activated signaling pathways are common factors in the pathogenesis of diabetes mellitus and osteoporosis [18, 19]. Excessive ROS or increased oxidative stress breaks the balance between antioxidant and oxidant levels, leading to accelerating lipid peroxidation, reducing the expression and activity of antioxidant enzymes, suppressing bone formation, stimulating apoptosis of osteoblast, facilitating bone resorption of osteoclasts, and eventually bone loss [8, 20].

A great variety of factors are involved in the pathogenesis of diabetic osteoporosis including insulin insufficiency, insulin resistance, calcium and phosphorus metabolic disorders and hyperglycemia. Nowadays, more and more publications reveal the importance of ROS in the development of diabetic osteoclasts [7, 21, 22]. Diabetes mellitus causes bone and mineral abnormalities, contributing to the change, in which the activation of the NLRP3 inflammasome is implicated.

NLRP3 is predominantly expressed in macrophages and, as a component of the inflammasome, detects the products of damaged cells [23]. NLRP3 plays an important role in caspase-1 activation and IL-1β secretion in response to endogenous and bacterial stimuli [24]. Therefore, in our study we first evaluated the secretion of IL-1β to reflect inflammatory levels in the osteoclasts. Although a variety of cytokines besides IL-1β are activated in the inflammatory responses, we chose IL-1β not only as the marker of inflammatory responses, but also as an indicator of NLRP3 activation. Since numerous publications indicated that the activation of the NLRP3 inflammasome play an important role in diabetic osteoclasts, we expected that an anti-inflammasome drug might serve as a candidate therapeutic drug for inflammasome-related diabetic osteoclasts.

RA is an ester of caffeic acid and 3,4-dihydro xyphenyllactic acid, extracted from natural plants, such as species of the subfamily Nepetoideae of the Lamiaceae and the Boraginaceae [25]. It has several biological activities including antibacterial, antiviral, antioxidant and anti-inflammatory action. Moreover, RA also has anti-diabetic [26], hepatoprotective [27], and anti-cancer [28] properties. In addition, RA was reported to inhibit inflammasome activation under high-glucose condition [28]. RA extract was regarded as a potential anti-diabetic candidate [26]. However, RA has only been proven to regulate glucose homeostasis and lipid profile in type 2 diabetes [29], and there is no report on the effect of RA on blood glucose levels in T1DM. Our study shows that RA could improve diabetic osteoporosis by inhibiting the activation of NLRP3 inflammasome without altering blood glucose levels. Moreover, previous publications indicated that RA as an antioxidant and anti-inflammatory agent could attenuate diabetes-induced

endothelial damage and cerebral ischemia [30, 31], which was consistent with our observation that RA could effectively improve diabetic complication, such as osteoporosis by inhibiting both oxidative stress and inflammation.

CONCLUSION

In summary, our data strongly reveal the protective role of RA in diabetic osteoporosis through inhibiting the activation of NLRP3 inflammasome *in vivo* and *in vitro*. Our findings highlight that RA might serve as a potential candidate drug for treating diabetic osteoporosis.

Competing interests: The authors declare that they have no conflict of interest.

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