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Rutin prevents the ovariectomy-induced osteoporosis in rats

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Abstract. – OBJECTIVE: Our study aimed to explore the anti-osteoporotic effect of rutin (quercetin-3-O-rutinose) on ovariectomized female rats.

MATERIALS AND METHODS: An ovariectomized (OVX) rat model of osteoporosis was employed to evaluate the anti-osteoporotic potency of rutin. One week after surgery, the rats were administered intragastrically with rutin or saline once daily respectively for 3 months. Bone mineral density (BMD) was measured by dual-energy x-ray absorptiometry (DEXA). The bone microstructure was analyzed by hematoxylin and eosin (HE) staining of the left tibia histomorphology. Estradiol, IL-6, and TNF-α were measured by ELISA kits.

RESULTS: The results showed that rutin significantly improved the bone mineral density (BMD) and increased the level of inflammatory factor of IL-6, TNF-α, and INF-γ in OVX rats. Rutin turned bone trabecula to be thickened and dense, and kept regular array. Moreover, rutin significantly improved the average thickness of trabecular bone and the average bone volume fraction.

CONCLUSIONS: Rutin possessed with significant anti-osteoporotic activity, which can be considered as an idealistic anti-osteoporotic candidate for human osteoporosis diseases.

Key Words

Rutin, Osteoporosis, Ovariectomized Rats, IL-6, TNF-α.

Introduction

Osteoporosis is a kind of metabolic disorder characterized by the imbalance between osteoblastic formation and osteoclastic resorption, resulting in skeletal fragility and susceptibility to fractures [1,2]. It is a stealthy and unpredictable disease, which become symptomatic until the advanced stages[3]. Ovarian hormone deficiency is a major risk factor for osteoporosis in postmenopausal women. It was predicted that, by 2050, more than a half of worldwide osteoporotic hip fractures would occur in Asia[4]. Some pharmacological treatments have antiosteoporotic effect, such as hormone therapy (HT), bisphosphonate and selective estrogen receptor modulators (SERMs)[5]. However, these medications were exposed some side effects, including gastrointestinal tolerance problems in bisphosphonate and the potential malignancies in HT[6,7]. Therefore, natural compounds with antiosteoporotic activity and few side effects are worth of exploring. Many studies[8-14] have been shown that some vegetables and fruits such as onions, beans and tea can inhibit bone resorption. They are strong antioxidants, which can help to prevent cardiovascular disease by inhibiting oxidation of low-density lipoproteins[15]. Onions is rich in flavonol glycosides, and the antioxidant effect is considered the strongest in vegetables[16]. Several independent evidence indicated that some particular natural products containing polyphenol molecules possible may be useful for these beneficial treatments. For example, the protective action against osteopenia or osteoporosis was discovered in catechin[17], rutin[18], green tea

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polyphenol[19], olive oil polyphenol[20] and apple polyphenol. Flavonoids represent a large number of phenolic compounds that commonly found in daily nutrition with proven health benefits as well as antiosteoporotic properties[21]. Flavonoids have been used widely implicated in the alleviation of postmenopausal osteoporosis. Studies have been shown that isoflavonoids (such as genistein and daidze) and flavonols (such as quercetin) in the form of rutin can prevent bone loss in ovariectomized (OVX) rats. Quercetin is the primary flavonol, which mainly found as a glycoside (rutin also called quercetin-3-O-rutinose). It binds to estrogen receptors and influences the development of cell lines from several hormone-dependent-cancers[22,23]. In foods, quercetin occurs mainly as its glycoside, rutin. Like quercetin, rutin displays antioxidative properties and inhibits the growth of cancer cell lines[24,25]. In the present study, we studied the effect of rutin on bone metabolism in the ovariectomized (OVX) rats.

Materials and Methods

Chemicals

Rutin was purchased from the National Institute for the Control of Pharmaceuticals and Biological Products (Beijing, China) and its purity was determined to be ≥ 98% by HPLC measurement. Unless otherwise stated, all other reagents were of analytical grade.

Animal Groups and Experiments

Fifty female Sprague-Dawley (SD) rats aged 8 months (purchased from Slacom, Shanghai, China, 340-350 g) were housed under controlled conditions including a room temperature of 22 ± 2°C with a 12 h light/dark cycle and allowed free access to food and distilled water. One week later, the acclimatized rats were anesthetized with chloral hydrate (300 mg/kg, i.p.) (Sinopharm, Shanghai, China) under aseptic conditions. Ten rats underwent bilateral laparotomy without removing the ovaries as Sham control group, the others were underwent bilateral ovariectomy and equally randomized into three groups: intragastric administered vehicle as model group (OVX), OVX with rutin (R) of graded doses (R5, n = 10, 5 mg/kg body weight/day and R10, n = 10, 10 mg/kg body weight/day) according to preliminary experiments. The administration of rutin lasted for 3 months. The uterus wet weight and uterus index of rats were recorded weekly during the experimental period. The procedures of the animal study, including the raising, feeding, and the whole surgical process, followed the APS's "Guiding Principles on the Care and Use of Animals" and were approved by the Committee of Animal Study at Shandong University.

Enzyme-Linked Immunosorbent Assay (ELISA)

After the completion of treatments, the animals were sacrificed under deep anesthesia with chloral hydrate (300 mg/kg, i.p.) and bloodletting via arteria femoralis. Blood samples were collected and centrifuged (4°C) at 3000 r/min for 15 min. The upper serum was separated immediately and was stored at -20°C for future analysis. According to the manufacturer's instructions, ELISA assay was performed to determine serum levels of osteocalcin, estrogen (E2), 1, 25-dihydroxycholecalciferol (1, 25$(OH)_2D_3$) and inflammatory cytokines (IL-6, TNF-α, INF-γ) with ELISA kits (BioSource International, Camarillo, CA, USA).

Bone Histomorphometric Analysis

Sections of un-decalcified femurs fixed in 10% neutral buffered formalin and embedded in paraffin wax were taken using a microtome and stained with Goldner's trichrome. The sections were mounted and observed for histopathological changes. Histomorphology of the left femur was analyzed for bone microstructure after hematoxylin and eosin (HE) staining (Beijing Solar Bioscience Technology Co., Ltd, Beijing, China). HE staining was performed according to the previous studies of Huang et al[26]. The histomorphometric study of the metaphysis of the proximal tibiae was performed as previously described with image analysis software (Image Pro Plus 6.1 for Windows; Media Cybernetics, Silver Spring, MD, USA)[27].

Bone Mineral Density

The total bone mineral density (BMD) of the right femur was measured using Lunar Prodigy Advance by dual-energy X-ray absorptiometry (GE Healthcare, Pittsburgh, PA, USA) equipped with appropriate software for bone density assessment in small laboratory animals as reported elsewhere[28]. Distal and proximal regions corresponded to the cancellous bone and central to the cortical bone[29]. Results are given in g/cm^2.

Statistical Analysis

These data were presented as means ± standard deviation (SD). Statistical comparisons among all groups were analyzed by using one-way analysis

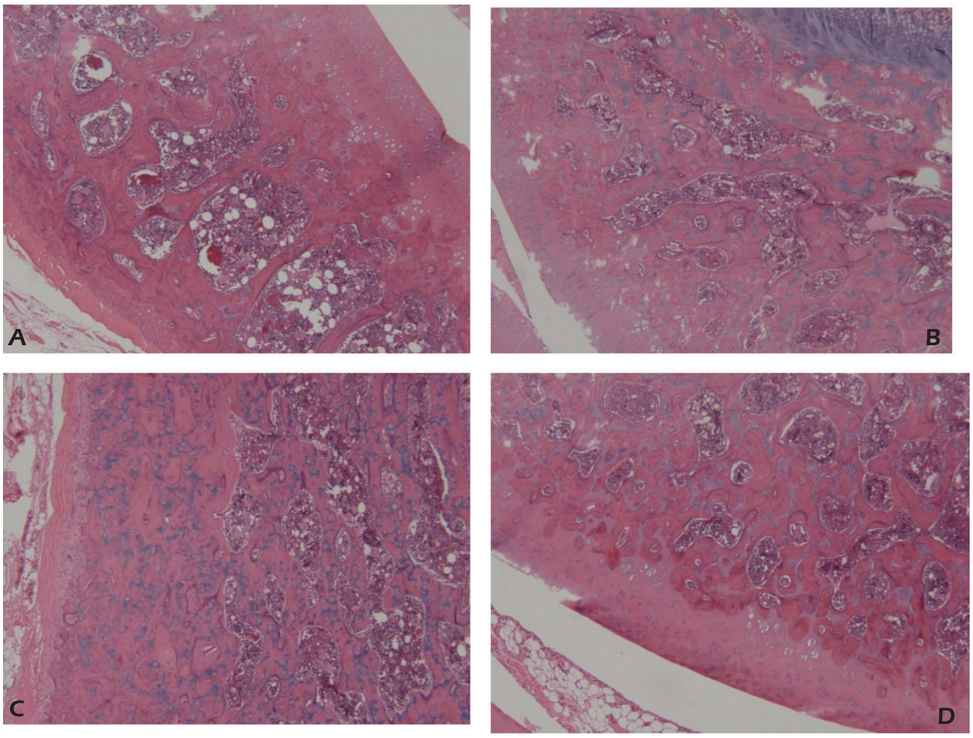

Figure 1. Body weight gain during the treatment period. The body weight of OVX group was significantly higher than other groups. The interventions of rutin did not significantly affect body weight gain throughout the period of experiment. Data were presented as means ± SD. Sham: sham control group; OVX: the ovariectomized rats group; R5: OVX rats with rutin at a dose of 5 mg/kg body weight/day; R10: OVX rats with rutin at a dose of 10 mg/kg body weight/day.

of variance (ANOVA). When significant levels ($p<0.05$) were revealed, pair-wise comparisons between groups were made using the Fisher's least significant difference (LSD) method. Statistical analysis software, SPSS 11.5 version (SPSS Inc., Chicago, IL, USA) was adopted for processing the data of the present study.

Results

Body Weight

No rats died during the study period in all groups. Five groups of rats had a similar initial mean body weight. During the 3-month-experimental period, body weights of rats in all groups were weekly monitored to study whether rutin altered body weight following ovariectomy. After operation, body weight gain was typically and consistently higher in the ovariectomized rats in each group compared with the Sham control group ($p<0.05$) (Figure 1). The body weight of OVX group was significantly higher than Sham control group ($p<0.05$) on week 4 after surgery. Except for the slightly lower body weight gain of the R10 (rutin in high dose) group, the interventions we used, R5 groups, did not significantly affect body weight gain throughout the period of the experiment.

Index of Uterus

The variation tendency of uterine index was similar to body weight. OVX caused significant atrophy of uterine tissue comparison with Sham control group ($p<0.05$). Rutin significantly increased the uterine weight comparison with OVX group ($p<0.05$), which appeared to be dose dependent (Table I).

Table I. Index of uterus during the treatment period.

Group	Weight (mg)	Index of uterus (mg/g)
Sham	326.8±16.4	2.15±0.75
OVX	81.6±6.3*	0.99±0.83*
R10	168.8±12.9*#	1.85±0.75*#
R5	159.4±13.2*#	1.69±0.94*#

Data were presented as means ± SD. Power, power values calculated using α=0.05; *p<0.05 vs. Sham; #p<0.05 vs. OVX.

Table II. The level of cytokines IL-6, IFN-γ and TNF-α.

Group	IL-6 (pg/ml)	TNF-α (pg/ml)	INF-γ (pg/ml)
Sham	369.72±38.82	38.92±17.95	0.948±0.135
OVX	583.82±41.73*	104.74±27.84*	2.582±0.852*
R5	482.83±28.89*#	82.84±21.37*	1.862±0.957*#
R10	427.95±38.48*#	63.95±24.53#	1.264±0.729#

Data were presented as means ± SD. Power, power values calculated using α=0.05; *p<0.05 vs. Sham; #p<0.05 vs. OVX.

Table III. The level of E_2, OCN, $1,25(OH)_2D_3$ in serum.

Group	E_2 (ng/l)	OCN (ng/ml)	$1,25(OH)_2D_3$ (ng/ml)
Sham	369.72±38.82	38.92±17.95	0.948±0.135
OVX	583.82±41.73*	104.74±27.84*	2.582±0.852*
R5	482.83±28.89*#	82.84±21.37*	1.862±0.957*#
R10	427.95±38.48*#	63.95±24.53#	1.264±0.729#

Data were presented as means ± SD. Power, power values calculated using α=0.05; *p<0.05 *vs*. Sham; #p<0.05 *vs*. OVX.

Effect of Rutin on Inflammatory Markers

ELISA was performed to evaluate serum cytokines level in different treatment groups.

As shown in Table II, the cytokines IL-6, IFN-γ and TNF-α were significantly elevated in OVX group compared to Sham control group (p<0.05). However, intervention with rutin reversed the upregulated levels of cytokine IL-6, IFN-γ and TNF-α on OVX rats in a dose-dependent manner.

Serum Parameters

Results of serum parameters in different groups of rats were shown in Table III. The results indicated a significant reduction in the levels of serum E_2 and 1, $25(OH)_2D_3$ in OVX rats compared with Sham control group, while intervention with rutin the level of E_2 and 1, $25(OH)_2D_3$ significantly increased. In contrast, statistically significant (p<0.05) elevated levels of serum OCN were observed in OVX rats when compared with Sham control group. Interestingly, rutin treatment reversed the upregulated serum OCN levels on OVX rats in a dose-dependent manner.

Table IV. Bone mineral density of the femur.

Group	BMD (g/cm²)
Sham	0.251±0.007
OVX	0.148±0.004 *
R10	0.198±0.008 *#
R5	0.176±0.005 *#

Data were presented as means ± SD. Power, power values calculated using α=0.05; *p<0.05 *vs*. Sham; #p<0.05 *vs*. OVX.

Bone Mineral Density of the Femur

OVX significantly decreased the right femur BMD comparison with the Sham control group (p<0.05). The administration of rutin to the OVX rats significantly recovered the BMD of femur. The 3-month treatment with rutin at higher doses increased the right femur BMD compared to the OVX group (p<0.05), which appeared to be dose dependent (Table IV).

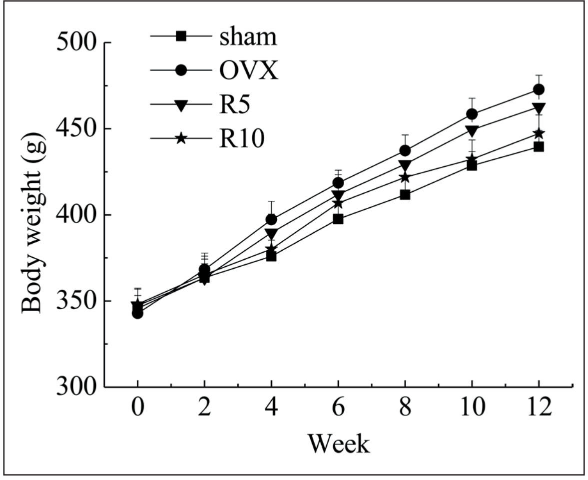

Figure 2. Histological sections of femur in rats of different groups (magnification 200×). The ovariectomized rat showed sparse, uniform thinning of the trabeculae resulting in the wideness of trabecular interspace, but the administration of rutin significantly protected bone from ovariectomy-induced osteopenia. Sham: sham control group; OVX: the ovariectomized rats group; R5: OVX rats with rutin at a dose of 5 mg/kg body weight/day; R10: OVX rats with rutin at a dose of 10 mg/kg body weight/day.

Rutin Decreased Trabecular Bone Loss

The bone architecture of the femur was analyzed with Harris hematoxylin and eosin. The rats of the Sham control group revealed normal compactness of the diaphysis and competent trabeculae (Figure 2A). The OVX rats showed sparse, uniform thinning of the trabeculae resulting in the wideness of trabecular interspace (Figure 2B). The low (R5) dosage rutin groups exhibited both restorative progress with mineralization and quite well-distributed osteocytes. Uniform trabeculae with dense matrix and shaft size were observed (Figure 2C). The group treated with high dose rutin (R10) showed nearly complete recovery with essential features of normal bone and complete formation of trabeculae, which was similar to the Sham control group (Figure 2D). These results demonstrated there were beneficial effects of rutin on the prevention of bone loss, which induced by ovariectomy.

Discussion

Osteoporosis is a kind of metabolic disorder characterized by the imbalance between osteoblastic formation and osteoclastic resorption, resulting in skeletal fragility and susceptibility to fractures. Ovarian hormone deficiency is a major risk factor for osteoporosis in postmenopausal women. It is well known that estrogen deficiencies are important risk factors in the pathogenesis of osteoporosis. Ovariectomy results in a dramatic decrease in uterine weight, bone mineral density, and biomechanical strength, and these changes are in part due to estrogen deficiency. Some studies have shown that several vegetables and fruits such as onions, beans and tea can inhibit bone resorption. Tea consumption was associated with a high bone mineral density in older women[30], which was assumed to be caused by catechins that counteracted the adverse bone metabolism effects of caffeine. Bone resorption inhibition occurred in rats on an onion-rich diet (the main source of quercetin in many diets)[30]. Being the glycosylated form of quercetin, rutin was often found in onions as main flavonols[31]. Onion extracts have been shown to inhibit bone resorption *in vitro* and *vivo*[32]. The present study was designed to evaluate the effect of rutin on the protection against ovariectomy-induced osteopenia in rats. The data showed that OVX decreased body and uterine weight compared with the sham control group. Administration of rutin increased both the body and uterine weight in the ovariectomized rat. In the histopathology examination, the OVX rats showed sparse, uniform thinning of the trabeculae resulting in the wideness of trabecular interspace. The administration of rutin exhibited restorative progress with mineralization along with quite well-distributed osteocytes. Histopathology examination of the femurs of rutin groups revealed ossification, mineralization, and calcified cartilaginous deposits, all of which indicate marked restorative action, thus suggesting that the protective action of rutin may be due to an increase in bone formation with a reduction in bone resorption. It is well-known that estradiol (E2), osteocalcin (OCN), calcium and phosphorus are widely accepted phenotype markers for bone formation[33]. A previous study[18] showed that rutin consumption was more efficient in restoring bone mineral loss in OVX rats than isofiavones, decreased a bone resorption marker (deoxypyridinoline) after urinary excretion, and increased an osteoblastic activity marker (osteocalcin). In the present study, the results indicated a significant reduction in the levels of serum E_2 and $1,25(OH)_2D_3$ in OVX rats when compared with Sham control group, while intervention with rutin significantly increased the level of E_2 and $1, 25(OH)_2D_3$. Interestingly, rutin treatment reversed the upregulated serum OCN levels on OVX rats in a dose-dependent manner. Considerable evidence has demonstrated that pro-inflammatory cytokines, such as interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), are involved in the regulation of bone turnover, by increasing bone resorption[34,35]. Ralston et al[36] demonstrated that interleukin-1 (IL-1), tumor necrosis factor (TNF), and IL-6 mRNAs are expressed more frequently in bone cells from untreated postmenopausal women than from those on estrogen replacement therapy. Those cytokines are also involved in the release of free radicals[37,38]. This pathway will, in turn, inhibit osteoblastic recruitment and the activity of mature cells[39] and increase osteoclastic resorption. In the present study, intervention with rutin reversed the upregulated levels of cytokine IL-6, IFN-γ and TNF-α on OVX rats in a dose-dependent manner. Our work demonstrated the usefulness and beneficial effects of rutin on the prevention of bone loss induced by ovariectomy. OVX significantly decreased the right femur BMD compared to the Sham control group, while the administration of rutin to the

OVX rats significantly recovered the BMD of femur in a dose-dependent manner. Gaumet et al[40] discovered that an increase in fecal and urinary calcium excretions, as well as a decrease in calcium absorption efficiency, might contribute to the reduction of BMD. Rutin could counteract the ovariectomy-induced osteopenia in rats in a dose-dependent manner.

Conclusions

The present study clearly demonstrates that rutin contributes to the prevention of bone loss and deterioration of trabecular microarchitecture induced by ovariectomization in rats. Furthermore, the results indicate that rutin is a potential alternative therapeutic agent for treatment of postmenopausal osteoporosis.

Conflict of interest statement
We declare that we have no conflict of interest.

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