Ann. N.Y. Acad. Sci. ISSN 0077-8923

ANNALS OF THE NEW YORK ACADEMY OF SCIENCES
Issue: *Resveratrol*

Evaluating resveratrol as a therapeutic bone agent: preclinical evidence from rat models of osteoporosis

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Resveratrol (RSV) is a naturally occurring plant polyphenol that has potential to attenuate osteoporosis with distinct pathologies. This review evaluates preclinical evidence regarding the efficacy and safety of RSV as a therapeutic bone agent using different rat models. Limitations of these animal models are discussed, and suggestions for strengthening the experimental design of future studies are provided. The ovariectomized rat model of postmenopausal osteoporosis reported that RSV supplementation attenuated estrogen deficiency–induced bone loss and trabecular structural deterioration. RSV safety was indicated by the absence of stimulation of estrogen-sensitive tissue. Providing RSV to rats aged >6 months attenuated age-related bone mass loss and structural deterioration but produced inconsistent effects on bones in rats aged <6 months. The hindlimb-suspension rat model of disuse osteoporosis reported that RSV attenuated bone loss in old rats, but higher doses and longer duration supplementation before mechanical loading were required for younger rats. Limitations common to studies using rat models of osteoporosis include requirements to include animals that are skeletally mature, longer study durations, and to adjust for potential confounding effects due to altered body weight and endocrine function. Strengthening experimental design can contribute to translation of animal results to clinically relevant recommendations for humans.

Keywords: resveratrol; rats; postmenopausal osteoporosis; senile osteoporosis; disuse osteoporosis

Introduction

Bone remodeling involves the coordinated and continuous processes of bone resorption and formation. Bone is resorbed by osteoclasts and then new bone is formed by osteoblasts. Excessive osteoclastic activity and/or inadequate osteoblastic activity results in bone loss.[1] Osteoporosis is characterized by progressive bone loss and structural deterioration that place bones at risk of fracture with minimal trauma.[2] Osteoporosis is a chronic disease with significant morbidity, mortality, and socioeconomic burden.[3] Furthermore, the prevalence of osteoporosis is expected to increase with longer life expectancy and with the aging of the global population.[4] In the United States, it is estimated that, by 2020, 61 million women and men age ≥50 years will develop osteoporosis.[5] Pharmacological agents are available to prevent/treat osteoporosis but often have adverse side effects.[6] This has generated interest in nonpharmacological strategies. Awareness of complementary and alternative therapies such as dietary supplements and functional foods for the treatment of osteoporosis has increased among consumers.[7]

Resveratrol (RSV), a polyphenolic (3,4′,5-trihydroxystilbene) compound, is naturally found in a variety of foods, including grapes, red wine, and peanuts.[8] In clinical studies, a RSV dose of 1000 mg/day for 4 weeks was reported to be well tolerated.[9] The low toxicity of RSV is attributed in part to its rapid metabolism and clearance from the body.[10] RSV has various properties, such as estrogenic, anti-inflammatory, antioxidant, and proliferative with the potential to influence bone.[10] *In vitro*, RSV treatment stimulated osteoblastogenesis and inhibited osteoclastogenesis.[11] The ability of RSV to both promote osteoblast-mediated bone formation and inhibit osteoclast-mediated bone resorption may be advantageous over pharmaceutics that act as either an anabolic or an antiresorptive,[6] since

doi: 10.1111/nyas.12840

osteoporosis represents a group of distinct pathological conditions rather than a single entity.[12] Type I osteoporosis, also referred to as postmenopausal osteoporosis, is due to estrogen deficiency at menopause causing rapid bone loss by increasing osteoclast-mediated bone resorption. Type II osteoporosis, also referred to as senile osteoporosis, is due to aging in both women and men causing gradual bone loss by reducing osteoblast-mediated bone formation.[13] Differences in the pathophysiology of senile and postmenopausal osteoporosis may explain the effectiveness of antiresorptive agents in younger, but not always in older, women.[14] Secondary osteoporosis includes disuse osteoporosis caused by mechanical loading.[12] Age-related bone loss is often accelerated by mechanical unloading due to physical inactivity and prolonged bed rest in the elderly.[15]

Clinical studies of osteoporosis are challenging. Expensive long-term studies are required because osteoporosis is a slow-progressing disease. Bone is affected by lifestyle factors and, therefore, experimental design must control for these confounding factors. Animal studies enable maintenance of a level of experimental control impossible to achieve in clinical research.[16] Rat models have the advantages of low cost, adaptability to experimental manipulation, and a shorter life span for assessing aging on bone. Rodent studies are commonly used to screen the efficacy and safety of pharmacological agents and therapeutic strategies before undertaking clinical trials in humans.[17] This review paper summarizes preclinical evidence from rat models of postmenopausal, senile, and disuse osteoporosis regarding the efficacy and safety of RSV as a therapeutic bone agent. The limitations of rat models are discussed and suggestions provided for overcoming these shortcomings.

RSV supplementation for postmenopausal osteoporosis

Occurrence of osteoporosis fractures in females to males is 6:1.[18] Higher risk of osteoporosis in women is attributed to their smaller bones and rapid bone loss after menopause.[18] In postmenopausal osteoporosis, declining estrogen increases bone remodeling and alters the balance toward resorption to promote rapid bone loss.[19] *In vitro*, RSV has been shown to exert estrogenic activity.[11] Five preclinical studies have been published using the ovariectomized (OVX) rat model to investigate RSV as a therapeutic agent on estrogen deficiency–induced bone loss.[20,22–25] RSV doses ranged from 0.7 to 80 mg/kg body weight (bwt)/day (Table 1). Zhao *et al.*[20] administered 20, 40, and 80 mg RSV/kg bwt/day to OVX rats for 12 weeks. RSV doses of 40 and 80 mg/kg bwt/day attenuated femoral neck bone mineral density (BMD) loss ($P < 0.01$) and trabecular structural deterioration ($P < 0.05$) compared to OVX rats. The highest (80 mg/kg bwt/day) RSV dose achieved a reduction in femoral trabecular spacing (117.2 $\pm$ 9.5 mm) comparable to rats provided estrogen replacement therapy (ERT; 100.4 $\pm$ 10.2 mm). Similar to the action of estrogen, RSV reduced bone resorption by inhibiting signals regulating the receptor activator of nuclear kappa B ligand pathway of osteoclast differentiation indicated by a dose-dependent upregulation of gene expression of osteoprotegerin and downregulation of cytokines in the femur (Fig. 1).[20] ERT has been reported to have serious side effects of increased risk of breast and uterus cancer.[21] Safety of RSV doses up to 80 mg/kg bwt/day was indicated by no significant stimulation of the uterus.[20] Other than assessing for estrogenic activity, studies investigating the effects of RSV supplementation on bone have not determined potential side effects. Cottart *et al.*[10] reviewed 13 clinical studies that included toxicology. RSV doses used in these studies ranged from 8 to 5000 mg for a duration of 4 days to 1 year. Reported side effects were mild and mainly involved gastrointestinal discomfort.[10]

Postmenopausal osteoporosis is associated with greater loss of trabecular than cortical bone, which predisposes individuals to spine (lumbar vertebrae) fractures.[26] Lin *et al.*[22] investigated the effect of RSV supplementation on vertebrae using the OVX rat model. Feeding 5, 15, or 45 mg RSV/kg bwt/day for 13 weeks reduced ($P < 0.05$) vertebral BMD compared to OVX rats. Serum alkaline phosphatase (ALP 37.9 $\pm$ 3.0 U/100 mL) and osteocalcin (1.27 $\pm$ 0.10 ng/mL), both markers of bone formation, were highest ($P < 0.05$) in rats provided 45 mg RSV/kg bwt/day. No significant effect on serum tartrate-resistant acid phosphatase, a bone resorption marker, suggested that RSV preserved estrogen-deficiency vertebral BMD loss by promoting bone formation.[22] Sehmisch *et al.*[23] reported OVX rats showed no significant effect on tibia BMD, trabecular and cortical bone, and strength at a dose

Table 1. The effects of RSV supplementation on bone mass, microstructure, and strength in rat models of postmenopausal, senile, and disuse osteoporosis[68]

Authors	Animal models and treatments	Main results	Potential mechanisms
	Postmenopausal osteoporosis model		
Zhao *et al.*[20]	OVX Wistar rats $n = 10$/group Age: 3–4 months Doses: 20, 40, 80 mg RSV/kg bwt/day Duration: 12 weeks	↓Femur neck BMD loss and ↓Femur trabecular bone loss 40 and 80 mg RSV/kg bwt ↓Femur trabecular spacing 80 mg RSV/kg bwt	↑Femur OPG gene expression ↓Femur cytokine gene expression
Lin *et al.*[22]	OVX Sprague–Dawley rats $n = 8$/group Age: 3 months Doses: 5, 15, 45 mg RSV/kg bwt/day Duration: 13 weeks	↓Vertebral BMD all doses	↑Serum ALP and osteocalcin NS serum TRAP
Sehmisch *et al.*[23]	OVX Sprague–Dawley rats $n = 11$/group Age: 3 months Doses: 50 mg RSV/kg bwt/day Duration: 12 weeks	Tibia NS BMD NS trabecular bone NS cortical bone NS strength	
Liu *et al.*[24]	OVX Wistar rats $n = 11$/group Age: 2.5 months Dose: 0.7 mg RSV/kg bwt/day Duration: 12 weeks	↓Femur BMC loss	
Mizutani *et al.*[25]	OVX SHRSP/Izm rats $n = 6$/group Age: 4 months Dose: 5 mg RSV/kg bwt/day Duration: 8 weeks	↓Loss of femur strength	
	Senile osteoporosis model		
Momken *et al.*[36]	Male Wistar rats $n = 6$–7/group Age: 4.5 months Dose: 400 mg RSV/kg bwt/day Duration: 6 weeks	↓Femur BMD loss ↓Femur strength loss	↑Plasma osteocalcin ↓Urinary DPD
Habold *et al.*[37]	Male Wistar rats $n = 5$/group Age: 5 months Dose: 400 mg RSV/kg bwt/day Duration: 6.5 weeks	NS tibia and femur BMD NS tibia and femur trabecular bone NS tibia and femur cortical bone	
Lee *et al.*[38]	Male Hooded Wistar rats $n = 7$/group Age: 6 months 20 mg RSV/kg bwt/day Duration: 12 weeks	NS tibia trabecular bone NS tibia cortical bone	↑Serum CTX NS serum ALP NS femur gene expression Sirtuin 1 ↑CCAAT/EBPα
Tresguerres *et al.*[40]	Male Wistar rats $n = 10$/group Age: 22 months Doses: 10 mg RSV/kg bwt/day Duration: 10 weeks	↓Femur cortical bone loss ↓Femur trabecular bone loss ↓Loss of femur strength	
Durbin *et al.*[41]	Male Fisher 344 × Brown Norway rats $n = 6$–7/group Age: 33 months Dose: 12.5 mg RSV/kg bwt/day Duration: 3 weeks	↓Loss of tibia trabecular connectivity NS femur and tibia BMD, BMC NS femur and tibia cortical bone NS femur and tibia strength	

Continued

Table 1. *Continued*

Authors	Animal models and treatments	Main results	Potential mechanisms
	Disuse bone osteoporosis model		
Durbin *et al.*[41]	HLS Male Fisher 344 × Brown Norway rats $n = 6$–7/group Age: 33 months Dose: 12.5 mg RSV/kg bwt/day Duration: 3 weeks (1 week before and during 2-week HLS)	NS tibia and femur cortical bone ↓Tibia trabecular BV/TV loss ↓Tibia trabecular number loss ↓Tibia trabecular spacing ↓Femur trabecular BV/TV loss ↓Femur trabecular thickness loss ↓Loss of femur spacing	↓Plasma C-reactive protein ↑Plasma osteocalcin
Durbin *et al.*[57]	HLS Male Fisher 344 × Brown Norway rats $n = 7$/group Age: 6 months Dose: 12.5 mg RSV/kg bwt/day Duration: 3 weeks (1 week before and during 2-week HLS)	↑Tibia BMC loss ↓Tibia cortical thickness ↑Tibia cortical porosity	↑Plasma lipid peroxidation ↓Plasma osteocalcin
Momken *et al.*[36]	HLS Male Wistar rats $n = 6$–7/group Age: 4.5 months Dose: 400 mg RSV/kg bwt/day Duration: 6 weeks (4 weeks before and during 2-week HLS)	↓Femur BMD loss ↓Loss of femur strength	↑Plasma osteocalcin ↓Urinary DPD
Harbold *et al.*[37]	HLS Male Wistar rats $n = 5$/group Age: 5 months Dose: 400 mg RSV/kg bwt/day Duration: 6.5 weeks (4.5 weeks before and during 2-week HLS)	↓Femur BMD loss ↓Femur trabecular bone loss ↓Femur cortical bone loss ↓Tibia BMD loss ↓Tibia trabecular bone loss ↓Tibia cortical bone loss	↓Bone marrow area
Wang *et al.*[59]	Spinal cord–injured male Sprague–Dawley rats $n = 10$–12/group Age: 6 weeks Dose: 400 mg RSV/kg bwt/day Duration: 10 days	↓Tibia BMD loss ↓Tibia BMC loss ↓Tibia trabecular bone loss ↓Loss of femur strength	↓Femur IL-6 gene expression ↓Femur *PPARγ* gene expression ↑Plasma osteocalcin ↓Urinary DPD

ALP, alkaline phosphatase; BMC, bone mineral content; BMD, bone mineral density; BV/TV, bone volume per unit to total volume; CCAAT/EBPα, CCAAT/enhancer-binding protein α; CTX, C-terminal telopeptide of type I collagen; DPD, dexoypyridinoline; HLS, hindlimb suspension; IL-6, interleukin-6; NS, statistically nonsignificant; OPG, osteoprotegerin; OVX, ovariectomized; PPARγ, peroxisome proliferator-activated receptor γ; RSV, resveratrol; TRAP, tartrate-resistant acid phosphatase.

of 50 mg RSV/kg bwt/day for 12 weeks. However, OVX rats provided a lower dose of 0.7 mg RSV/kg bwt/day for 12 weeks resulted in higher ($P < 0.05$) femur calcium content and epiphysis bone mineral content (BMC) compared to OVX rats provided no RSV supplementation.[24] In another study, loss of femur strength was attenuated in OVX rats provided 5 mg RSV/kg bwt/day for 8 weeks. This was indicated by a 33% higher breaking energy in OVX rats provided RSV compared to no RSV supplementation and similar femur strength to sham-operated rats.[25] Four of five published studies using the OVX rat model provided evidence that RSV supplementation protects against estrogen deficiency–induced bone mass loss, trabecular structural deterioration, and/or mechanical strength (Table 1). RSV doses of >40 mg/kg bwt were most effective with regard to safety, based on absence of estrogenic activity indicated by no hyperplastic effect on the uterus, reported for a dose of up to 80 mg/kg bwt.[20,22–25]

However, the OVX rat is considered a poor animal model for studying cortical bone loss owing to their lack of Haversian remodeling. However, cortical bone loss in the Haversian system plays a minor role in postmenopausal osteoporosis.[26] In OVX rats, primary bone loss was due to trabecular bone loss, which is similar to menopause in women.[27] In published studies, the age of OVX rats ranged from 2.5

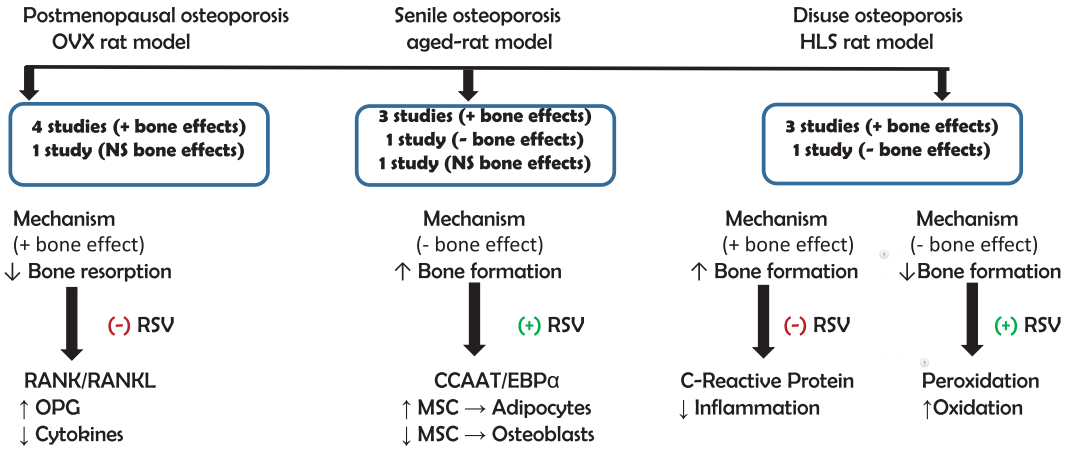

Figure 1. Summary of preclinical studies of the effects of resveratrol supplementation on bone using different rat models and potential mechanisms. HLS, hindlimb suspension; MSC, mesenchymal stem cells; NS, nonsignificant; OPG, osteoprotegerin; OVX, ovariectomized; RANKL, receptor activator of nuclear kappa B ligand; RSV, resveratrol.

to 4 months (Table 1).[20,22–25] Although rats reach sexual maturity at age 2.5 months, their skeleton is considered mature only after age 10 months.[28] The skeletally mature rat is an appropriate animal model of postmenopausal osteoporosis because there is a gradual transition from modeling to remodeling similar to age-related bone loss in humans.[27] If skeletally immature rather than mature OVX rats are used, bone protection may be due to RSV supplementation enhancing bone growth (modeling) rather than attenuating bone loss induced by estrogen deficiency at menopause. Wronski and Yen[29] suggested that longitudinal bone growth can be minimized by using rats aged 9–12 months and by performing measurement of skeletal sites with limited longitudinal growth such as the lumbar vertebrae.

Postmenopausal osteoporosis in women is characterized by rapid bone loss for 5–10 years after menopause followed by a steady state of age-related bone loss.[12] Published studies provided RSV to OVX rats for 8–13 weeks (Table 1).[20,22–25] However, femur, neck, and lumbar vertebrae trabecular bone requires 39 weeks postovariectomy to achieve steady state in the OVX rat model.[28] Therefore, the published OVX rats studies of RSV supplementation were limited to early rapid bone loss in postmenopausal osteoporosis. Another consideration is that OVX can promote weight gain that may increase mechanical loading and provide protection against the later stage of age-related bone loss in postmenopausal osteoporosis.[30] This can be minimized by weight monitoring and pair feeding. Additional preclinical studies with experimental designs that include skeletally mature animals and longer duration supplementation will strengthen preclinical evidence in support of RSV as a therapeutic bone agent for postmenopausal osteoporosis.

RSV supplementation for senile osteoporosis

Epidemiological data have consistently demonstrated that the incidence of bone fracture increases with age.[31] The burden of osteoporosis is expected to increase with the aging of the global population.[4] Senile osteoporosis differs from postmenopausal osteoporosis because bone remodeling is slow rather than rapid and involves both trabecular and cortical bone loss predisposing individuals to hip fractures.[32] However, age-related bone loss occurs in the later stage of postmenopausal osteoporosis. The etiology of senile osteoporosis include declining hormones and bone cell differentiation. In the bone marrow, pluripotent mesenchymal stem cells (MSCs) are capable of differentiating into either osteoblasts or adipocytes. In the process of aging, bone accumulates adipocytes at the expense of osteoblasts.[33] Fewer osteoblasts produce an imbalance in bone remodeling that favors bone resorption.[34] There are few pharmaceutical anabolic bone agents despite the higher risk of osteoporotic fractures and greater morbidity and mortality due to

hip fractures in the elderly.[35] *In vitro*, RSV treatment stimulated osteoblastogenesis.[11]

Male rats age 4.5 months provided 400 mg RSV/kg bwt/day for 6 weeks had higher femur BMD ($P < 0.001$) and bone strength ($P < 0.05$) compared to rats provided no RSV.[36] Anabolic activity was indicated by a positive correlation between femur BMD and the bone formation marker plasma osteocalcin ($R^2 = 0.9$, $P < 0.05$), and antiresorptive activity indicated by a negative correlation ($R^2 = 0.83$, $P < 0.05$) between femur strength and bone resorption marker urinary dexoypyridinoline (DPD).[36] In contrast, male rats age 5 months also provided 400 mg RSV/kg bwt/day for 6.5 weeks exhibited no significant effect on femur and tibia BMD or trabecular and cortical bone.[37] In a longer duration 12-week study, male rats age 6 months fed 20 mg RSV/kg bwt/day also had no significant effect on tibia trabecular and cortical bone.[38] A potential negative bone effect was indicated by higher serum ($P = 0.02$) C-terminal telopeptide of type I collagen, a bone resorption marker, without an accompanying increase in the bone formation marker ALP. Imbalance toward higher bone resorption was due to adipocyte differentiation at the expense of osteoblasts, indicated by no significant change in femoral expression of Sirtuin1, a transcription factor that inhibits MSC differentiation into adipocytes while promoting differentiation into osteoblasts and a tendency ($P = 0.06$) for upregulation of gene expression of CCAAT/enhancer-binding protein alpha (EBPα), which promotes adipogenesis.[38]

Investigating senile osteoporosis using the rat model may require animals to be age >6 months since age-related bone loss does not occur in the long bones of male rats until age 24–27 months.[39] Male rats age 22 months provided 10 mg RSV/kg bwt/day for 10 weeks had higher ($P < 0.05$) femoral cortical and trabecular bone and strength compared to rats provided no RSV.[40] Since long bone epiphyseal growth plates in male rats can remain open past 30 months,[27,28] we investigated RSV supplementation using male rats age 33 months. Feeding 12.5 mg RSV/kg bwt for 3 weeks had no significant effects on femur and tibia BMD, BMC, cortical bone, and strength, but increased tibia trabecular connectivity.[41] Trabecular bone loss occurs more rapidly than cortical bone loss.[28] Therefore, significant effects of RSV supplementation on trabecular bone, but minimal effects on BMD, cortical bone, and strength, may have been due to the short duration of the study.

Three of five published studies reported that RSV supplementation attenuated bone mass loss, structural deterioration, and/or mechanical strength (Table 1). However, mechanisms of action were not investigated. Tou[68] reviewed potential molecular mechanisms of RSV on bone. RSV stimulation of Sirtuin1 and inhibition of CCAAT/EBPα is bone beneficial since senile osteoporosis results from differentiation of MSC to adipocytes over osteoblasts. However, as previously discussed, mature rats provided 20 mg RSV/kg bwt for 12 weeks had no effect on Sirtuin1 and upregulated CCAAT/EBPα (Fig. 1).[38] Male rats age <6 months reported inconsistent findings of negative, positive, or no significant bone effects (Table 1).[36–38,40,41] A drawback of using rats as a model of age-related bone loss is that the rat skeleton retains lifelong growth in some bones.[28] Therefore, failing to adjust for longitudinal bone growth can lead to inappropriate conclusions since bone mass is directly related to skeletal size.[42] All studies were conducted on male rats. Longitudinal growth ceases in the tibia at age 15 months in female rats compared to 30 months for the long bone epiphyseal growth plates to close in male rats.[43] To reduce study length, the senescence-accelerated mouse, a model for age-related spontaneous osteopenia, may provide a useful tool for investigating RSV supplementation in senile osteoporosis.[44] Additional studies that include longer duration supplementation, appropriate-age rats, monitoring of longitudinal bone growth, adjusting for bone size, and selecting the appropriate skeletal sites for performing measurements can strengthen preclinical evidence in support of RSV as a therapeutic bone agent for senile osteoporosis.

RSV supplementation for disuse osteoporosis

The elderly often experience bed rest and inactivity that decrease mechanical loading and accelerate age-related bone loss.[15] Osteocytes embedded in bone play a central role in remodeling by sensing external mechanical loads and transmitting this information to osteoblasts and osteoclasts.[45] In disuse osteoporosis, mechanical unloading produces transient rapid bone turnover due to increased resorption

and then sustained decreased bone formation.[34] Approximately 30% of mechanical unloading–induced bone loss is attributable to increased bone resorption and 70% to decreased bone formation.[46] Mechanical unloading promotes osteocyte apoptosis, which signals osteoclast recruitment and bone resorption.[47] Osteocytes also increase sclerostin secretion in response to mechanical unloading. This inhibits Wnt/β-catenin signaling, which suppresses osteoblast activity and, in turn, bone formation.[48] Sustained decreased bone formation may be due to mechanical unloading favoring adipocyte over osteoblast differentiation.[49] Disuse osteoporosis produces faster loss of trabecular than cortical bone. This results in decreased trabecular number, thickness, and connectivity and thins cortical bone.[50] Mechanical loading produces bone loss in weight-bearing bone, placing these bones at the highest risk of fracture. In spinal cord injury, the most frequent fractures occur in the knee region and lower limbs.[51,52] Accepted rat models of disuse osteoporosis are spinal cord resection, which includes both mechanical unloading and neurological factors, and hindlimb suspension (HLS). Mechanical unloading using the HLS rodent model has been reported to induce similar bone changes to bed rest in humans, such as increased number and volume of adipocytes in the bone marrow stroma.[53,54]

We investigated RSV supplementation effects on the long bones by providing male rats age 33 months 12.5 mg RSV/kg bwt 1 week before and during 2-week HLS. RSV supplementation had no significant effect on tibia or femur cortical bone measurements.[41] However, bone loss is faster in trabecular than cortical bone owing to the higher surface-to-volume ratio.[28,50] In the HLS rat model, the earliest bone loss occurs in tibia trabecular bone after 14-day mechanical unloading.[55] In the tibia, HLS rats provided RSV increased trabecular bone volume per unit to total volume by 33% and trabecular number by 10%, and decreased trabecular spacing by 10% compared to no RSV, and showed no significant differences compared to ambulatory (AMB) rats. In the femur, HLS rats provided RSV increased trabecular bone volume per unit to total volume by 22% and trabecular thickness by 6.3%, and decreased trabecular spacing by 11% compared to no RSV, and showed no significant differences compared to AMB rats. Mechanical unloading increases reactive oxygen species and proinflammatory cytokines.[56] We found a negative relationship ($R^2 = 0.87$, $P < 0.01$) between plasma osteocalcin and C-reactive protein. This suggested that RSV supplementation prevented osteoblast loss by decreasing inflammation induced by mechanical unloading (Fig. 1).[41] On the basis of this study, RSV supplementation protected against disuse osteoporosis in old rats.

Disuse osteoporosis also occurs in younger individuals with inactivity, bed rest, and spinal cord injury. Male rats age 6 months provided 12.5 mg RSV/kg bwt/day 1 week before and during 2-week HLS resulted in detrimental bone effects, indicated by lowest ($P < 0.05$) tibia BMC and lower (7%) cortical thickness and increased cortical porosity (22%) compared to HLS rats provided no RSV.[57] A negative correlation ($R^2 = 0.69$, $P = 0.02$) between plasma osteocalcin and plasma lipid peroxidation suggested decreased bone formation due to increased oxidation (Fig. 1). Under certain circumstances, RSV produces singlet oxygen that can promote lipid peroxidation.[58] Using a different model, a dose of 400 mg RSV/kg bwt/day provided to young spinal cord injury rats had antioxidant effects, indicated by increased ($P < 0.05$) serum total antioxidant capacity and reduced ($P < 0.05$) femur malondialdehyde concentration compared to rats provided no RSV.[59] HLS male rats aged 4.5 months provided 400 mg RSV/kg bwt/day for 4 weeks before and during 2-week HLS, increased ($P < 0.001$) femur BMD, and mechanical strength compared to no RSV supplementation.[36] The three-point bending testing used in this study is a good indicator of the mechanical strength of cortical bone.[60] RSV had anabolic activity, indicated by the higher ($P < 0.05$) plasma osteocalcin, and antiresorptive activity, indicated by decreased urinary DPD ($P < 0.05$). Similarly, Habold *et al.*[37] reported that male rats age 5 months provided 400 mg RSV/kg bwt/day for 4.5 weeks before and during the 2-week HLS had higher ($P < 0.05$) tibia and femur BMD compared to no RSV supplementation. Femoral BMD of HLS rats provided RSV was similar to AMB rats. Additionally, distal femur and proximal tibia metaphysis trabecular and cortical bone were higher ($P < 0.05$) in HLS rats provided RSV compared to no RSV, and bone microstructure was comparable to AMB rats.[37] The authors suggested that decreased bone marrow area in rats provided

RSV indicated reduced MSC differentiation to adipocytes. Together, the results indicated that HLS male rats provided 400 mg RSV/kg bwt/day increased bone formation by reducing differentiation of osteoblasts into adipocytes. However, specific measurements of adipoctyes and osteoblast content in bone marrow are needed, since HLS also increases endocortical resorption, which increases the bone marrow cavity.[61] The importance of dose was further demonstrated in a spinal cord injury rat model of disuse osteoporosis. Young (aged 6 weeks) spinal cord–injured male rats provided 400 mg RSV/kg bwt/day for 10 days resulted in higher ($P < 0.05$) tibia BMD, BMC, and trabecular bone compared to no RSV supplementation. There were no significant differences in trabecular bone measurements of bone volume to total volume and spacing between spinal cord–injured rats provided RSV and sham-operated rats. Femoral strength was greater ($P < 0.05$) in spinal cord–injured rats provided RSV compared to no RSV supplementation.[59] Reduced inflammation by RSV supplementation was indicated by downregulation of gene expression of femoral interleukin-6 ($P < 0.05$) and reduced bone marrow adipocytes was indicated by downregulation of femoral PPARγ ($P < 0.05$), a key transcription factor regulating MSC differentiation into adipocytes. Anabolic activity was indicted by the higher plasma osteocalcin and antiresorptive activity was indicated by decreased urinary DPD.[49]

Published studies using HLS rats to investigate RSV as a therapeutic agent for disuse osteoporosis included male rats age 33 months and 4.5–6 months (Table 1).[36,37,41,57] Three of four published studies using the HLS rat model reported that RSV supplementation attenuated BMD loss, microachitectural deterioration, and/or loss of bone strength induced by mechanical unloading (Table 1). Beneficial bone effects of RSV supplementation were observed in older rats (age 33 months), whereas younger (age 4.5–6 months) HLS animals required higher doses and longer RSV supplementation before mechanical unloading. Bone-protective effects observed in young rats (age 4.5–6 months) provided RSV were likely due to enhanced bone accretion before mechanical unloading since peak bone mass is achieved after age 10 months.[28] Studies provided HLS rats RSV supplementation for a duration of 3–6.5 weeks (Table 1).[36,37,41,57] According to Sievanen,[63] the most important determinant of bone loss is the duration of disuse. In HLS rats, bone cavity marrow enlargement and reduction of cortical bone requires ∼6 weeks, and this can reach 10% loss at 26 weeks.[62] To strengthen existing preclinical evidence, study experimental designs should include longer duration RSV supplementation to assess cortical bone loss. Another important consideration is selection of bone sites to measure, since the magnitude of bone response to disuse is affected by the function of the bone in the skeleton (i.e., weight-bearing bones) and by specific location within the bone (i.e., epiphysis, metaphysis, diaphysis, proximal or distal). For growing animals, including a baseline control or forelimbs as an internal control to compare with disuse hindlimbs at the end of the experiment can assist in distinguishing between bone loss and bone growth.[64]

Bone protective effects in younger (age 4.5–6 months) HLS rats were observed at the higher RSV doses. Published studies investigating RSV as a therapeutic agent for disuse osteoporosis included doses that ranged from 12.5 to 400 mg/kg bwt/day (Table 1).[36,37,41,57] Using body surface–area conversion, this translated to ∼122–3892 mg/kg bwt in a 60-kg human.[65] Doses above 100 mg/kg bwt are difficult to achieve through the diet and, therefore, require supplementation.[65] According to Blanchard *et al.*,[66] it is not appropriate to rely on body surface scaling. Instead, performance of physiologic, pharmacokinetic, and toxicology studies are required to determine RSV doses that are physiologically relevant and pharmacologically active in humans.

One limitation, however, is that HLS rat studies investigating RSV supplementation on disuse osteoporosis have used males. The anatomy of the rat allows the testes to enter the abdomen through the inguinal canal when the hindlimb is elevated, resulting in reduced testosterone after 7-day HLS.[66] Since reductions in steroid hormones influence bone measurements, a solution is to perform ligation of the inguinal canal.[66] Another consideration is that, during the first days of HLS, food consumption can drop as much as 50% and, therefore, group mean feeding may be needed initially or throughout the study,[64] particularly if RSV is being provided in the diet. HLS of rats can also induce stress, which affects bone.[67] Stress during HLS can be assessed by weight loss and appearance of porphryin around

the eyes and nose of rats. Postmortem indicators of stress include adrenal hypertrophy, thymus atrophy, and elevated corticosterone levels.[64] Additional preclinical studies using HLS rat models that include these considerations in their study design can strengthen existing preclinical evidence in support of RSV as a therapeutic bone agent for disuse osteoporosis.

Conclusions

Preclinical evidence provides a foundation for designing future human studies to investigate the efficacy and safety of RSV supplementation to reduce osteoporosis risk in the population. The ability of RSV to act as both an anabolic and antiresorptive agent makes it a promising therapeutic agent for osteoporosis with distinct pathologies. Several studies using rat models of postmenopausal, senile, and disuse osteoporosis have reported bone-protective effects, but some negative bone findings indicate that determining safe doses and life stages when RSV is administered requires further investigation. Few studies have been conducted, and study limitations exist that do not allow definitive recommendations to be made regarding RSV supplementation without further research. Limitations of rat models include differences from the human skeleton, such as lack of a Haversian remodeling and longitudinal bone growth beyond sexual maturity. Also, short-term studies using skeletally immature animals have frequently been used to investigate aging effects on bone. Weaknesses of OVX and HLS models include potential for altered food intake, body weight, and endocrine function that can confound bone effects of RSV. Additionally, there is lack of agreement regarding an appropriate method of RSV dose translation from animal species to humans. Suggestions for strengthening the experimental design of studies were provided. Common to studies using different rat models of osteoporosis were a need to include animals that are skeletally mature, longer study duration to allow slower cortical bone loss to be assessed, and use of RSV doses that are physiologically relevant and achievable by humans. This is important because rat models plays a critical role in osteoporosis research by providing evidence that will contribute toward translation of therapeutic benefits of RSV into clinical practices and dietary guidelines to reduce bone loss.

Acknowledgments

The author was supported by the West Virginia University Agriculture and Forestry Experimental Station Hatch Grant WVA00665. The author appreciates funding provided by the conference organizers to participate as a speaker in the 3rd International Resveratrol Conference hosted by the University of Hawaii, Hilo.

Conflicts of interest

The author declares no conflicts of interest.

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