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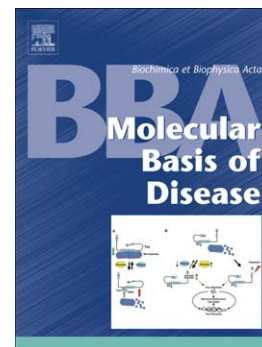
Resveratrol Supplementation Affects Bone Acquisition and Osteoporosis:  
Pre-Clinical Evidence Towards Translational Diet Therapy

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**Resveratrol Supplementation Affects Bone Acquisition and Osteoporosis: Pre-Clinical  
Evidence Towards Translational Diet Therapy**

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**ABSTRACT**

Osteoporosis is a major public health issue that is expected to rise as the global population ages. Resveratrol (RES) is a plant polyphenol with various anti-aging properties. RES treatment of bone cells results in protective effects, but dose translation from *in vitro* studies to clinically relevant doses is limited since bioavailability is not taken into account. The aims of this review is to evaluate *in vivo* evidence for a role of RES supplementation in promoting bone health to reduced osteoporosis risk and potential mechanisms of action. Due to multiple actions on both osteoblasts and osteoclasts, RES has potential to attenuate bone loss resulting from different etiologies and pathologies. Several animal models have investigated the bone protective effects of RES supplementation. Ovariectomized rodent models of rapid bone loss due to estrogen-deficiency reported that RES supplementation improved bone mass and trabecular bone without stimulating other estrogen-sensitive tissues. RES supplementation prior to age-related bone loss was beneficial. The hindlimb unloaded rat model used to investigate bone loss due to mechanical unloading showed RES supplementation attenuated bone loss in old rats, but had inconsistent bone effects in mature rats. In growing rodents, RES increased longitudinal bone growth, but had no other effects on bone. In the absence of human clinical trials, evidence for a role of RES on bone health relies on evidence generated by animal studies. A better understanding of efficacy, safety, and molecular mechanisms of RES on bone will contribute to the determination of dietary recommendations and therapies to reduce osteoporosis.

**Keywords:** resveratrol, bone mass, ovariectomized, mechanical unloading, age, osteoblasts, osteoclasts

## 1. Introduction

Resveratrol (RES) is a polyphenolic (3,4',5-trihydroxystilbene) compound naturally present in red wine and a variety of plant foods such as grapes, cranberries, and nuts [1]. There is a growing body of evidence that RES is an effective therapeutic agent for age-related degenerative diseases such as osteoporosis [2-3]. Osteoporosis is a skeletal disorder characterized by low bone mass, structural deterioration, decreased bone strength, and increased risk of bone fractures [4]. Osteoporosis is a major public issue with a worldwide estimated 9 million bone fractures annually. Additionally, the prevalence of osteoporosis is expected to increase as the global population ages [5].

Throughout life, bone is remodeled by a process involving bone resorption which removes old bone followed by replacement with new bone by the process of bone formation. During the growth stages of childhood, adolescence, and into young adulthood, bone formation exceeds the rate of bone resorption resulting in bone mass acquisition. After this stage, at ~20-30 years of age, bone resorption exceeds bone formation resulting in gradual and progressive bone loss [6]. Therefore, maximizing peak bone mass (PBM) during the growth stage is an important factor for preventing future risk of osteoporosis. This is particularly important for women, who are at greater of risk of osteoporosis than men, due to rapid bone loss resulting from declining estrogen at menopause [7]. Both women and men experience age-related bone loss that is often accelerated by mechanical unloading associated with physical inactivity and prolonged bed rest [8]. Disuse-related bone loss is also observed in young patients confined to prolonged bed rest as a result of injury or immobilization due to spinal cord injury.

RES has estrogenic, anti-inflammatory, antioxidant, and proliferative properties that can influence bone metabolism [9]. No toxicity has been reported for RES intakes of up to 500 mg/d in animals and humans [9-10]. Due to its multiple bioactivities and low toxicity, RES offers the promise of being an efficacious and safe therapeutic agent for osteoporosis. However, due to the lack of human clinical trials, evidence of a therapeutic role of RES on bone relies on *in vitro* studies and animal models of bone loss. The aim of this review is to evaluate the pre-clinical evidence of RES supplementation to enhance bone, to reduce risk of osteoporosis, and to determine potential mechanisms of action. A better understanding of the effects of RES on bone will contribute toward the development of dietary recommendations and therapies for preventing bone loss leading to osteoporosis.

## **2. Bone Remodeling**

Bone consists of cortical (compact) and trabecular (spongy) components. Cortical bone accounts for ~80% of skeletal mass and is located in the diaphyseal regions of long bones; whereas, trabecular bone is located inside cortical bone in the proximal and distal epiphysis region of long bones and vertebrae [6 Stagi] (Figure 1A). Bone is constantly remodeled in a process where old bone is removed (bone resorption) and replaced by new bone (bone formation). The process of bone remodeling is summarized in Figure 1B. The cell lineages important in bone turnover are osteoblasts and osteoclasts [11]. Osteoclasts are derived from haematopoietic progenitors (i.e. monocyte/macrophage) in the bone marrow. Receptor activator of nuclear kappa B ligand (RANKL) produced by osteoblasts bind to RANK receptors located on the surface of haematopoietic cells promotes differentiation into osteoclasts [11]. Activated osteoclasts attach to the bone surface and release proteolytic enzymes that digest connective tissue proteins and solubilize bone mineral. Production of enzymes such as tartrate-resistant acid

of the phosphatase (TRAP) and collagen degradation products such as deoxypyridinoline (DPD) and C-terminal telopeptide of type I collagen (CTX) during osteoclastogenesis provide useful surrogate clinical markers of bone resorption. To counterbalance bone resorption, osteoblasts also produce osteoprotegerin (OPG) that inhibit osteoclastogenesis by binding to RANKL and blocking interaction with the RANK receptor [12]. Osteoblasts fill the cavity produced by osteoclast-mediated resorption by synthesizing and mineralizing new bone [11]. Hence, skeletal integrity requires a balance between bone-forming osteoblasts activity and bone-resorbing osteoclast activity. Imbalances where bone resorption exceeds formation results in bone loss [12].

Osteoporosis is the result of increased osteoclast activity and/or decreased osteoblast activity during remodeling. Osteoblasts are derived from pluripotent mesenchymal stem cells (MSCs) in the bone marrow. MSCs differentiate into either osteoblasts, adipocytes or chondrocytes depending on the activation of specific transcription factors [11]. Expression of the transcription factor, peroxisome proliferator activated receptor gamma (PPAR $\gamma$ ), is the main determinant of MSC differentiation into adipocytes [13]. Several transcription factors are required for MSC differentiation into osteoblasts. Runt-related transcription factor 2 (Runx2) is considered the master regulator of osteoblast differentiation [14] and Osterix downstream of Runx2 is also essential [15]. MSCs committed to the osteoblast lineage form osteoprogenitors. Entering a proliferation phase, osteoprogenitors undergo morphological changes into pre-osteoblasts that are capable of synthesizing bone matrix and alkaline phosphatase (ALP) [11]. Pre-osteoblasts mature into osteoblasts that regulate bone matrix mineralization and produce osteocalcin [11]. Circulating ALP and osteocalcin provide useful surrogate clinical markers of

bone formation. Osteoblast differentiation ends with the formation of osteocytes that regulate bone responses to mechanical stimuli and bone mineralization [16] (Figure 1B).

The activity of transcription factors can be influenced by various local and systemic factors that include bone morphogenetic proteins [17], insulin-like growth factor (IGF) [18], the canonical wingless (Wnt)/ $\beta$ -catenin signaling pathway [19], mechanical forces [20], estrogen and other hormones [21]. The natural food component RES has both structural and functional similarities to estrogen [22]. Furthermore, dietary RES activates Sirtuin1 (Sirt1) known as the longevity gene [23]. Backesjo et al. [24] reported that Sirt1 activation decreases MSC differentiation into adipocytes while promoting differentiation into osteoblasts. Much of the knowledge about molecular mechanisms underlying RES as a dietary treatment for osteoporosis has been derived from *in vitro* studies.

### **3. *In Vitro* Studies**

Mobasheri and Shakibaei [25] reviewed evidence from *in vitro* studies conducted using immortal tumor-derived cells, primary MSCs, pre-osteoblasts, and osteoclast progenitors concluded that RES enhanced bone mass by promoting osteoblastogenesis and by inhibiting osteoclastogenesis. Therefore, RES may have advantages over current pharmacological therapies which act either by promoting osteoblast-mediated bone formation or by inhibiting osteoclast-mediated bone resorption. Multiple actions include direct stimulation of osteoblast proliferation and differentiation indicated by increased DNA synthesis and ALP activity in RES-treated osteoblastic MC3T3-E1 cells. The ability of the anti-estrogenic drug, tamoxifen to antagonize these effects indicated that RES stimulated osteoblastogenesis by acting as an estrogen agonist [26]. Dai et al. [27] demonstrated that treating human bone marrow-derived

MSC with RES increased gene expression of the key osteogenic transcription factors, Runx2 and Osterix. RES was also demonstrated *in vitro* to act on various signal transduction pathways. RES activated the estrogen-mediated extracellular signal-regulated kinase 1/2 (ERK) signaling pathway regulating osteoblast differentiation and proliferation [27]. RES activated AMP-activated protein kinase (AMPK) which regulates osteoblast differentiation and inhibits bone resorption by acting as a negative regulator of RANKL [28]. Zhou et al. [29] showed that RES augmented Wnt signaling which stimulated osteoblastogenesis and bone formation. Treating human bone marrow-derived MSC with RES promoted differentiation of MSC towards osteoblasts by up-regulating Runx2 gene expression through the activation of Sirt1 [30-31]. Also, activation of Sirt1 by RES was shown to promote binding to PPAR $\gamma$  which repressed MSC differentiation into adipocytes [24][32]. Additionally, RES suppresses osteoclastogenesis by acting through Sirt1 to bind to RANK which inhibited binding to RANKL [11][33]. Potential molecular mechanisms underlying RES effects on bone are summarized in Figure 2.

Based on evidence derived from *in vitro* studies, RES by acting on both osteoblasts and osteoclasts offers a promising natural therapeutic agent for osteoporosis. Whether effects on bone cells translates to bone tissue cannot be determined *in vitro*. Also, cell culture systems do not take bioavailability into account. *In vivo* evidence indicates that RES has low bioavailability due to rapid metabolism in the body [34]. Therefore, this review focuses on evaluating pre-clinical evidence of RES supplementation to enhance bone, prevent osteoporosis, and to determine potential mechanisms.



#### 4. Animal Studies

The pathophysiology of osteoporosis differs depending on the etiology. Age-related osteoporosis produces gradual bone loss that is predominantly due to reduced osteoblasts; whereas, postmenopausal osteoporosis produces rapid bone loss that is predominantly due to increased osteoclast activity [7] (Figure 1C). The therapeutic value of RES has been investigated using various animal models that include the ovariectomized (OVX) animal model of rapid bone loss due to estrogen-deficiency and the hindlimb unloaded (HLU) animal model of bone loss due to mechanical unloading. Animals of varying ages have been used in order to determine the effects of providing RES during the growth stage on PBM and during aging on bone loss associated with senile osteoporosis.

##### 4.1. Resveratrol and Estrogen Deficiency-Related Bone Loss

Declining estrogen is a major risk factor in osteoporosis [35]; therefore, hormone or estrogen replacement therapy (ERT) has been used as treatments. However, ERT has serious side effects which include increased risk of cardiovascular disease, breast cancer, and uterine cancer [36-37]. *In vitro*, RES has been shown to exert estrogenic as well as estrogenic-independent effects on bone cells [25]. In a pre-clinical study, Liu [38] provided mature OVX Wistar rats a daily oral gavage of 0.7 mg/kg bwt RES for 12 weeks. RES attenuated OVX-induced loss of femur calcium (Ca) and epiphyseal bone mineral density (BMD), but not BMD loss in the mid-diaphysis (Table 1). The femur mid-diaphysis consists predominantly of cortical bone while the epiphysis consists predominantly of trabecular bone which is more sensitive to estrogen deficiency [39]. Another study providing a higher dose of 5 mg/kg bwt/d RES in the diet for a shorter-duration of 8 weeks to mature spontaneously hypertensive stroke prone OVX

Izm rats showed no significant effect on femur Ca content and bone breaking load strength, but preserved breaking energy [40], a strength measurement that takes into account stiffness provided by minerals and toughness provided by collagen in the bone matrix [41]. OVX rats provided dietary RES improved bone collagen indicated by significantly higher femur hydroxyproline content (Table 1).

In contrast, Sehmisch et al. [42] reported that mature OVX Sprague-Dawley rats provided diets supplemented with 5 or 50 mg/kg bwt/d RES for a duration of 12 weeks had no effect on tibiae total BMD, cortical or trabecular bone, and bone strength (Table 1). The absence of effects may be because other estrogenic compounds were controlled in this study by providing a phytoestrogen-free diet and RES has been reported to have synergistic effects when combined with other phytochemicals [25]. Also, bone measurements were performed on the tibia rather than the femur. Lin et al. [43] performed measurements on both the femur and tibia of mature OVX Sprague-Dawley rats fed 0, 5, 15 or 45 mg/kg bwt/d RES or ERT (0.03 mg/kg bwt diethylstilbestrol) in the diet for 13 weeks. At the higher doses of 15 and 45 mg/kg bwt/d, RES attenuated BMD loss in whole femur and in estrogen sensitive regions of the femur. The highest (45 mg/kg bwt/d) RES dose completely prevented OVX-induced whole tibial BMD loss. All doses of RES prevented OVX-induced BMD loss in vertebrae, a site consisting predominantly of trabecular bone. RES effects on bone were equal to ERT and RES had no adverse effects on the uterus indicated by the absence of endometrial hyperplasia (Table 1). Estrogen deficiency bone loss is due to increased osteoclastic activity without a corresponding increase in osteoblastic activity [44] (Figure 1C). Lin et al. [43] reported that providing RES to OVX rats promoted bone formation indicated by higher serum ALP, but had no effect had on bone resorption marker, TRAP. More recently, Zhao et al. [45] investigated RES supplementation on bone including

mechanistic studies. OVX Wistar rats were administered RES doses of 0, 20, 40 or 80 mg/kg bwt/d or ERT (0.8 mg/kg bwt estradiol valerate) by stomach tube for a duration of 12 weeks. In agreement with other studies, RES supplementation had no significant effect on femur diaphysis BMD, but was protective at trabecular bone sites. RES doses of 40 and 80 mg/kg bwt/d preserved BMD at the femoral neck while the highest dose of 80 mg/kg bwt/d preserved BMD at the distal femur (Table 1). RES doses of 40 and 80 mg/kg bwt/d also protected against OVX-induced structural deterioration indicated by preservation of trabecular area, thickness, and number. The highest RES dose produced a similar degree of decreased trabecular spacing as ERT. A dose-dependent down-regulation of femur gene expression of OPG and cytokines, interleukin-6 and tumor necrosis factor alpha (TNF $\alpha$ ) indicated that RES reduced bone resorption by inhibiting signaling pathways regulating RANKL. *In vitro*, primary bone cells isolated from the femoral heads of canine bone fragments treated with RES inhibited RANKL-induced activation of nuclear factor of kappa B (NF $\kappa$ B), a key transcription factor regulating inflammation [33].

Collectively, the OVX rodent model showed that RES supplementation protected against estrogen deficiency induced bone mass loss and trabecular structure deterioration (Table 1). High doses of RES produced similar effects to ERT without stimulating endometrial hyperplasia. Pre-clinical evidence supports the therapeutic value of RES in estrogen-deficiency bone loss, but senile osteoporosis differs from postmenopausal osteoporosis since both male and females are affected and bone loss is gradual [7]. As the population ages, morbidity, mortality, and the financial costs attributed to osteoporosis are expected to increase [46]. Therefore, it is important to determine an efficacious and safe therapy for age-related bone loss.

#### 4.2 *Resveratrol and Age-Related Bone Loss*

In postmenopausal osteoporosis, trabecular bone loss predisposes women to spine and wrist fractures; whereas, in senile osteoporosis, cortical bone loss predisposes the elderly to hip fractures [47-48]. Age-related bone loss is due to reduced osteoblasts more than greater osteoclast activity which characterizes estrogen-deficiency bone loss [35] (Figure 1C). During aging there is an accumulation of adipocytes at the expense of osteoblasts in the bone marrow [7][49]. Increased adipocytes in bone results in oxidative stress due to higher susceptibility to lipid oxidation [7][49]. *In vitro*, RES treatment of murine osteoclast progenitors suppressed RANKL-induced osteoclast differentiation by inhibiting reactive oxygen species production [50].

In a pre-clinical study, Tresguerres et al. [51] provided Male Wistar rats 10 mg/kg bwt/d RES for a duration of 10 weeks. Enhanced bone structure was indicated by increased cortical thickness, increased trabecular bone volume and number, and reduced trabecular spacing (Table 1). However, there were no significant effects on bone turnover markers, plasma osteocalcin and CTX. Rats were 22 month old at the start of the study and age-related bone loss does not occur in the long bones of male rats until age 24-27 months [52]. To investigate the efficacy of RES in age-related bone loss, Fischer 344 x Brown Norway male rats aged 33 months were provided a daily oral gavage of 12.5 mg/kg bwt RES [53]. Old rats provided RES showed no significant effects on BMD, bone mineral content (BMC), bone mineral area (BMA), and strength, but increased femur phosphorus (P) content and tibia trabecular connectivity. The RES supplementation duration of 3 weeks was short. In a longer study, male C57BL/6NIA mice (age 12 months) were provided a phytoestrogen-free diet supplemented with 8 or 31 mg/kg bwt/d RES for 18 months [54]. Feeding RES improved whole femur trabecular tissue mineral density

and bone volume per unit of total volume (BV/TV). Pooling of RES groups to increase statistical power from n=4-5 to 8-10 mice/group resulted in significantly higher femur trabecular thickness, cortical tissue mineral density, and bone strength. Based on results of these few studies in different ages and species, RES supplementation prior to age-related bone loss over a long-duration enhanced long bone microstructure (Table 1). To determine potential mechanisms, Durbin et al [53] assessed oxidative stress, inflammation, and bone turnover. There was no significant effect on serum measurements oxidative stress. Anti-inflammatory actions of RES supplementation in old male rats were indicated by reduced serum C-reactive peptide concentration which preserved osteoblasts as indicated by higher serum osteocalcin and ALP concentrations, but no effects on bone resorption markers, TRAP, DPD, and CTX.

Low bone mineral mass in the elderly is a function of the amount of bone loss due to aging and insufficient PBM attainment during skeletal growth [35]. Epidemiological studies indicated that a 10% increase in PBM can reduce bone fracture risk in postmenopausal women by 50% [55-56]. Therefore, maximizing PBM during the bone acquisition stage is important for preventing future risk of osteoporosis.

#### 4.3. *Resveratrol and Bone Acquisition*

Skeletal growth involves both longitudinal and cross-sectional growth. Periosteal cortical bone formation coupled with endosteal cortical bone resorption regulates cross-sectional bone growth [57]. To study bone growth, weanling female Sprague-Dawley rats were randomly assigned to a daily oral gavage of 0, 1, 4, 10, 40, 100  $\mu\text{g/d}$  RES or estradiol (100  $\mu\text{g/d}$ ) dissolved in ethanol for a duration of 6 days. Despite estrogenic activity, RES had no significant effect on

tibia cross-sectional area, medullary area, cortical bone area, periosteal bone formation rate or periosteal mineral apposition rate [58] (Table 1).

Lengthening of vertebrae and long bones is regulated by chondrocyte proliferation in the epiphyseal growth plate. Therefore, longitudinal growth ceases upon growth plate fusion [59]. *In vitro*, RES treatment of chondrocytes obtained from an adult rat femur protected against the catabolic effect of pro-inflammatory cytokine, interleukin-1 $\beta$  [60]. To determine the effects of RES on longitudinal growth *in vivo*, Karimian et al. [61] provided a daily oral gavage of 200 mg/kg bwt RES to pubertal female New Zealand white rabbits until growth plate fusion occurred. After 16 weeks, rabbits provided RES supplementation had longer tibia and vertebrae, more chondrocytes, and increased growth plate area compared to control rabbits (Table 1). RES supplementation delayed growth plate fusion by suppressing the replacement of avascular cartilage with vascularized bone indicated by the down-regulated gene expression of vascular endothelial growth factor, a signaling molecule in vascularization, and laminin, a cartilage protein.

In rabbits and humans, the epiphyseal growth plate fuses at sexual maturation, but in rats and mice longitudinal bone growth continues after sexual maturation [59]. Mature (age 6 months) male Fischer 344 x Brown Norway rats provided a daily oral gavage of 12.5 mg/kg bwt RES for 3 weeks had lengthen tibiae [62]. Anti-inflammatory actions of RES indicated by reduced plasma C-reactive peptide concentration prevented chondrocyte destruction. The absence of RES effects on femur longitudinal growth may be due to slower growth compared to the tibia [63]. Despite enhanced tibia length and width there was no significant effects on bone mineralization and microarchitecture (Table 1).

The role of RES supplementation on PBM remains unclear due to inconsistent study findings and the few pre-clinical studies that have been conducted to date. However, Chen et al. [64] found beneficial effects of RES supplementation on bone following nutritional deprivation during the growth stage. Immature male Sprague-Dawley rats were restricted to 60% of their typical food intake. After 4 weeks, rats were randomly assigned to be re-fed a normal (14% kcal) or high (59% kcal) fat diet supplemented with a daily oral gavage of 100 mg/kg bwt RES for a duration of 8 weeks. Providing RES reversed spine, femur, and whole body BMD, BMC, and BMA losses due to caloric restriction (Table 1). Catch-up growth was more effective when RES was provided a normal versus a high fat diet. Anti-inflammatory actions of RES preserved osteoblasts indicated by reduced serum TNF $\alpha$  concentration and enhanced bone formation indicated by higher serum osteocalcin concentration. Based on the Chen et al. [64] study, RES supplementation may be beneficial for treating secondary osteoporosis characterized by bone loss due to conditions such as nutritional deficiencies, inactivity, bed-rest, and immobilization.

#### 4.4. *Resveratrol and Disuse-Related Bone Loss*

Mechanical unloading due to bed rest, paralysis, and microgravity accelerates bone loss in weight-bearing bones [65]. Bone loss related to mechanical unloading has been attributed to suppression of bone formation and to the stimulation of bone resorption [8]. Aguirre et al. [66] showed that mechanical unloading of mice increased osteocyte apoptosis in cortical and trabecular bone which then acted as a signal for osteoclast recruitment resulting in bone resorption and bone loss. The deleterious effects of mechanical unloading on bone was attributed to increased reactive oxygen species and pro-inflammatory cytokines [67]. Nakamura et al. [68] reported that inhibiting NF $\kappa$ B activity prevented bone loss in mechanically unloaded

mice. *In vitro*, suppressing NF $\kappa$ B in osteoclast precursors decreased bone resorption by inhibiting RANKL [68]. Thus, due to its anti-inflammatory and antioxidant properties, RES has the potential to be an effective therapy for disuse-related bone loss.

#### 4.4.1. Mechanical Unloading

In the elderly, decreased mechanical loading due to inactivity and bed rest accelerates age-related bone loss [8]. To investigate the effects of RES supplementation, rats were subjected to HLU, a model that has been reported to induce similar bone changes as bed rest in humans [69]. Durbin et al. [53] provided old male Fisher 344 x Brown Norway rats a daily oral gavage of 12.5 mg/kg bwt RES for 1 week prior to HLU and throughout the 2 weeks of HLU. RES supplementation ameliorated femoral BMD, BMC, Ca, and P loss due to mechanical unloading (Table 1). RES supplementation also protected against microarchitecture deterioration indicated by complete prevention of decreased femoral trabecular number and partial prevention of decreased trabecular BV/TV and increased trabecular separation induced by mechanical unloading. There were no significant effects on cortical bone. During mechanical loading, loss of cortical bone is gradual and continues over a longer period of time compared to trabecular and bone loss which is more rapid and severe [70]. RES supplementation preserved mechanical strength in the femur, but not tibia strength. In the tibia, mechanical unloading induced bone thinning rather than bone loss as indicated by loss of trabecular thickness and connectivity rather than trabecular number. RES supplementation attenuated loss of tibial BMC, BMA, cortical area, trabecular BV/TV, thickness, connectivity, and increased trabecular spacing induced by HLU. According to Morey et al. [71], during HLU suppression of bone formation rather than acceleration of bone resorption predominates (Figure 1C). In the Durbin et al. study [53], old



HLU male rats provided RES exhibited elevated plasma osteocalcin and ALP, but had no effects on plasma markers of bone resorption, TRAP, DPD, and CTX. Inflammation was indicated by higher plasma TNF $\alpha$  concentration in HLU compared to ambulatory rats. Providing RES to old HLU male rats decreased plasma TNF $\alpha$  although concentrations remained elevated compared to ambulatory rats. Based on these results, RES supplementation attenuated inflammation induced by mechanical unloading and this in turn, prevented the loss of osteoblasts as indicated by higher osteoblast activity.

Disuse-induced bone loss is not restricted to the elderly, but also occurs in young individuals confined to bed rest. To investigate this Durbin et al. [62] chose mature (age 6 months) male Fisher 344 x Brown Norway rats to ensure that bone loss was due to mechanical unloading rather than aging. Using the same study design as for old rats, mature rats were provided 12.5 mg/kg bwt/d RES to for 1 week prior to HLU and throughout the 2 weeks. HLU did not attenuate loss of trabecular BV/TV, number, and connectivity in the femur mid-diaphysis. Unexpectedly, HLU mature male rats provided RES had detrimental bone effects indicated by significantly lowered tibia BMC, Ca, cortical thickness, and increased cortical porosity compared to HLU rat provided no RES (Table 1). A negative correlation between plasma lipid peroxidation and plasma osteocalcin concentrations ( $r^2=0.69$ ,  $P=0.02$ ) in HLU mature rats provided RES indicated decreased bone formation was due to increased oxidation. Under certain conditions, RES acts as a pro-oxidant by producing singlet oxygen that promotes lipid peroxidation [72-73].

In another study, a higher dose of RES was provided for a longer-duration prior to HLU. Harbold et al. [74] provided mature (age 5 months) male Wistar rats 400 mg/kg/d RES for 4.5

weeks prior to HLU and throughout the 2 weeks of HLU. RES supplementation completely prevented tibial BMD loss and partially prevented BMD loss in the femur. Providing RES attenuated loss of trabecular number, BV/TV and increased trabecular spacing due to HLU in the distal femur and proximal tibia metaphysis (Table 1). Similar to the Durbin et al. [62] study, RES supplementation had no effect on mid-diaphysis cortical bone. Bone strength was not determined in this study. The Momken et al. [75] study which included bone strength measurements found that mature male Wistar rats provided with a liquid meal consisting of 400 mg/kg bwt/d RES for 4 weeks prior to HLU and during the 2 weeks of HLU prevented the loss of femoral BMD and preserved bone strength (Table 1). Providing RES increased bone formation in HLU rats as indicated by the higher plasma osteocalcin and decreased bone resorption indicated by reduced urinary DPD.

As discussed above, RES supplementation benefited old HLU rats [53], but detrimental bone effects were observed in mature HLU rats [62]. However, other studies that provided higher RES doses to mature rats for a longer duration prior to HLU showed bone protective effects (Table 1). Based on the study results, optimizing bone prior to mechanical unloading protects against bone loss. Gafni et al. [76] suggested that bone responds more to current rather than past conditions. This has important implications for RES supplementation as a treatment for bone injury and repair.

#### 4.4.2. *Resveratrol and Injury Related Bone Loss and Bone Injury Repair*

Bone fractures occur in 50% of individuals with complete spinal cord injury [77]. Disuse contributes to bone loss in immobilization-related spinal cord injury, but differs from bed rest, in that circulating bone resorption markers are higher and bone loss is more severe [78]. Wang et

al. [79] investigated the effects of RES supplementation using a spinal cord injury model produced by complete surgical transection of the lower thoracic cord of mature male Sprague-Dawley rats. Twelve hours after surgery, rats were provided 400 mg/kg bwt/d RES intragastrically for a duration of 10 days. RES supplementation attenuated tibial BMC and BMD loss and preserved bone microstructure indicated by higher trabecular bone volume, number, thickness, and lower trabecular spacing in the metaphyseal region of the proximal tibia compared to control rats. Histomorphometric evaluation of the tibiae of rats provided RES showed less eroded bone surface, decreased osteoclast surface, increased osteoblast surface, and higher tibia mineral apposition rates. Higher serum osteocalcin and lower urinary DPD concentrations indicated that RES supplementation preserved against bone loss by promoting bone formation and by inhibiting bone resorption. Biomechanical strength measurements performed on the femur showed RES supplementation preserved bone strength. Gene expression analysis of the femur revealed multiple actions. RES actions on the RANK/RANKL/OPG axis suppressed osteoclastogenesis and promoted osteoblastogenesis indicated by down-regulation of gene expression of the RANKL to OPG ratio and bone resorption marker, TRAP and by up-regulation of bone formation marker, osteocalcin (Table 1). Oxidative stress is considered a hallmark of spinal cord injury [80]. Antioxidant actions were indicated by reduced serum malondialdehyde and increased serum total antioxidant capacity and total sulfhydryl concentrations in spinal cord injury rats provided RES compared to control rats. Almedia et al. [81] reported that higher lipid oxidation in osteoblasts diminishes Wnt signaling which activates transcription factors promoting MSC differentiation into osteoblasts and suppresses gene expression of PPAR $\gamma$ , the transcription factor that promotes differentiation of MSC into adipocytes. Providing RES to spinal cord injured rats restored Wnt1 signaling, IGF-1, IGF-1 receptor, and down-regulated

femur PPAR $\gamma$  gene expression indicating RES promoted osteoblast and inhibited adipocyte differentiation.

To investigate the role of RES supplementation on bone repair, Casarin et al. [82] produced a calvarial defect rat model by surgically inserting a screw-shaped titanium implant into the tibia of mature male Wistar rats. Following surgery, rats were provided 10 mg/kg bwt/d RES for 30 days. Higher torque force indicated better retention of the titanium implants in the RES-treated group. Histomorphometric evaluation showed RES supplement improved bone repair indicated by reduced spacing in the bone margins of the calvarial defect. Up-regulation of gene expression of bone morphogenetic protein and osteopontin in calvarial samples indicated that RES supplementation improved bone repair by promoting bone ossification and bone tissue maturation [82]. Collectively, the study results indicated the RES supplementation attenuates bone loss induced by immobilization resulting from spinal cord injury and promotes bone repair.

## **5. Resveratrol Dose and Bioavailability Considerations**

RES is often administered dissolved in ethanol due to poor solubility in water. When converting doses provided to rodents based on body weight to equivalent doses in humans, this translates into hundreds of liters of wine per day [83]. RES doses reported to be effective for ameliorating bone loss in rat and mice studies ranged from 0.7 to 400 mg/kg bwt (Table 1). Using body surface area as described by Regan et al. [83], this translated to doses ranging in a 60 kg human from ~7 to 3900 mg/kg bwt. RES doses above 100 mg/kg bwt cannot be achieved dietary and therefore, require supplementation. Blood concentrations provide another method for estimating effective doses. However, RES rapid metabolism and clearance from the body leads to low plasma RES concentrations of between 0 to 26 nM [84] which is a dose several fold

lower than required *in vitro* to stimulate bone cell differentiation, proliferation, and activation [85]. Therefore, efficient delivery routes that allow for a sustained release of RES at bone sites are being investigated. Li et al. [85] tested a three-dimensional porous polycaprolactone (PCL) scaffold deliver method engineered to provide controlled release of RES *in situ*. *In vitro*, rat bone marrow stoma cell treated with RES using PCL scaffolding exhibited increased ALP activity and mineralization. Similarly, human bone marrow-derived MSC treated with RES using PCL scaffolding exhibited increased ALP activity and mineralization [86]. *In vivo*, calvarial defect male Sprague-Dawley rats were treated with PCL scaffolding containing RES [85]. After 8 weeks, bone extracted from the skull showed that RES promoted bone regeneration as indicated by histomorphologic and X-ray evidence of a greater area of bone regeneration. Bone formation was indicated by elevated bone sialoprotein concentration. Supplementation and development of novel delivery systems can assist in overcoming issues such as high dose requirements and low bioavailability which limits translation of RES for human clinical use.

## 6. Conclusions

There is a growing body of evidence supporting the efficacy of RES supplementation for attenuating bone loss. Shown in Figure 2, RES influences estrogen-dependent and independent signaling transduction pathways which modulated gene expression of transcription factors, Runx2 and Osterix regulating osteoblasts differentiation and transcription factors, RANKL and NFkB regulating osteoclast differentiation and activation. Ability of RES to act on both osteoblasts and osteoclasts through multiple mechanisms suggests that RES can prevent bone loss associated with different etiologies and pathologies. Table 1 summarizes the bone physiological effects of RES supplementation in different rodent models and potential mechanisms of action. RES supplementation prevented OVX-induced loss of bone mass,

trabecular bone by suppressing inflammation which preserved bone formation. Providing RES to aging rats prior to age-related bone loss attenuated loss of cortical and trabecular bone by decreasing inflammation and preserving bone formation. In HLU rat models, beneficial or detrimental bone effects of RES supplementation depended on dose and timing of exposure.

Collectively, the evidence from animal models supports a therapeutic value of RES supplementation on bone. However, few of the animal studies attempted to investigate the molecular mechanisms underlying RES anti-inflammatory effects on bone formation. Further mechanistic focused studies would improve understanding as would experimental designs using comparable doses, timing of exposure, and treatment duration. Still, determination of a therapeutic RES dose will ultimately require human clinical studies due to inherit limitations of extrapolating doses found to be effective and safe in animals to humans. Meanwhile, evidence generated by animal studies will provide the necessary foundation for future clinical trials.

## **7. Disclosures**

The author reports no disclosures or conflicts of interest

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ACCEPTED MANUSCRIPT

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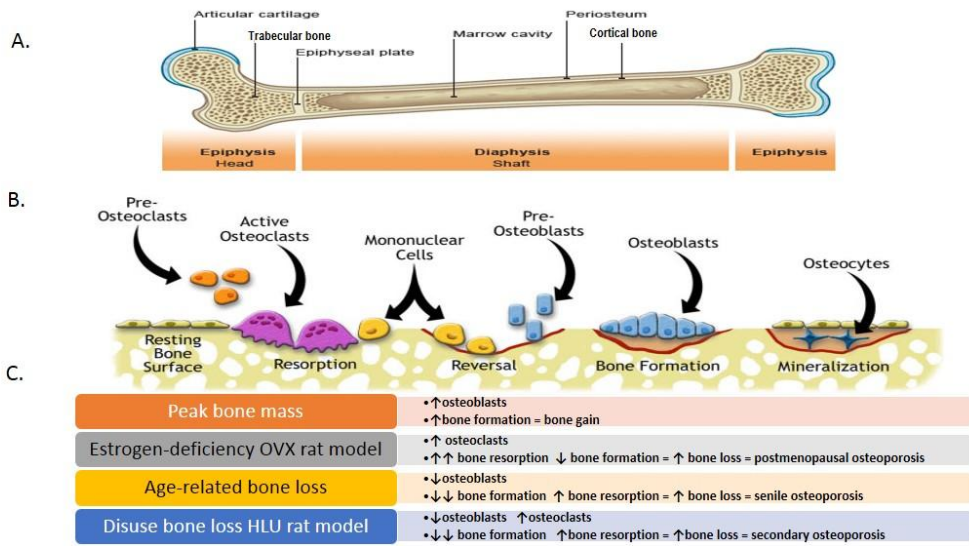
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**Figure Legends**

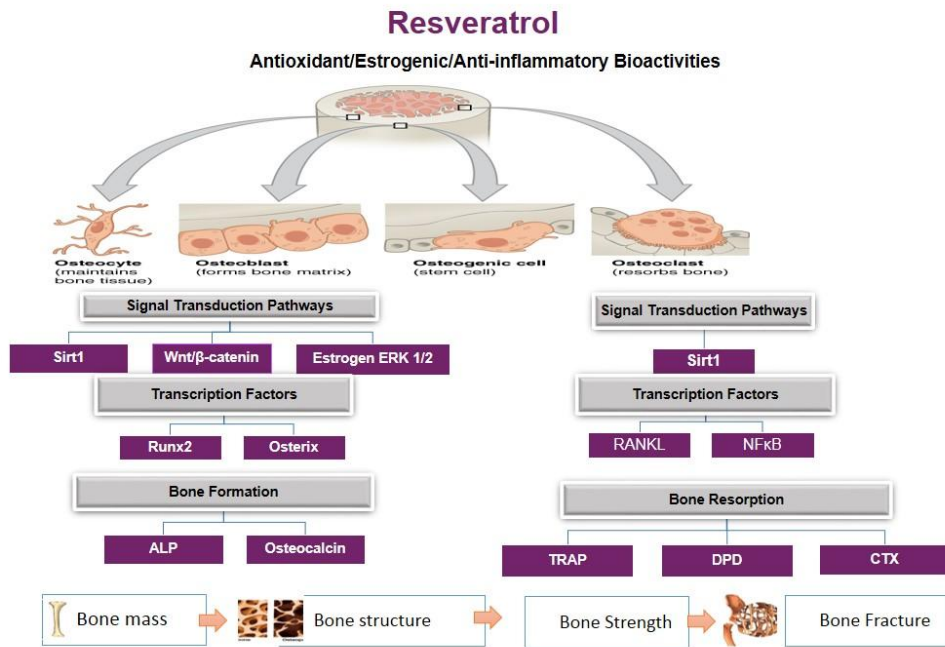
Figure 1. Summarizes steps in normal bone remodeling and the results of imbalances in bone turnover. A) femur anatomical sites, B) cell lineages involved in the process of bone remodeling, C) bone loss and acquisition due to alterations in bone formation and bone resorption. Symbol ↓ decrease, ↑ increase, ↑↑ predominant. Adapted from the University of Michigan bone remodeling <http://www.umich.edu/news/Releases/2005/Feb05/bone.html> and Openstax College bone structure <http://cnx.org/content/m46281/latest/>

Figure 2. Schematic of potential molecular mechanisms of resveratrol on osteoblasts and osteoclasts. Abbreviations are ALP, alkaline phosphatase; CTX, C-terminal telopeptide of type I collagen; DPD, deoxypyridinoline; ERK, extracellular signal-regulated kinase 1/2; NFκB, nuclear factor kappa B; Runx2, runt-related transcription factor 2; Sirt1, Sirtuin1; TRAP, tartrate-resistant acid of the phosphatase; Wnt; canonical wingless/β-catenin signaling pathway. Adapted from Openstax College bone structure <http://cnx.org/content/m46281/latest/>



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Fig 1



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Fig 2

**Table 1.** Summary of pre-clinical studies of resveratrol supplementation on bone mineralization, microarchitecture, strength, growth, and remodeling in different rodent models.

Authors	Treatment	Bone Results	Bone Remodeling
<i>Estrogen-Deficiency Bone Loss Models</i>			
Liu et al. [38]	OVX Wistar rats (n=11/group) Age: ~2.5 months RES dose: 0.7 mg/kg bwt/d Duration: 12 wks	↑femur epiphysis BMD ↑femur Ca	
Mizutani et al. [40]	OVX SHRSP/Izm rats (n=6/group) Age: 4 months RES dose: 5 mg/kg bwt/d Duration: 8 wks	NS femur Ca ↑femur breaking energy	<i>Preserved bone protein matrix indicated by</i> ↑femur hydroxylproline
Sehmisch et al. [42]	OVX Sprague-Dawley rats (n=11/group) Age: 3 months RES doses: 5, 50 mg/kg bwt/d Duration: 12 wks	NS tibia total BMD NS tibia cortical bone NS tibia trabecular bone NS tibia bone strength	
Lin et al. [43]	OVX Sprague-Dawley rats (n=8/group) Age: 3 months RES doses: 5, 15, 45 mg/kg bwt/d Duration: 13 wks	↑femur BMD 15, 45 mg/kg bwt/d RES ↑tibia BMD 45 mg/kg bwt/d RES ↑vertebrate BMD all doses	<i>Promoted bone formation indicated by</i> ↑serum ALP NS serum TRAP
Zhao et al. [45]	OVX Wistar rats (n=10/group) Age: 3-4 months RES doses: 20, 40, 80 mg/kg bwt/d Duration: 12 wks	↑femur neck BMD 40, 80 mg/kg bwt/d RES ↑femur distal BMD 80 mg/kg bwt/d RES ↑femur trabecular bone 40, 80 mg/kg bwt/d RES	<i>Suppressed inflammation indicated by</i> ↓femur IL-6, TNF $\alpha$
<i>Age-Related Bone Loss Models</i>			
Tresguerres et al. [51]	Male Wistar rats (n=10/group) Age: 22 months RES doses: 10 mg/kg bwt/d Duration: 10 wks	↑cortical bone ↑trabecular bone	NS plasma osteocalcin NS plasma CTX
Durbin et al. [53]	HLU male Fisher 344 x Brown Norway rats (n=6-7/group) Age: 33 months RES dose: 12.5 mg/kg bwt/d Duration: 3 wks	NS bone mass ↑femur P content ↑tibia trabecular connectivity NS bone strength	<i>Suppressed inflammation &amp; promoted bone formation indicated by</i> ↑serum C-reactive peptide ↑serum osteocalcin and ALP

			NS TRAP, DPD, and CTX
Pearson et al. [54]	Male C57BL/6NIA mice (n=8-10/group) Age: 12 months RES dose: 8 and 31 mg/kg bwt/d Duration: 18 months	↑femur trabecular bone ↑femur cortical bone ↑femur strength	
<i>Bone Acquisition Models</i>			
Turner et al. [58]	Female Sprague-Dawley rats (n=5-6) Age 21 d RES dose: 1,4,10,40,100 µg/d Duration: 6 days	NS cross-sectional growth measurements	
Karimian et al. [61]	Female New Zealand white rabbits (n=12/group) Age: 12 weeks RES dose: 200 mg/kg bwt/d Duration: 16 wks	↑tibia and vertebrae length ↑tibia chondrocytes ↑growth plate area ↓growth plate fusion	<i>Suppressed vascularization indicated by</i> ↓VEGF ↓laminin
Durbin et al. [62]	Male Fisher 344 x Brown Norway rats (n=7/group) Age: 6 months RES dose: 12.5 mg/kg bwt/d Duration: 3 wks	↑tibia length and width NS tibia mineralization NS tibia microarchitecture	<i>Suppressed inflammation indicated by</i> ↓plasma C-reactive peptide
Chen et al. [64]	Male Sprague-Dawley rats (n=6/group) Age: 6 weeks RES dose: 100 mg/kg bwt/d Duration: 8 wks	↑spine, femur, whole body bone mass	<i>Suppressed inflammation &amp; promote bone formation indicated by</i> ↓serum TNFα ↑serum osteocalcin
<i>Disuse Bone Loss Models</i>			
Durbin et al. [53]	HLU male Fisher 344 x Brown Norway rats (n=6-7/group) Age: 33 months RES dose: 12.5 mg/kg bwt/d Duration: 3 wks (1 wk prior 2 wks during HLU)	↑femur mineralization ↑femur trabecular bone NS femur cortical bone ↑femur strength ↑tibia bone mass ↑tibia trabecular bone ↑tibia cortical area NS tibia strength	<i>Suppressed inflammation &amp; promote bone formation indicated by</i> ↓serum TNFα ↑plasma ALP and osteocalcin NS plasma TRAP, DPD, CTX
Durbin et al. [62]	HLU male Fisher 344 x Brown Norway rats (n=7/group) Age: 6 months RES dose: 12.5 mg/kg bwt/d Duration: 3 wks (1 wk prior 2 wks during HLU)	NS femur mineralization NS femur cortical bone NS femur trabecular bone NS femur strength ↓tibia mineralization ↓cortical bone	<i>Promote oxidation &amp; decrease osteoblasts indicated by</i> ↑plasma lipid

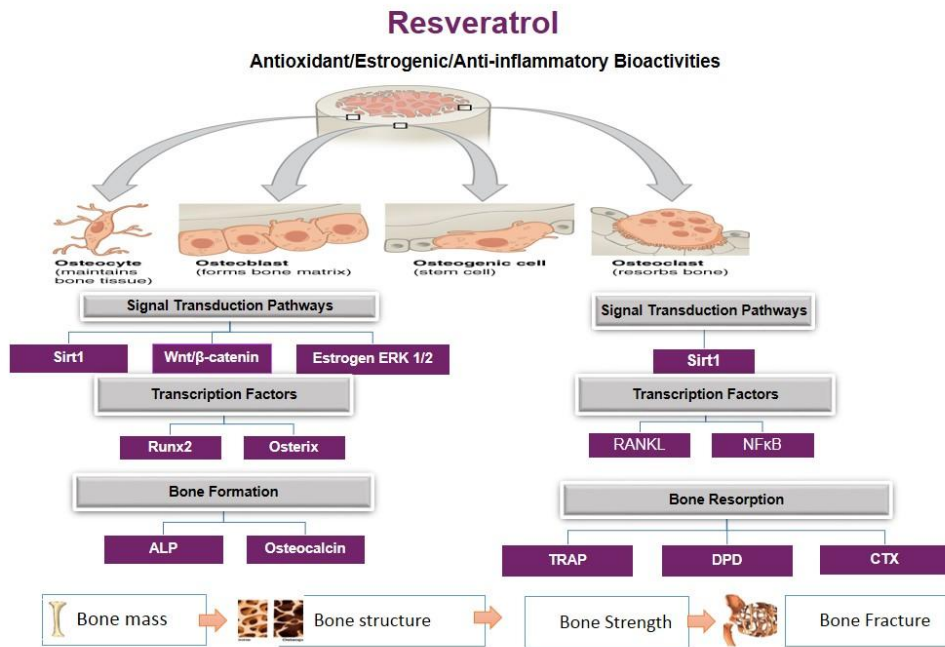


		NS trabecular bone	peroxidation ↓plasma osteocalcin
Harbold et al. [74]	HLU male Wistar rats (n=5/group) Age: 5 months RES dose: 400 mg/kg bwt/d Duration: 6.5 wks (4.5 wks prior 2 wks during HLU)	↑femur BMD ↑femur distal trabecular bone NS femur cortical bone ↑tibia BMD ↑tibia proximal trabecular bone NS tibia cortical bone	
Momken et al. [75]	HLU male Wistar rats (n=6-7/group) Age: ~4.5 months RES dose: 400 mg/kg bwt/d Duration: 6 wks (4 wks prior 2 wks during HLU)	↑femur BMD ↑femur strength	<i>Promoted bone formation &amp; suppressed bone resorption indicated by</i> ↑plasma osteocalcin ↓urinary DPD
Wang et al. [79]	Spinal cord injured male Sprague-Dawley rats (n=10-12/group) Age: 6 weeks RES dose: 400 mg/kg bwt/d Duration: 10 days	↑tibia bone mass ↑tibia trabecular bone ↑femur strength	<i>Promote bone formation &amp; suppress bone resorption indicated by</i> ↑plasma osteocalcin ↓urinary DPD ↑bone osteoblast surface ↓bone osteoclast surface ↑femur osteocalcin gene expression ↓femur TRAP gene expression
Casarin et al. [82]	Calvarial defect male Wistar rats (n=15/group) Age: 2.5 months RES dose: 10 mg/kg bwt/d Duration: 30 days	↓calvarial defect ↑retention of implants	<i>Promote bone maturation &amp; ossification indicated by</i> ↑calvarial sample BMP ↑calvarial sample osteopontin

Abbreviations: ALP, alkaline phosphatase; BMC, bone mineral content; BMD, bone mineral density; BMP, bone morphogenetic protein ; Ca, calcium; CTX, C-terminal telopeptide of type I collagen; DPD, deoxypyridinoline; HLU, hindlimb unloaded; IL-6, interleukin-6; OVX, ovariectomized; NS, statistically non-significant; RES, resveratrol; SHRSP, spontaneously hypertensive stroke prone;

TNF $\alpha$ , tumor necrosis factor alpha; TRAP, tartrate-resistant acid of the phosphatase, VEGF, vascular endothelial growth factor.

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Graphical abstract

**Highlights**

- RES supplementation shown to be bone protective in various animal models
- RES anti-inflammatory actions prevent estrogen deficiency and age-related bone loss
- RES improved bone by promoting bone formation and by inhibiting bone resorption
- RES effect osteoblasts/osteoclasts through transcription factors/signaling pathways

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