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Pharmacological Activation of WNT- B Catenin Signaling Pathway As A Potential Therapeutic Target In Osteoporosis

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Abstract

WNT- β Catenin signaling is emerging as a key pathway in the regulation of bone mass and strength. The aim of the current study was to investigate the effects of a drug claimed to stimulate WNT signaling pathway; namely resveratrol, on ovariectomy (OVX)-induced osteoporosis in rats. The present study was conducted on 40 female Wistar albino rats that were divided into 4 groups of 10 rats each, Group I: sham operated, Group II: non-treated OVX rats, groups III and IV were OVX rats treated with resveratrol and resveratrol plus a compound that inhibits WNT; namely C59, respectively.

At the end of the experimental period, the following parameters were assessed: urinary deoxypyridinoline (DPD), serum osteocalcin, calcium and phosphorous concentrations and serum alkaline phosphatase activity. Biochemical assessment of β catenin in fourth lumbar vertebrae (LV4) was carried out. The tibia, left femur and third lumbar vertebrae (LV3) were weighed and biomechanical study on LV3 was carried out. Immune histochemical studies of right femur and the forth-lumbar vertebrae (LV4) were carried out to detect apoptotic osteoclastic and osteoblastic cells. Evaluation of cortical bone morphometric indices was done by CT-Scanning technique. The present results demonstrated that resveratrol protected against biochemical, histological, biomechanical and histomorphometrically osteoporotic changes. C59 blocked resveratrol-induced changes in assessed parameters suggesting that the effect of resveratrol was mediated mainly through activation of WNT signaling pathway. It can be concluded that the use of drugs stimulating WNT signaling pathway could be effective in ameliorating OVX-induced osteoporotic changes.

Keywords: Wnt/ β Catenin; C59; Resveratrol; Osteoporosis; Ovariectomy

Introduction

Drugs used in the treatment of osteoporosis are traditionally classified as antiresorptive and anabolic; the majority of those currently licensed falling into the former category. Although these agents are effective in stabilizing or increasing bone mass density and reducing the risk of fractures, their exact mechanism of action frequently is not known, and they do not increase bone formation. Alternative drug therapies that either block the function of the bone-resorbing osteoclasts or enhance the anabolic function of osteoblasts could prove beneficial to patients with osteoporosis [1].

Identification of specific molecular targets for pharmacological intervention provides opportunities for design of therapeutic modalities with improved safety and/or efficacy [2]. Many of the mechanisms responsible for coordination of activity between osteoclasts and osteoblasts and how they are dysregulated in osteoporosis are now being clarified. This leads to the possibility that bone remodeling can be manipulated by pharmacological means to reverse the changes caused by osteoporosis [3].

Among the recently discovered molecular signals mediating bone formation, and that can be a target for osteoporosis treatment, is the WNT / β-catenin signaling pathway [4]. In recent years, the role of this pathway in bone biology has gained considerable attention. Specific human pathologies of bone including osteoporosis have been associated with aberrant WNT signaling.

WNT glycoproteins bind to the receptor frizzled (Fzd) and their co-receptor low-density lipoprotein receptor-related protein 5/6 (Lrp5/6) complex, leading to stabilization and accumulation of β-catenin in the cytoplasm [7,8]. Cells respond to WNT/ β-catenin signaling by an increase in the levels of beta-catenin. Accumulation of this nuclear transcriptional co-adapter, β-catenin, has been shown to be critical to the elaboration of osteoblast development downstream of osteogenic WNT/

β-catenin signaling [9]. WNT signaling plays a key role in bone tissue by determining the differentiation of stem cells into mature osteoblasts rather than into chondrocytes and adipocytes [10]. Its regulation is predominantly driven by the production of two WNT signaling antagonists including sclerostin (SOST), which inhibits β-catenin signaling via binding LRP5 [11].

Given the numerous findings showing that the WNT signaling pathway regulates osteogenesis, this pathway has arisen as an attractive therapeutic target for treating several osteogenic disorders including osteoporosis. Potential therapeutic approaches attempt to stimulate the WNT signaling pathway by upregulating the intracellular mediators of the WNT signaling cascade and inhibiting the endogenous antagonists of the pathway [12].

Inhibitors of the negative regulators of WNT/β-catenin signaling ("inhibiting the endogenous inhibitors") are potential candidates for the prevention and treatment of bone loss. Inhibiting SOST appears to be the most attractive strategy because SOST is the only component of the WNT pathway expressed almost exclusively by osteocyte [13]. Among the candidate drugs reported to inhibit SOST is resveratrol [14]. a phytoestrogen that naturally occurs in many plant species [15]. Resveratrol has been identified as a potent activator of Sirtuin 1 (SIRT1)+. Indeed, SIRT1 has been found to repress SOST levels [17]. Thus, the aim of the current study was to investigate the effects of resveratrol on OVX-induced osteoporosis in rats.

Materials and Methods

Animal grouping

The current study was conducted on 40 female Wistar albino rats that were approximately 90 days of age, at the beginning of the study, with regular 4-days estrous cycle and body weight ranging from 200-250 grams. Rats were fed a standard diet and acclimatized for 2 weeks before the experiments. The animal protocol was reviewed and approved by the Ethical Committee of Faculty of Medicine-Alexandria University. The regularity of the cycle was verified by daily vaginal smear examination [18]. Bilateral OVX was performed using a dorsal approach [19]. Sham operated rats were subjected to the same surgical procedure except that the ovaries were not removed. Upon recovery from anesthesia, animals were grouped into five groups each of 10 rats:

Group I: (Sham rats) sham -operated ¬rats that received 1 ml 2 % aqueous solution of gum acacia daily orally for 8 weeks, following sham operation, and served as a control for group II.

Group II: (OVX rats) rats in which osteoporosis was induced by bilateral OVX and received 1 ml 2 % gum acacia daily orally for 8 weeks following OVX.

Group III: (OVX-Resveratrol treated rats) OVX rats that received resveratrol (Sigma- St. Louis, MO) suspended in 2 % gum acacia in a dose of 20 mg/kg b.wt daily orally [20]. for 8 weeks following OVX.

Group IV: (OVX-Resveratrol + C59-treated) OVX rats treated with resveratrol in the same regimen as group III together with the WNT inhibitor; C59 in a dose of 20 mg/kg i.p [21]. All drugs were given daily by oral gavage syringe. The food consumption of OVX rats was restricted to that of control rats (pair-feeding) to minimize the increase in body weight associated with OVX [22].

Biochemical measurements

At the end of the experimental period, urine was collected after placing each rat in a metabolic cage and, to avoid urea degradation, urine samples were maintained frozen. Urinary deoxypyridinoline (DPD) (a biochemical indicator of collagen degradation and reflects the extent of bone resorption) was measured by an enzyme-linked immunosorbent assay (ELIZA) kit (Pyrilinks, Metra Biosystems, Inc., Mountain View, CA) [23] and normalized, with the content of creatinine measured enzymatically (Sigma, St. Louis, MO) [24].

Each animal was weighed, and blood samples were collected, using capillary tubes introduced into the medial retro-orbital venous plexus early in the morning after an overnight fast. Blood samples were centrifuged for 15 min at 3000 rpm. Sera were separated and stored at –80°C for determination of: serum glucose concentration, serum osteocalcin concentration (biochemical indicator of bone formation) by an ELIZA kit (Biomedical Technologies Inc., Stoughton, MA) [25], serum calcium (Ca) [26] and phosphorous(P) concentrations [27] and serum alkaline phosphatase (ALP) activity [28] by colorimetric method using commercial kits (Sigma Chemical Co., St. Louis, MO).

The femurs were removed and soaked in ice-cold 0.25 M sucrose solution. The right femur was separated into diaphysis and metaphysis (not containing epiphyseal tissues). β-catenin protein level was assessed in femoral diaphyseal tissue by Total β-catenin ELISA kit (Enzo Life Sciences, Inc) [29].

Histological examination:

After the blood samples were collected, animals were sacrificed by exsanguination. Failure to detect ovarian tissue and observation of marked atrophy of the uterine horns confirmed the success of OVX. The left femur and fourth lumbar vertebrae (LV4) were defleshed and fixed immediately in buffered formalin for histological evaluation. After fixation, the bones were embedded undecalcified in 8% formate. Five-micron thick undecalcified paraffin sections were prepared with an HM 360 microtome (Microm, Walldorf, Germany). They were deparaffinized in xylene. The sections were sampled in the median plane of the vertebrae and in the midsagittal plane of the femur and stained with H&E stain [30]. Immune staining, to detect apoptotic osteoclastic and osteoblastic cells, was carried out by anti-Fas antibodies (monoclonal mouse antihuman

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CD95(Fas ligand =antibody to Fas) using Dakota get retrieval solution of high PH, code number S 33308, Detection system Dako LSAB system, code number K0679 [31,32]. The reaction of the primary antibody was visualized using peroxidase conjugated streptavidin and DAB. Apoptosis was considered to be present when brown cytoplasmic stain was detected in osteoblasts or osteoclasts.

Bone-biomechanical-measurements

After isolation and freeing from muscular tissue, tibia, left femur, and third lumbar vertebra (LV3) were weighed. The LV3 vertebra was separated and trimmed to exclude the spinous, transverse, and articular processes. Bone strength was measured using a three-point bending test [33]. LV3 were embedded in rectangular epoxy blocks fitting the grips of the torsional testing machine (SEY 10, Magnetic Electromotor AG, Wardenship, Switzerland). Load (newtons N) was applied mid-way between the two supporting ends of the tested LV3. The central part of the vertebral body was loaded along the longitudinal axis. Specimens were tested in a saline bath at 37 °C. Each specimen was submerged in the saline bath for 3 min before testing to allow equilibration of temperature. Mechanical properties of LV3, including the maximum load [ultimate force] N), and stiffness (N/mm) were recorded by a plotter (Perkin-Elmer Corp., model 165, Hitachi Ltd., Tokyo, Japan).

Measurement of cortical bone morphometric indices

The left femurs were scanned using a spiral CT-scanning machine. For each bone sample two axial sections, one at 40% of the length from the distal end in the diaphysis (cortical) and other at the distal part of the metaphysis (cancellous) were taken. Medullary width combined cortical thickness (CCT), periosteal area (PA), cortical area (CA) and the ratio of cortical area to periosteal area (CA/PA) were calculated [34].

Statistical analysis

Data were fed to the microcomputer program Statistical Package for Social Science SPSS version 17.0. Results were expressed as a mean ± standard error means (S.E.M.) Tabulation and analysis of data was done using analysis of variance (ANOVA) test. Significance of differences between the groups studied was determined with Least Significant Difference (LSD) test. Statistically significant differences were assumed at P less than or equal 0.05.

Results

Mortality

One rat died in each of groups II & IV, while no rats died in the rest of the groups.

Histological results

Immunostaining of bone sections using anti-Fast antibody revealed positively stained osteoblasts denoting increased apoptotic activity in osteoblasts of resveratrol + C59 –treated OVX rats (Figure 1a). Negatively stained osteoblasts (i.e. no apoptosis) could be detected in OVX rats treated with resveratrol, whereas osteoclasts became positively stained (Figure 1b). H&E sections of LV4 in resveratrol + C 59 –treated OVX rats showed thinning out of bone trabeculae, widening of bone marrow spaces and loss of bone architecture (Figure 2a). Treatment of OVX rats with resveratrol markedly restored bone mass compared to non-treated OVX rats (Figure 2b).

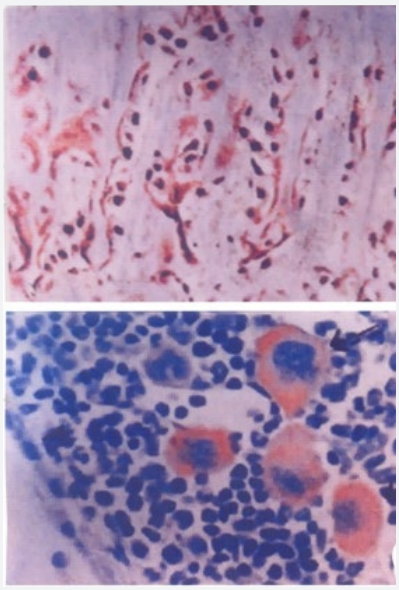

Figure 1: Histological sections of right femur stained by anti-Fas protein-antibody. (a)positively stained osteoblasts with deep brown cytoplasmic staining in reveratrol+ C 59 - treated OVX rats (Magnification X 250), (b) positively stained osteoclasts with deep brown cytoplasmic staining (long arrows) and negatively stained osteoblasts in resveratrol-treated OVX rats (Magnification X 400).

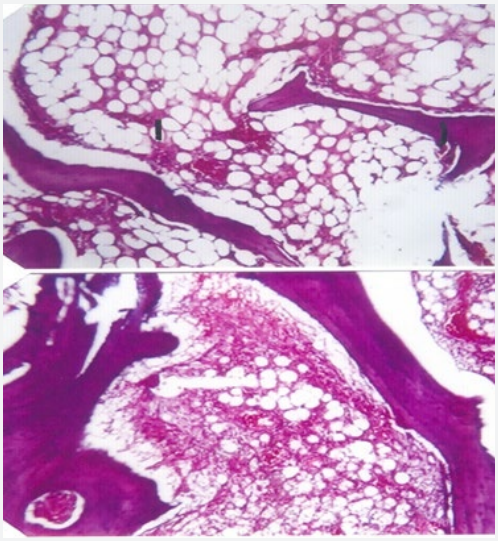

Figure 2: Histological sections of forth-lumbar vertebrae stained by H&E (a)Thinning of bony trabeculea in resveratrol + C59-treated OVX rats (Magnification X 250),(b) Restoration of normal thickness of bony trabeculea in resveratrol-treated OVX rats (Magnification X 250).

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Body weight, bone mass and Biomechanical results

A non-significant increase in body weight was observed in OVX rats compared to sham-operated rats. Neither resveratrol nor resveratrol + C59 resulted in a significant change in body weight compared to non-treated OVX rats. A significant decrease in the examined bone mass of tibia, femur and LV3 and in biomechanical properties of LV3 was seen in non-treated OVX rats compared to sham operated rats.

Table 1: Body weight, bone mass and biomechanical properties of bones, (Mean ± S.E.M), eight weeks following ovariectomy (OVX) in different studied groups.

Rat group	Body weight (g)	Bone mass(g)			LV3 biomechanical properties	
		Tibia	Femur	LV 3	Maximum load(N)	Stiffness (N/mm)
I-sham-operated n=10	186.91±8.29	0.61±0.03	0.74±0.02	0.28±0.01	280.3±16.5	2476±83.8
II-OVX non-treated n=9	193.46±7.43	0.49±0.01#	0.53±0.01#	0.21±0.01#	164.4± 10.9#	1890±62.0#
III-OVX + resveratrol n=10	187.12±8.86	0.58±0.01*	0.68±0.02*#	0.27±0.01*	227.2± 11.2*	2224±89.0*
IV-OVX + resveratrol and C59 n=9	190.64±6.21	0.51±0.01#	0.57±0.02#	0.22±0.01#	180.2±9.1#	1910±72.2#
F value	1.18	24.85	19.42	30.25	26.19	36.53
P	>0.05	<0.001	<0.001	<0.001	<0.001	<0.001

#: Significant as compared to sham-operated group.
*: Significant as compared to OVX non-treated group.
n: number of rats in each group.
S.E.M: Standard Error Mean

A significant increase in tibial, femoral and LV3 mass as well as a significant increase in the biomechanical properties of LV3 was seen in resveratrol -treated group compared to non-treated OVX rats. Whereas no significant difference in these studied parameters could be observed in the group that received resveratrol + C59 compared to non-treated OVX rats (Table 1).

Biochemical results

Table 2: Body weight, bone mass and biomechanical properties of bones, (Mean ± S.E.M), eight weeks following ovariectomy (OVX) in different studied groups.

Rat group	Bone β catenin (ng/mg)	Serum Ca (mg/dl)	serum P (mg/dl)	serum osteocalcin (ng/ml)	serum ALP (U/L)	urinary DPY/ creatinine (nmol/ mmol)
I-sham-operated n=10	63.2± 6.3	9.38±0.1	5.75±0.35	72.52±5.13	285.34± 11.21	29.52 ± 4.16
II-OVX non-treated n=9	35.5± 4.3#	9.65±0.20	8.73±0.4#	98.16±4.8#	399.87±18.0#	59.14± 7.35#
III-OVX +resveratrol n=10	50.45±8.3#*	9.38±0.4	7.27±0.21#	80.93±6.74*	334.37± 9.57*	42.52 ± 5.15*
IV-OVX +resveratrol and C59 n=9	39.8± 7.8#	9.64±0.49	7.95±0.20#	94.17±8.77#	379.63±15.17#	52.67±6.89#
F value	40.72	0.93	15.21	31.83	39.78	44.13
P	<0.001	>0.05	<0.001	<0.001	<0.001	<0.001

#: Significant as compared to sham-operated group.
*: Significant as compared to OVX non-treated group.
n= number of rats in each group.
S.E.M: Standard Error Mean

A significant decrease in bone β catenin in group II compared to group I could be observed. A significant higher mean value in β catenin in resveratrol-treated group (III) vs. non-treated OVX rats could be observed. Whereas, no significant change in bone β catenin was observed in resveratrol + C59- treated group compared to non-treated OVX group (Table 2). No significant difference among groups I to IV was observed in mean serum Ca concentration. Serum P concentration was significantly higher in the OVX group than in the sham group. Neither resveratrol nor resveratrol + C 59 had a significant effect on serum P concentration compared to non-treated OVX rats. Serum ALP activity, serum osteocalcin concentration as well as urinary DPD/creatinine were significantly higher in the OVX group than in the sham group and were significantly decreased compared to non-treated OVX rats by resveratrol, but not by resveratrol + C59 (Table 2).

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Cortical bone morphometric results

Evaluation of cortical bone morphometric indices by CT-Scanning technique showed an increased medullary width, decreased: combined cortical thickness (CCT), periosteal area (PA), cortical area (CA) & CA/PA ratio, in OVX animals when compared with sham operated. Resveratrol treatment significantly prevented these bone resorption variables and this prevention of bone resorption was abolished in resveratrol + C59 OVX rats (Table 3, Figure 3).

Table 3: Cortical bone morphometric indices: medullary width, combined cortical thickness (CCT), periosteal area (PA), cortical area (CA) & CA/PA ratio; measured at 40% length from the distal end of femur bone (Mean ± S.E.M), eight weeks following ovariectomy (OVX) in different studied groups.

RAT GROUP	Medullary width (mm)	CCT (mm)	PA (mm^2)	CA (mm^2)	CA/PA
I-sham-operated n=10	1.97 ± 0.004	1.47±0.006	11.35±0.052	7.21 ±0.061	0.59±0.002
II-OVX non-treated n=9	2.73 ± 0.006#	0.81±0.002a	8.95 ±0.054a	3.53±0.020a	0.40±0.001#
III-OVX +resveratrol n=10	2.42 ± 0.010 #*	1.16±0.021*	10.250.081*	6.40±0.072*	0.52±0.002*
IV-OVX +resveratrol and C59 n=9	2.52 ± 0.004#	0.98±0.006#	9.32±0.06#	4.13±0.046#	0.45±0.003#
F value	39.21	32.64	49.53	36.45	44.65
P	<0.001	<0.001	<0.001	<0.001	<0.001

#: Significant as compared to sham-operated group.

*: Significant as compared to OVX non-treated group.

n= number of rats in each group.

S.E.M: Standard Error Mean

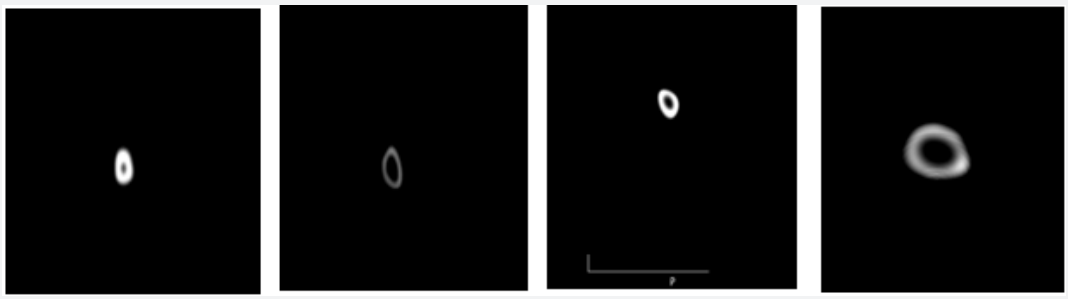

Figure 3: Bone histomorphometry of left femur (a) Normal control, (b) OVX rats, (c) reseveratrol- treated OVX rats and (d) resveratrol + C59- treated OVX rats.

Discussion

In the present study, OVX resulted in a significant osteopenia response at multiple skeletal sites, as determined by bone histology, bone mass and bone biomechanical test. OVX produced the expected increase in bone remodeling compared to sham-operated rats supporting the validity of the method. In fact, the OVX rat model has proven to be extremely useful in the mechanistic analysis of a variety of pharmacological agents with clinical potential to treat postmenopausal osteoporosis [35]. Administration of resveratrol to OVX rats decreased the osteoporotic process as assessed histologically, biomechanically and biochemically.

The role played by WNT / β catenin signaling pathway in bone remodeling has been proven in the current study by the demonstration of a significant decrease in β catenin level in OVX rats. These findings consolidate current view that activation of WNT /β-catenin signaling in osteocytes leads to bone gain [36].

An important finding in the current study was that activation of WNT /β catenin signaling pathway by resveratrol is mainly responsible for this significant osteogenic response. Several lines of evidence in the present study support this statement. First, treatment of rats with resveratrol significantly induced β catenin in OVX rats. Correlated with these results, administration of resveratrol decreased the osteoporotic process as assessed histologically, biomechanically and biochemically and furthermore, C59, an inhibitor of WNT, when administered with resveratrol, markedly attenuated resveratrol-induced osteogenic response, suggesting that this effect of resveratrol is probably through the activation of WNT The important role played by WNT /β catenin signaling pathway in bone formation has been highlighted by a number of studies, where induction of this pathway has been found to promote bone formation while inactivation of the pathway leads to osteopenia states [13,37].

Supporting the role of WNT /β catenin signaling pathway in osteogenesis, several studies demonstrated that manipulating the intracellular mediators of the WNT signaling pathway is another potential approach to promote osteogenesis. For instance, inhibiting glycogen synthase kinase 3-β (GSK3β) from phosphorylating β catenin would stabilize the cytoplasmic level of β catenin, allowing further progression through the WNT /β catenin signaling pathway downstream. Lithium, a commonly used medication for bipolar disorder, is a well characterized example of a GSK3β inhibitor. Animal studies have shown

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that the administration of lithium chloride for 4 weeks in LRP5 knockout mice restored bone mass to normal levels and increased the bone mass of wild-type mice [38]. Additionally, mice treated with lithium after bone injury exhibited enhanced fracture healing [39].

Another recent study demonstrated that pulsed electromagnetic fields stimulation is able to partially prevent estrogen deficiency-induced bone loss in OVX rats via potential WNT / β catenin signaling [40]. Our results demonstrating bone anabolic action of resveratrol via inducing WNT /β catenin signaling pathway are in accordance with an in vitro study indicating that resveratrol promotes osteogenesis by augmenting WNT /β catenin signaling [41].

Resveratrol-induced activation of WNT has been reported to be via inhibition of SOST, given the physiologic role of SOST as a negative regulator of the WNT /β catenin pathway [14]. Indeed, studies have shown that subcutaneous injection of SOST-neutralizing antibody markedly increased bone formation in OVX rats after 5 weeks of treatment [42]. In fact, the anabolic effect of anti-SOST antibody was so effective that it not only reversed bone loss by estrogen deficiency, but it increased the bone mass compared with no ovariectomized rats. The bone-forming effects of anti-SOST antibody were similarly observed in aged male rats after 5 weeks of treatment [43].

The ability of resveratrol to inhibit SOST and thus activates WNT signaling pathway is most likely mediated by being a SIRT1 inducer. This is based on the fact that SIRT1 has been found to be a WNT activator [44]. Previous studies demonstrated an antiresorptive effect of resveratrol, but they related this bone protective action to resveratrol's anti-inflammatory effect [45]. Indeed, a randomized controlled trial in men with metabolic syndrome - a condition linked to low-grade inflammation that can reduce bone density and lead to osteoporosis -suggested that high dose resveratrol supplementation positively affects bone, primarily by stimulating formation or mineralization.

The investigators however, failed to detect an anti-inflammatory effect of resveratrol and they opened a question to the exact mechanism of action of resveratrol in protection against bone resorption [46]. Our findings suggest that resveratrol can have an anti-osteoporotic effect via activation of the WNT /β catenin signaling pathway, and that this drug might be beneficial for osteoporosis by promoting bone formation. However, additional research would be required to assess whether the bone-protective effects shown in this study would be evident in populations at risk for osteoporosis over the course of long-term treatment.

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