Influence of Resveratrol Against Ovariectomy Induced Bone Loss in Rats: Comparison With Conjugated Equine Estrogen Tibolone and Raloxifene✍

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OBJECTIVE: We examined the effect of resveratrol, a phenolic compound found in the skins of most grapes, on bone loss in ovariectomized rats.
STUDY DESIGN: A total of 42 young Wistar-albino rats, of which 35 animals were submitted to bilateral oophorectomy, and 7 rats were submitted to the same surgical incision but without oophorectomy were studied. The rats were assigned to six groups of 7 animals each. For 35 consecutive days the following treatments were given: Group 1, sham; group 2, ovariectomized (OVX); group 3, OVX plus resveratrol; group 4, OVX plus conjugated equine estrogen; group 5 OVX plus tibolone; group 6, OVX plus raloxifene. Immediately 40 days after the ovariectomy bone mineral density (BMD) of the lumbar spine and femur by using dual-energy X-ray absorptiometry. The BMD of lumbar region (R1), total femur region (R2) and three neighbor subregions of femur (R3, R4, R5) were drawn.
RESULTS: There were no significant difference between OVX and resveratrol groups for R2, R3 and R4 values. Compared with the OVX group, CEE group had lower values for R3 but similar values for R2 and R4. Raloxifene group had significantly higher value than the OVX, resveratrol, CEE and tibolone groups for R2. For R3, raloxifene had significantly higher values than in the ones in resveratrol, CEE and tibolone. For R4 region, raloxifene had significantly higher values than CEE group.
CONCLUSION: Resveratrol administered to ovariectomized rats for 35 days had not a beneficial effect on the development of osteopenic skeletal changes.

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Key Words: Resveratrol, Tibolone, Raloxifene, Conjugated Equine Estrogen, Bone Mineral Density, Ovariectomy, Rat

The estrogen deficit associated with the menopause is the main cause of the changes in bone remodeling that lead to the disorder known as postmenopausal osteoporosis.[1,2] Many of these changes can be eliminated or reduced with estrogen replacement therapy (ERT). Despite its benefits, treatment with estrogens have been demonstrated to cause serious adverse effects including prolonged menstruation, stroke and gallbladder disease, as well as endometrial and breast cancers. [3-6]

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In the past few years research efforts have been directed toward the search for substitutes for estrogen treatment that may act in a similar manner on the skeleton without the adverse side effects.[7] As a result, there is growing interest among patients in natural alternatives to conventional hormonal therapy (HT). One of these alternatives is phytoestrogens.[8] They are naturally occurring plant-derived nonsteroidal compounds that are functionally and structurally similar to steroidal estrogens, such as estradiol.[8-10] Several beneficial health effects have been associated with phytoestrogens, including a protective effect against the development of breast and prostate cancers and preventive actions in osteoporosis.[9] For this reasons, dietary consumption of phytoestrogens has become a common treatment for menopausal symptoms.[8,11] Resveratrol (3,5,4' trans-trihydroxystilbene) powerful phytoestrogens, present in the skins of grapes and other plant foods and wine, which demonstrate a broad spectrum of pharmacological and therapeutic health benefits.[12-14] Resveratrol is (Fig. 1) structurally similar to synthetic estrogens, such as diethylstilbestrol and 17 β-estradiol benzoate 14 and binds equally to estrogen receptors α and β (ER-α and ER-β).[13] Recent research demonstrates that trans-resveratrol binds to human estrogen receptors and increases estrogenic activity in the body.[12-15] However, the effects of resveratrol on bone have not yet been established. The similarity in structure between

resveratrol and the synthetic estrogen diethylstilbestrol prompted us to investigate whether resveratrol might exhibit estrogenic activity, a property that is known to produce a anti-osteoporotic benefit. There are no available publications on the influence of that drug on the development of osteopenia in women; therefore, the aim of this study was to examine the effect of resveratrol (5 mg/kg po) administered for 35 days on the development of osteopenia induced by bilateral ovariectomy in rats.

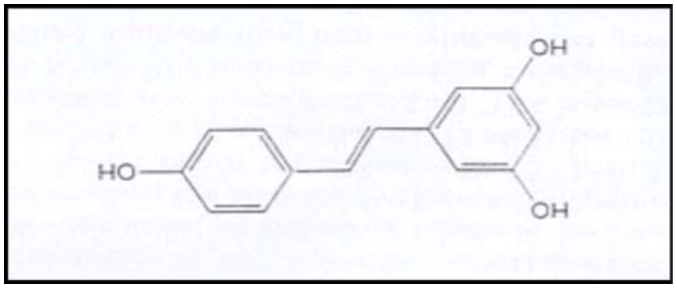

Figure 1. Structure of resveratrol (trans-3,5,4'-trihydroxystilbene).

Materials and Methods

Forty-two virgin, adult, albino Wistar rats weighing 212 ± 32 gm (mean ± SD) at 3 months of age, were studied. The animals were housed in plastic cages with a metal grid lid, at a room temperature of 20°C and artificial light from fluorescent lamps. The permission for the animal tests and experiments was given by the Bioethical Board of Inonu University Medical faculty. The rats, except for in the sham operation group were bilaterally ovariectomized under intraperitoneal ketamine (50mg/kg) and xylazine HCl (10mg/kg) anesthesia, using the dorsal approach. Rats in the sham operation group underwent a surgical procedure similar to the other groups but the ovaries were not removed. Five days after the surgical procedure, the 35 castrated and 7 noncastrated sham-operated animals were assigned to six groups as follows: group 1, consisting of the 7 noncastrated sham-operated animals; group 2, control, consisting of 7 rats that received only 0.1 % etanol; group 3, consisting of 7 rats that received resveratrol (Sigma Chemicals, USA) at a dose of 5 mg/kg per day po; group 4, consisting of 7 rats that received conjugated equine estrogens (CEE) at a dose of 0.1 mg/kg per day po; group 5, consisting of 7 rats that received tibolone at a dose of 0.25 mg/kg per day po; and group 6, consisting of 7 rats that received raloxifene at a dose of 1.0 mg/kg per day. The animals were fed on standard laboratory rodent chow and distilled water ad libitum. Each morning all the animals were weighed immediately before administration of the tested preparations. That enabled us to determine an increase in rat's body weight and to administer a proper resveratrol, CEE, tibolone and raloxifene dose, with respect to body weight. Administration of drugs started 5 days after bilateral ovariectomy and continued for 35 days. After distribution of the animals, medication was administered daily by gavage, between 9 AM and 11 AM, during 35 consecutive days, with use of a metal gavage probe. Serum obtained by cardiac puncture on the day of death. Serum alkaline phosphatase and calcium levels were determined with use of an autoanalyzer (Olympus Diagnostica GmbH, IRISH branch). Immediately 40 days after the ovariectomy or sham-surgery, we measured body weight and bone mineral density by using dual-energy X- ray absorptiometry and then animals were killed and the weight of the uterus determined.

Bone mineral density measurements: In the all rats, bone mineral density (BMD) of the lumbar vertebrae and femur were measured with dual-energy X-ray absorptiometry (DEXA)[16] (QDR 4500/W, Hologic Inc., Bedford, MA, USA) and results were evaluated by the same examiner DEXA equipment uses switched pulsed stable dual-energy radiation with 70 kV and 140 kV. Subregion analysis soft-ware was used to analyze of the all regions. BMD was used, in g/cm^2, in the lumbar vertebrae and four region of the right femur. Lumbar region (R1), total femur region (R2) and three neighbor subregions of femur (¼ distal, R3; 1/4 proximal, R4; ½ middle, R5) were drawn (Fig. 2). Lumbar region is drawn as 17 mm length and 5 mm thickness and is located uplevel of superior iliac corner. Femur is drawn as a 3 mm thickness. The coefficient of variation expressed as a percentage standard deviation of the mean was 8%, 10%, 16%, 13% and 15% for R1, R2, R3, R4 and R5 respectively.

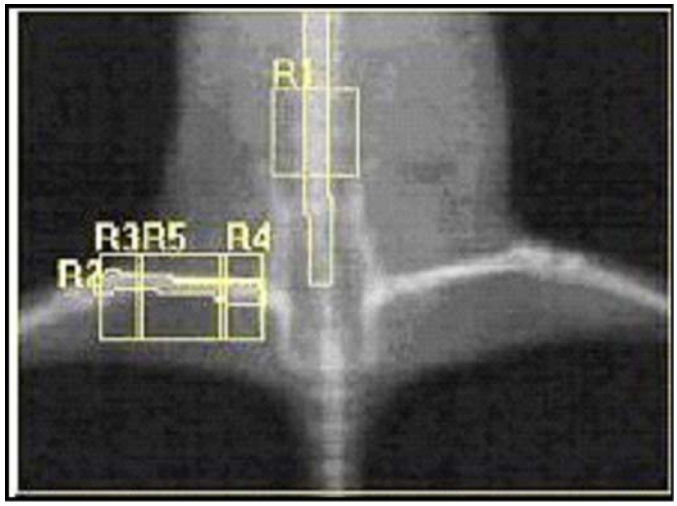

Figure 2. For BMD measurement, region of the interest was placed at the lumbar region (R1), total femur region (R2) and three neighbor subregions of femur (¼ distal, R3; 1/4 proximal, R4; ½ middle,R5).

Statistical analysis: A comparison of the results of the different groups was performed with use of the Kruskal-Wallis analysis of variance and post-hoc multiple comparison test. The results are given in the text as means ± STD. For all comparisons, statistical significance was defined as $p < 0.05$.

Results

Our study examined the influence of resveratrol on BMD in a rat osteoporosis model which was prepared by ovariectomy. Forty days after ovariectomy, bone mineral density of the ovariectomized control rats became significantly lower than the sham operated controls. This shows that our rat osteoporosis model is useful to evaluate the effect of resveratrol, CEE, tibolone and raloxifene on BMD. Table 1 shows femoral bone mineral density values for the different groups. Lumbar region (R1), total femur region (R2) and three neighbor subregions of femur (¼ distal, R3; 1/4 proximal, R4; ½ middle, R5) bone mineral density was expressed as the mean of the values obtained for the lumbar vertebrae and femur. There was no significant difference between all groups for R5 measurement. For that reason, only values of measurement of R1, R2, R3 and R4 areas were included for statistical analysis. There was no significant difference between OVX and resveratrol groups for R1, R2, R3 and R4 regions. Compared with the OVX group, CEE group had lower values for R3 but similar values for R2 and R4 regions. Compared with the OVX, resveratrol, CEE and tibolone groups, raloxifene group had significantly higher value for R2 region, but similar values for sham group. For R3 region, raloxifene had significantly higher values than in the ones in resveratrol, CEE and tibolone groups. For R4 region, raloxifene had significantly higher values than CEE group, but similar to all others. Compared with the sham operation group, tibolone treated rats had significantly lower values for R2 and R3 regions, but R4 values were insignificant. In ovariectomized rats treated with resveratrol, CEE, raloxifene or tibolone, only raloxifene prevents the loss of bone mass attributed to oophorectomy. No significant differences in the levels of serum ALP and calcium levels were detected between the all groups.

Figure 3 shows the mean body weights and uterine weights obtained for each study group. It may be seen that oophorectomy led to a significant weight increase compared with the weight reached by sham operated rats. The oophorectomized group treated with CEE also showed moderate but insignificant weight increase. However, there were no significant differences between the weights of oophorectomized rats treated with resveratrol and the sham operated control group. Oophorectomized rats treated with tibolone or with the raloxifene showed weights lower than those of the ovariectomized control group. The body weight of resveratrol treated rats were significantly lower than ovariectomized control rats, but higher than tibolone and raloxifene treated rats. The difference between resveratrol and CEE groups were not significant. There were no significant differences between tibolone and raloxifene treated rats.

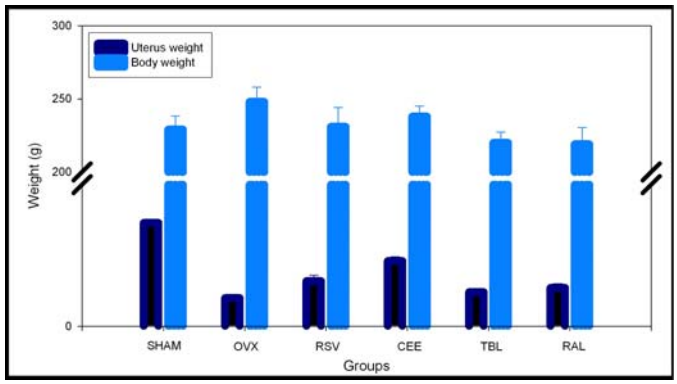

Figure 3. Histograms depicting uterus and body weight of SHAM, OVX, RSV, CEE, TBL and RAL groups. Body and uterus weight expressed as gram. OVX represents 'ovariectomized', RSV represents 'resveratrol', CEE represents 'conjugated equine estrogen', RAL represents 'raloxifene' SHAM represents 'sham operated animal' and TBL represents 'tibolone' in the histograms.

Table 1. Lumbar region (R1), total femur region (R2) and two neighbor subregions of femur (¼ distal, R3; 1/4 proximal, R4; ½) BMD values of rats all groups.

Groups	Bone mineral density (gm/cm^2)			
	R1	R2	R3	R4
I.Sham	.250±0.01	.215±0.02	.205±0.02	.237±0.01
II.OVX	.251±0.01	.190±0.01[a]	.191±0.02	.205±0.01[i]
III.Resveratrol	.239±0.01	.178±0.01	.171±0.02	.205±0.03
IV.CEE	.224±0.02	.170±0.02	.157±0.03	.200±0.03
V.Tibolone	.247±0.01	.186±0.01	.168±0.01	.216±0.03
VI.Raloxifene	.258±0.02	.215±0.03[b,c,d,e]	.210±0.03[f,g,h]	.233±0.03[j]

* *The mean difference is significant at the .05 level.*

Values represent mean ± SD. Sham-operated control group, n = 7; OVX group, n = 7 oophorectomized rats treated with 0.1% etanol); Resveratrol group, n = 7 (oophorectomized rats treated with resveratrol 5 mg/kg/day); CEE group, n = 7 (oophorectomized rats treated with conjugated equine estrogen 0.1 mg/kg/day); Tibolone group, n = 7 (oophorectomized rats treated with tibolone 0.25 mg/kg/day); Raloxifene group, n=7 (oophorectomized rats treated with raloxifene 1 mg/kg/day).

For R2 region:a < 0.05, compared to sham; b < 0.05, compared to ovx; c < 0.05, compared to resveratrol; d< 0.05, compared to CEE;. e < 0.05, compared to tibolone.
For R3 region: f < 0.05, compared to resveratrol; g < 0.05, compared to CEE; h < 0.05, compared to tibolone.
For R4 region: i < 0.05, compared to sham; j< 0.05, compared to CEE.

Oophorectomized control rats showed a uterus weight significantly lower than sham operated control rats. Uterus weight in resveratrol, CEE, tibolone and raloxifene groups were also significantly lower than that of sham operated control rats. The difference between all groups for uterine weights was statistically significant except for values of tibolone and raloxifene. There were no significant differences between the uterus weights of oophorectomized rats treated with tibolone and raloxifene. Uterine weights of sham-operated rats were higher than all groups, and uterine weights of estrogen-treated rats were higher than all groups except sham-operated rats. Ovariectomized rats treated with resveratrol had significantly higher uterine weight than those of the ovariectomized control, tibolone and raloxifene groups.

Discussion

Although conventional HT has been the first line of defense against postmenopausal osteoporosis, concerns have recently arisen regarding the long-term risk of breast cancer and coronary disease, despite the antifracture efficacy of HT. Resveratrol is a natural phytoalexin present in grapes and red wine, which possesses a variety of biological activities including, anti-inflammatory, anticarcinogenic and antioxidative activities.[17,18]. At least some of these activities are mediated through the interaction of resveratrol with ER-α and Er-β.[13, 14] Both ERs have been detected in bone cells (osteoblasts, osteoclasts, osteocytes).[19] Human and mouse genetic studies suggest a predominant role of ER-α in bone metabolism.[19] Resveratrol may act directly on osteoblasts by genomic mechanisms involving activation or inhibition of nuclear oestrogen receptors.[20] Resveratrol may help to prevent bone resorption and bone loss by enchancing osteoblastic production of osteoprotegerin (OPG), which is an important inhibitor of the terminal differentiation and activation of osteoclasts. A recent study has found that genistein upregulates the production of OPG by human osteoblasts.

A recent study has found the oophorectomized rat to be a suitable experimental model for postmenopausal osteoporosis that faithfully reproduces the changes observed in humans and has the added benefit that the effects are detectable only a few months after intervention.[22,23] Folwarczna et al.[24] reported that estrogen deficiency occuring 30 days after bilateral ovariectomy in sexually mature female rats caused also changes in the osseous system associated with bone loss. In agreement with the results of previous studies[22-24] bilateral oophorectomy in our rats proved to be a good experimental model for postmenopausal osteoporosis.

This study shows that 40 days after oophorectomy there is a significant decrease in femoral bone mineral density of ovariectomized control rats compared with values of BMD observed in sham operated controls. This deficit in BMD was greater in cortical bone than in trabecular bone and has also been described by other authors.[25,26] Treatment with resveratrol and CEE do not canceled the effects of oophorectomy with respect to decreases in the levels of reached bone mass, and treated rats showed significant differences from sham-operated animals. Liu et al.[27] demostrated that epiphysis BMD in the resveratrol treated OVX animals was significantly greater than that in the OVX group. However, the femoral midpoint BMD was not significantly different among the OVX and OVX + resveratrol groups. Our results concordance with Liu's study regarding femoral midpoint BMD values. Several short term studies have examined the effects of phytoestrogen-rich foods on BMD in peri or postmenopausal women, and two of them suggest a positive effect at the lumbar spine at higher intakes.[28,29] In another study, habitual intake of high levels of soy products resulted in a significant increase in BMD compared to the lowest intake group, even after adjustment for weight and years since menopause.[30]

A recent study, while no significant differences in calcium, magnesium and phosphorus content were found between the femurs of ovariectomized control and ovariectomized rats treated with resveratrol, the femur hydroxyproline content in the resveratrol group was significantly higher than those of the ovariectomized group.[31] On the other hand, there are no references in the literature where resveratrol, CEE, tibolone or raloxifene have been administered to ovariectomized rats with the aim of observing the effects on bone mineral density. In an experimental study, oral administration of resveratrol to weanling rats had no significant effect on estrogenic responses such as serum cholesterol or messenger RNA for insulinlike growth factor I.[32]

The beneficial effects of ERT, selective oestrogen receptor modulators, or tibolone in the prevention and treatment of osteoporosis in postmenopausal women have been well established.[33,34] However, evidence to substantiate the ability of naturally occurring compounds, such as resveratrol, to reduce bone loss in the postmenopausal era have received limited attention in randomised long-term trials. The biological action of resveratrol is complex and their cellular actions are determined by many factors including the relative levels of ER-α and β and corepressors present in any given cell type.[13,14,35]

The increased risks over benefits of HT reported in a large prospective trial by the Women's Health Initiative[36] have fueled interest in therapy with SERMs. In the search for the ideal molecule that is an estrogen agonist in bone several selective estrogen receptor modulators (SERMs) have been developed and tested in nonhuman primates. Raloxifene is a SERMs used to increase bone density, with a molecular mechanism, not well known. Initial studies of the effects of raloxifene on bone metabolism in ovariectomized rats showed significantly lower rates of bone remodelling markers[34], while

BMD remained unchanged. Lees et al.[37] reported that raloxifene inhibited bone turnover and maintained spine BMD in ovariectomized cynomolgus monkeys. The effect of raloxifene on bone mass has been assessed in the Multiple Outcomes of Raloxifene Evaluation and after 24 months of treatment it was found a significant increase of the BMD in all raloxifene treated groups.[38] Biochemical markers of bone turnover were suppressed by raloxifene to normal premenopausal ranges through 3 years.[39] Estrogen and the raloxifene have also been shown to prevent bone loss in OVX rats.[40] On the other hand, estrogen was superior in preventing ovariectomy-induced spinal bone loss.[41] The mechanisms underlying the effects on bone appear to be different in the estrogenic versus the raloxifene. Estrogen acts on bone via ER-α and β as well as nonreceptor-mediated mechanisms. However, the interaction of SERMs with ER-α is distinct for each compound, including ER-β ligand conformation and interaction with transcriptional machinery.[42] Studies in vitro show that raloxifene modulates the bone homeostasis inhibiting osteoclastogenesis and the bone resorption with dose-dependent activity.[43] Yang et al.[44] have shown that the TGFβ–CAT expression was significantly up-regulated by raloxifene. Raloxifene also stimulates the production of OPG and inhibits the production of IL-6 from cultured human osteoblasts.[45] These characteristics could explain the different BMD values between the bone effects of raloxifene and of CEE.

Histomorphometric data show that ovariectomy induces bone loss and accelerated skeletal metabolism in OVX rats.[46,25] Osteopenia and increased indices of bone resorption and formation were detected in OVX rats as early as 14 days.[46,25] Wronski et al. reported that ovariectomy increased osteoclast and osteoblast surface and numbers. In addition, OVX rats exhibited a greater rate of longitudinal bone formation at 5 weeks postovariectomy and this growth was also significantly increased by ovariectomy at 14 days.[46,25] However, ovariectomy-induced osteopenia are largely confined to cancellous bone in rats, whereas bone strength in humans is known to be dependent also on cortical bone.[47] Estrogens prevent bone loss also by regulating the production of cytokines that modulate osteoclastic bone resorption, including interleukin- 6 (IL-6) and OPG by cells of the osteoblastic lineage.[48] Estrogens also control the rate of programmed cell death of mature bone cells, having pro-apoptotic effects on osteoclasts and antiapoptotic effects on osteoblasts.[49] OPG is a negative regulator of osteoclast mediated bone resorption. In vitro studies have demonstrated that estrogens stimulate the OPG production by osteoblasts.[43,45,50] Present study has demonstrated that 1 mg /kg raloxifene is an efficient means of preventing the loss of bone mass, which is maintained homogeneously at values significantly higher than those obtained in ovariectomized rats without treatment and CEE treated rats and similar to those obtained in animals sham. R2 and R4 values for estrogen-treated animals tended to be lower than that for OVX rats, but this difference was not significant. R2 and R4 values after CEE treatment was not different from that OVX, which is contrary to a previous report. This discrepancy may be explained by the result of the duration of CEE administration in this study (35 days) compared with the previous study. Studies have shown that estrogen was shown to prevent bone loss in the proximal tibia, vertebra, and femoral neck after 10 months in OVX rats.[40,51] Another plausible possibility of this finding is that CEE do not immediately depress bone formation, and continue filling the existing remodeling sites for some time. Initial rapid phase of bone loss in OVX rats is coincident with the maximal increase in bone turnover.[46,25] At later times postovariectomy, bone loss and bone turnover both subside.[46,25] Nozaki et al.[52] reported that in the initial 2 year period after oophorectomy, 0.625 mg/day of CEE alone could not prevent acute bone loss suggesting that additional therapy for the prevention of osteoporosis may be needed. When CEE administered to ovariectomized cynomolgus monkeys for 2 yr neither spinal BMD nor midshaft femur strength was improved.[53] Genant et al.[54] reported that conjugated equine estrogen in doses of less than 0.6 mg/d are inadequate to prevent the vertebral mineral loss women for 24 months after oophorectomy. Studies were made using rhesus monkeys demonstrated that visceral fat deposition increased after menopause[55] and estrogen inhibited food intake in females in a dose-dependent manner.[56] If CEE treatment causes a decrease in food intake this negative energy balance may cancel positive effects of CEE on BMD.

The advantages of raloxifene and some of its analogs over estrogens in the prevention of postmenopausal osteoporosis have been reported by many authors.[23,34,57] In this study, raloxifene prevents the loss of bone mass attributed to oophorectomy, but this is accompanied by an increase in uterus weight. On the other hand, raloxifene treated animals had lesser increase in uterus weight than that observed after treatment with resveratrol and CEE.

In the present study, treatment of oophorectomized rats with resveratrol, tibolone and raloxifene caused less hypertrophy of the uterus than CEE treatment did. Treatment of oophorectomized rats with resveratrol showed that resveratrol caused an increase in weight of the uterus. In an animal study, oral administration of resveratrol to rats gave a slight increase in uterine wet weight.[32] In another study resveratrol was shown to bind ER in cytosolic extracts from MCF-7 and rat uteri.[13] Recent study showed that estrogen deficiency 30 days after performing bilateral ovariectomy caused a statistically significant increase in the body weight of the examined female rats.[24] Body weight determinations indicated that treatment with resveratrol attenuates the weight increase attributable to oophorectomy. No significant differences in the levels of

serum ALP and calcium levels were observed between the different groups, in agreement with the results of previous studies.[34,58]

The results of the current study suggest that the resveratrol and CEE do not attenuate the reduction in bone mineral density generally observed after oophorectomy. Raloxifene also prevents the loss of bone mass attributed to oophorectomy, and this is accompanied by minimally increase in uterus weight and lower increase in body mass than that observed after treatment with CEE. Finally, the results of the our study suggest that resveratrol does not act as a potent partial estrogen agonist on bone with at a dose of 5 mg/kg for 35 days.

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