

Περιοδικό της Ελληνικής Κτηνιατρικής Εταιρείας

Τόμ. 70, Αρ. 2 (2019)



Red wine polyphenols modulate bone loss in the ovariectomized rat model of postmenopausal osteoporosis

C. PASSALI, A. PATSAKI, P. LELOVAS, N. ALIGIANNIS, M. MAKROPOULOU, S. KOURKOULIS, N. PAPAIOANNOU, S. MITAKOU, A.L. SKALTSOUNIS, I. DONTAS

doi: [10.12681/jhvms.21749](https://doi.org/10.12681/jhvms.21749)

Copyright © 2019, C. PASSALI, A. PATSAKI, P. LELOVAS, N. ALIGIANNIS, M. MAKROPOULOU, S. KOURKOULIS, N. PAPAIOANNOU, S. MITAKOU, A.L. SKALTSOUNIS, I. DONTAS



Άδεια χρήσης [Creative Commons Αναφορά-Μη Εμπορική Χρήση 4.0.](https://creativecommons.org/licenses/by-nc/4.0/)

Βιβλιογραφική αναφορά:

PASSALI, C., PATSAKI, A., LELOVAS, P., ALIGIANNIS, N., MAKROPOULOU, M., KOURKOULIS, S., PAPAIOANNOU, N., MITAKOU, S., SKALTSOUNIS, A., & DONTAS, I. (2019). Red wine polyphenols modulate bone loss in the ovariectomized rat model of postmenopausal osteoporosis. *Περιοδικό της Ελληνικής Κτηνιατρικής Εταιρείας*, 70(2), 1541–1550. <https://doi.org/10.12681/jhvms.21749>

Red wine polyphenols modulate bone loss in the ovariectomized rat model of postmenopausal osteoporosis

C. Passali¹, A. Patsaki¹, P. Lelovas¹, N. Aligiannis², M. Makropoulou², S. Kourkoulis³,
N. Papaioannou¹, S. Mitakou², A. L. Skaltsounis², I. Dontas¹

¹ Laboratory for the Research of the Musculoskeletal System, School of Medicine, National & Kapodistrian University of Athens, Greece

² Department of Pharmacognosy and Chemistry of Natural Products, School of Pharmacy, National & Kapodistrian University of Athens, Greece

³ Unit of Biomechanics, Department of Mechanics, School of Applied Mathematical and Physical Sciences, National Technical University of Athens, Greece

ABSTRACT. The aim of this study was to examine the effect of Red Wine Polyphenols (RWPs) extract on bone mineral density (BMD) in the ovariectomized (OVX) rat model. Thirty-five 10-month-old Wistar rats were separated into controls (CTRL), OVX and OVX plus RWPs in their drinking water (dose, 50 mg/kg body weight per day), starting immediately after OVX for 6 months. Body and uterine weight, BMD of the tibia at baseline, 3 and 6 months post-OVX, and 3-pointing bending of the femur, were examined. Statistical comparison of the total tibia BMD within groups during the study period showed a significant reduction in the OVX and OVX+RWPs groups both from baseline to 3 and 6 months and from 3 to 6 months, whereas in the CTRL group, there was no significant change. For the proximal tibial metaphysis, comparison of BMD percentage changes from baseline to 3 months and 6 months and from 3 to 6 months revealed highly statistical differences between OVX and OVX+RWPs groups ($P = 0.000$). OVX induced a significant reduction of biomechanical parameters as expected; the 3-point bending test showed that the maximum force before fracture, energy absorption and fracture stress significantly increased in the OVX group treated with RWPs compared with the nontreated OVX rats ($P = 0.048$, $P = 0.002$ and $P = 0.003$, respectively). Dietary intake of RWPs for 6 months significantly prevented trabecular bone loss and improved bone strength in estrogen-deficient ovariectomized rats.

Keywords: Rats, Osteoporosis, Polyphenols, Bone mineral density, Three point bending

Corresponding Author:

Christina C. Passali, Laboratory for the Research of the Musculoskeletal System,
10 Athinas Street KAT Hospital, 14561, Kifissia, Greece.
E-mail address: cpassali@gmail.com

Date of initial submission: 29-09-2018

Date of revised submission: 16-10-2018

Date of acceptance: 26-10-2018

ΠΕΡΙΛΗΨΗ. Σκοπός αυτής της μελέτης ήταν να εξεταστεί η επίδραση του εκχυλίσματος πολυφαινολών του κόκκινου κρασιού (RWPs) στην οστική πυκνότητα (BMD) σε μοντέλο ωθηκεκτομημένων επίμυων. Τριάντα πέντε Wistar επίμυες, ηλικίας 10 μηνών, χωρίστηκαν στις εξής ομάδες: ομάδα ελέγχου (CTRL), ωθηκεκτομής (OVX) και την ομάδα ωθηκεκτομής που έλαβε το εκχύλισμα RWPs (OVX+RWPs) σε συγκέντρωση 50 mg/kg σωματικού βάρους ανά ημέρα, ξεκινώντας αμέσως μετά από την ωθηκεκτομή για 6 μήνες. Προσδιορίστηκε η οστική πυκνότητα πριν από οποιαδήποτε παρέμβαση, στους 3 και 6 μήνες. Οι επίμυες ευθανατώθηκαν και τα μηριαία οστά εξετάστηκαν με την δοκιμασία κάμψης τριών σημείων. Η σύγκριση των τιμών οστικής πυκνότητας ολόκληρης της κνήμης μεταξύ των ομάδων, ανέδειξε στατιστικά σημαντική μείωση στις ομάδες OVX και OVX+RWPs τόσο από την αρχική μέτρηση στους 3 και 6 μήνες, όσο και από τους 3 στους 6 μήνες, ενώ στην ομάδα CTRL δεν υπήρξε σημαντική αλλαγή. Η ποσοστιαία μεταβολή της οστικής πυκνότητας στην εγγύς μετάφυση της κνήμης από την αρχική μέτρηση στους 3 και 6 μήνες και από τους 3 στους 6 μήνες, αποκάλυψε στατιστικά σημαντικές διαφορές μεταξύ των ομάδων OVX και OVX + RWPs ($P = 0.000$). Η ωθηκεκτομή προκάλεσε σημαντική μείωση των εμβιομηχανικών παραμέτρων όπως αναμενόταν, η δοκιμασία κάμψης τριών σημείων έδειξε ότι η μέγιστη φόρτιση πριν από τη θραύση, η απορρόφηση ενέργειας και η τάση θραύσης αυξήθηκε στατιστικά στην ομάδα OVX που υποβλήθηκε σε θεραπεία με RWPs σε σύγκριση με τους OVX επίμυες ($P = 0.048$, $P = 0.002$ και $P = 0.003$ αντίστοιχα). Η διατροφική πρόσληψη RWPs για 6 μήνες απέτρεψε την ταχεία οστική απώλεια και βελτίωσε την αντοχή των οστών σε ωθηκεκτομημένους επίμυες.

Λέξεις ενρετηρίασης: Επίμυες, Οστεοπόρωση, Πολυφαινόλες, Οστική πυκνότητα, Κάμψη τριών σημείων

INTRODUCTION

Osteoporosis is a severe condition of low bone mineral density (BMD). Patients with osteoporosis have a high fracture hazard, leading to physical impairment, significant pain and even untimely death (Lloret et al., 2016; Thulkar et al., 2016; Weisz, 2017). Preventive treatments include maintaining or increasing bone mineral density (BMD) when its loss is induced by decreasing levels of sex steroid levels with age in both males and females (Wang et al., 2017). Hormone replacement therapy (HRT) has been established as the method of choice to delay bone turnover and BMD loss, at all skeletal sites in early and late postmenopausal females (Cao et al., 2014; Ran et al., 2017). HRT significantly decelerates fracture rate while raising the risk of venous thromboembolism, coronary heart disease and stroke, thus, poor compliance has emerged (L’Hermite, 2017; Miller et al., 2016; Oster et al., 1982).

Polyphenols have gained growing interest within the last years, due to their antioxidant function and mild estrogenic effect (De França et al., 2013). Antioxidants curtail inflammation by anti-inflammatory signaling, and regulate osteoblastogenesis, osteoclastogenesis and osteoimmunological activity.

A few studies have displayed that grape extracts have been promising in preserving or enhancing bone calcium retention (Yahara et al., 2005; Zhang et al., 2014); some have shown benefits by reducing bone turnover, but the effect of grape consumption on bone

health has not been fully determined (Hohman & Weaver, 2015; Yahara et al., 2005). So far, there is no evidence backing the protective effect of red grape consumption on bone density, despite their antioxidant composition. The objective of this study was to examine the effect of Red Wine Polyphenols (RWPs) extract on bone mineral density and strength in the ovariectomized rat model of postmenopausal osteoporosis.

MATERIAL AND METHODS

Laboratory animals

Thirty-five ten-month-old virgin female Wistar rats were acquired from the laboratory animal center of the Hellenic Pasteur Institute (Athens, Greece). They were housed in the registered animal house of the Laboratory for Research of the Musculoskeletal System of the School of Medicine, National & Kapodistrian University of Athens, Greece. The experimental protocol was approved by the General Directorate of Veterinary Services with the authorization code 262/13-1-2011, consistent with Greek legislation (Presidential Decree 160/1991, in compliance to the Directive 86/609/EEC in force at the time).

The rats' body weight ranged from 260 to 330 g. They were housed by three to four in conventional open-top cages (transparent polycarbonate 45 x 30 x 20 cm; IFFA) with shredded wood bedding. The animals were maintained on a 12:12 h light/dark photoperiod, 55 - 65% relative humidity, 19 - 22°C temperature and 15 air changes per hour.

Experimental groups

The rats were acclimatized to the laboratory environment 1 month before commencing any examination or treatment. After the adjustment period, using a randomization procedure stratified according to body weight, the 11-month-old animals were assigned to three groups (Figure 1). Eleven of the 35 rats consisted the sham-operated non-ovariectomized control group (CTRL, n=11). Twenty four rats underwent bilateral

ovariectomy by the ventral approach; of these, 13 rats consisted the ovariectomized group without treatment (OVX, n=13) and the other 11 rats were treated with RWPs extract (OVX+RWPs, n=11). A sample size of rats was derived from a sample size calculation using data from our previous studies that investigated the protective effect of dietary interventions to ovariectomy-induced osteoporosis in rats (Dontas et al., 2011; Dontas et al., 2006).

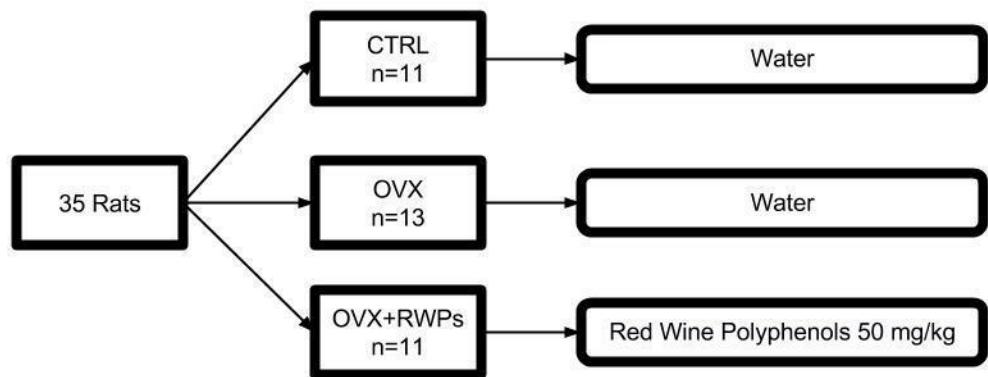


Figure 1. Study design.

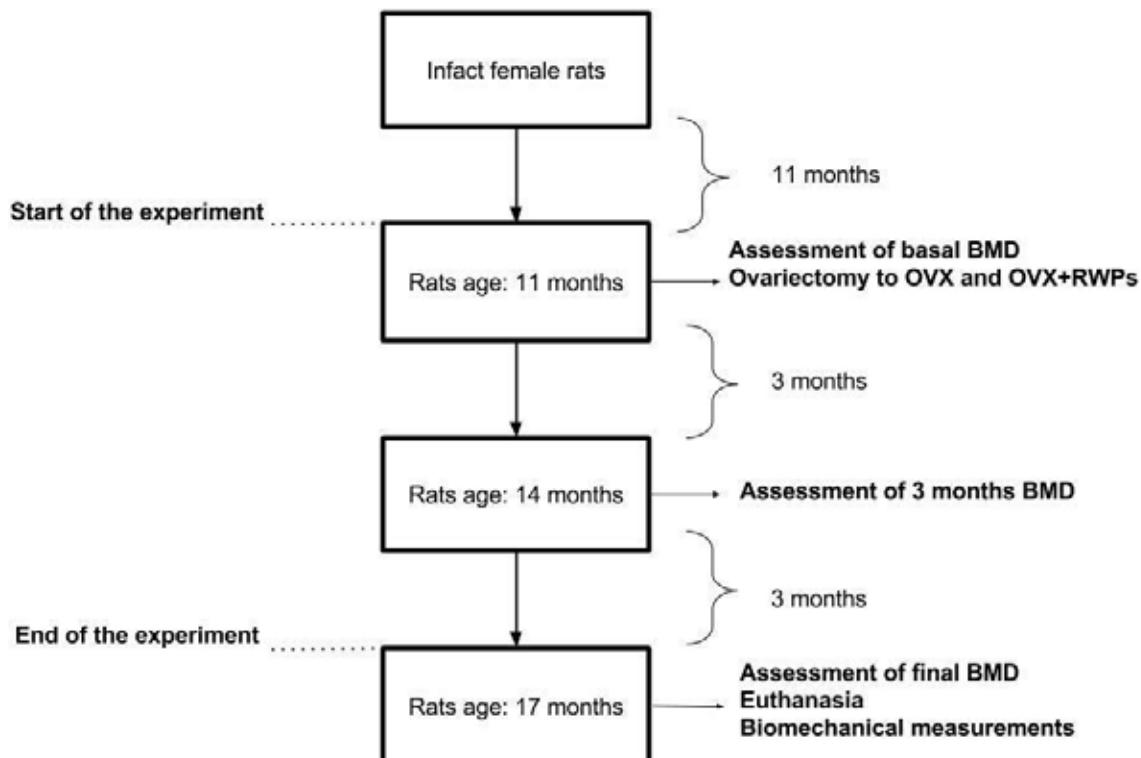


Figure 2. Flow-chart of experimental protocol.

Body weight and food intake

After ovariectomy, all rats had *ad libitum* access to a pelleted chow of soybean-free/low phytoestrogen rat diet (pellet, type 4RF21 GLP, Mucedola, Settimo Milanese, Italy). This diet was used to prevent confounding from residual isoflavones (Pawlowski et al., 2014). Rats were fed according to the mean food in-

take of the CTRL group, in order to avoid ovariectomy-induced obesity and obesity-related bone density changes. Adjustments of food intake took place twice weekly.

Body weight was recorded weekly throughout the 6-month study period; mean values \pm SD for final weights are presented in Table 1.

Table 1. Body weight, fat and uterus weight.

Group	CTRL ^a Mean (SD)	OVX ^b Mean (SD)	OVX+RWPs ^c Mean (SD)	P Anova
Body Weight, g	330.90 (33.33) ^{b,c}	377.07 (35.83) ^a	372.90 (17.43) ^a	0.001
Fat, g	22.94 (5.19) ^b	29.72 (7.74) ^a	29.75 (5.67)	0.023
Uterus, g	0.41 (0.09) ^{b,c}	0.14 (0.03) ^a	0.21 (0.29) ^a	0.002

Note: superscript letters (a, b, c) indicates significant difference ($P < 0.05$) after Bonferroni correction.

Groups: a: Control (CTRL), b: Ovariectomy (OVX), c: Ovariectomy treated with RWPs extract (OVX+RWPs).

Extract

Administration

CTRL and OVX rats had *ad libitum* access to filtered tap water in drinking bottles; RWPs extract was administered to the RWPs group through their drinking water (dose 50 mg/kg body weight/day), immediately after ovariectomy. Treatment was continued for 6 months. Water and extract ingestion was documented twice a week in all rat cages. The bottles with RWPs were shaken twice a day to prevent the gathering of precipitate.

Analysis

Vitis vinifera species comprise numerous varieties and Muscat is one of the oldest and most widespread in the world. In the present study, the red wine was obtained from the Muscat Hamburg (MHW) variety and used for the preparation of an extract rich in polyphenols using macroporous resin (XAD-4) technology.

The MHW was provided by the Tyrnavos Cooperative Winery & Distillery, Thessaly region, Greece. After concentration under reduced pressure, the dry

residue was calculated at 2.54 g per 100 ml of wine. In laboratory scale, two different procedures were used for the recovery of the extract enriched in MHW polyphenols (MHWPE). Column mode: A 40 \times 3 cm glass column packed with 50 mL of resin XAD-4 was used. MHW (400 ml) was passed through the column at a flow rate of 2 mL/ min. Total duration of the adsorption step was 200 min. and desorption duration 45 min. The temperature was 20°C throughout all the experimental procedures. After reaching adsorptive equilibration, the resin column was first washed with H₂O and then desorbed with 100 ml of EtOH 95°. After desorption, the alcoholic solution with a high content of polyphenolic compounds was condensed to remove EtOH. Finally, the crude extract was obtained by drying at 40 °C to achieve a constant weight (3.11 g). Therefore, the percentage of recovered polyphenolic fraction (MHWPECM) was calculated at 0.78 g/100 ml MHW.

Bath mode: 400 ml of MHW and 50 mL of XAD-4 were placed in a flask of 250 ml and stirred for 12 hours in room temperature. After filtration, the resin was washed with water and the polyphenolic fraction

recovered using 100 ml of EtOH 95°. Finally, 1.93 g of crude extract (MHWPEBM) was obtained after removal of solvent in a yield of 0.48 g/100 ml MHW.

The polyphenolic content in fractions obtained from MHW using column and bath mode (MHWPECM and MHWPEBM) was calculated by Folin-Ciocalteau and found to be 92.5 and 88.6 mg GAE/g of extract, respectively. These results showed that column mode was more effective for the recovery of polyphenolics from MHW and this procedure was used for the preparation of MHWPE in pilot scale.

For pilot scale processes, a column of 150 × 38 cm filled with 125 L of XAD-4 resin was used. MHW (300 L) was fed into the resin through a 0.5 in. diameter PVC pipe using a Wilden diaphragmatic pump. The flow rate was 2500 mL/min (150 L/h) and the total duration of the adsorption-desorption process was 180 min. Finally, EtOH (150 L) was evaporated under vacuum in a QVF glass 200 L evaporator and consequently in a Buchi 20 L rotary evaporator. The dry extract (MHWPECM 2.25 Kg) was used for the *in vivo* evaluation of its activity on osteoporosis. The MHWPECM dry extract was finally diluted with distilled water to obtain 0.6 g/L concentration and kept at 4 °C.

Ovariectomy

All animals except the CTRL group underwent ovariectomy. Ovariectomy was performed when the rats were 11 months old, using the technique defined by Waynfirth (Waynfirth & Flecknell, 1992). They were anesthetized by an intramuscular injection of dexmedetomidine (0.25 mg/kg) and ketamine hydrochloride (50 mg/kg), administered analgesia (carprofen 4 mg/kg) and chemoprophylaxis (enrofloxacin 10 mg/kg) subcutaneously, laparotomized via a ventral midline incision using aseptic procedures and their ovaries were removed bilaterally. The incision was sutured by single interrupted sutures in layers.

Bone densitometry

Prior to surgery, baseline bone mineral density (BMD) of all rats were obtained. BMD measurements of the entire and proximal tibia were also carried out 3 and 6 months post-OVX; their values were estimated using the technique defined by Patsaki et al (2016) and Duntas et al (2006; 2011).

Euthanasia, specimen collection and examination

The animals were anesthetized (3 mg/kg xylazine,

100 mg/kg ketamine hydrochloride, intramuscular) and euthanised after exsanguination from the abdominal aorta. At necropsy, the animals' abdominal cavity was examined for any abnormality and in the OVX groups for successful ovariectomy. The uteri were carefully removed free of surrounding tissues and their wet weight was immediately measured. The abdominal and perirenal fat tissue was also removed and immediately weighed. The femurs were dissected from the animals, cleaned of soft tissue and stored at -20°C to determine bone mechanical properties.

Bone biomechanical testing

Prior to biomechanical testing, the frozen femurs were thawed for 1 hour before the test and were rehydrated in physiologic saline. The femurs' length was measured with a micrometer and the middle of the diaphysis was marked. The femur was placed in the MTS INSIGHT 1kN testing frame (MTS, Eden Prairie) on two rounded supports at a distance of 20 mm from each other. The tests were quasi-static under displacement control mode at a rate of 1 mm/min. The force was applied to the middle of the diaphysis, until fracture. During loading, force and deflection were recorded. For the latter the RTSS_HR Video Extensometer (Limess, Krefeld, Germany) and the TestWorks 4 Software (WR Medical Electronics CO, Maplewood, USA) were used. From the force-deflection curve, femoral biomechanical parameters were determined, including maximum force (F_{max}), stiffness, energy absorption (W_{abs}).

In order to calculate also the fracture stress, σ_{max} , characterizing the material independently of the specimen's geometry, the characteristics of the bones' cross section had to be determined. In this context, the proximal one-half portion of the broken femur was embedded into specially designed plastic cups containing dental resin. The bone-dental resin complex was polished using LaboPol 5 (Struers), so as to trace the full cross-sectional shape. After deburring using Image-Pro Plus Processing Software, the photography session followed using LEICA MZ6 modular stereomicroscope. Picture processing was done with the stereoscope using the Solid Works software, enabling determination of: the cross section's geometrical centre, the loading axis and its eccentricity, the cross section's area, the inner and outer radii, the mean and minimum thickness of the cross section, the second moments of area with respect to the principal axes, the neutral axis and the critical point of the section. Using these characteristics the bones' fracture stress

(σ_{\max}) was determined by applying the Bernoulli-Euler technical bending theory.

Statistical analysis

Data are stated as mean \pm standard deviation (S.D.) for continuous parameters and as proportions for categorical variables. The Kolmogorov-Smirnov test was used for normality analysis of the variables. The comparison of variables at separate time points took place with the use of the one way analysis of variance (ANOVA) model. The repeated Measures ANOVA model was used for the comparison of dissimilar time quantity of variables for each assembly individually. Pairwise multiple comparisons were conducted with Tukey critical difference.

To indicate the trend in the first 6 months of treatment, the mean percentage changes after 3 and 6 months respectively were estimated. Comparison of the percentage change from baseline during the observation period between three groups was analyzed with the one way ANOVA model. Pairwise comparisons were performed using the Bonferroni, Kruskal-Wallis and Mann-Whitney tests in case of abuse of normalcy. Pearson or Spearman's correlation coefficients were used to examine the relation among quantitative variables. All tests were two-sided, statistical significance set at $P < 0.05$. All analyses were conducted

with SPSS vr 17.00 (Statistical Package for the Social Sciences, SPSS Inc., Chicago, Ill., USA).

RESULTS

Body weight and uterus weight

Administration of the plant extract did not present any adverse effect on the treated animals as observed by monitoring their body weights, fluid and food consumption.

Comparison of the animals' mean body weights at the end of the experiment showed a significant weight gain of OVX and OVX+RWP animals. This resulted in a significant difference between OVX and CTRL ($P = 0.002$), as well as between OVX+RWP and CTRL ($P = 0.008$), whereas there was no difference between OVX and OVX+RWP animals.

The uteri of OVX animals were macroscopically visibly atrophic, and their mean uterine weight was significantly lower in contrast to CTRL ($P = 0.002$). The mean uterine weight of OVX+RWP animals was identical to that of OVX and significantly lower in comparison with the CTRL ($P = 0.03$).

Comparison of the animals mean fat values at the end of the experiment showed a significant weight gain of OVX ($P = 0.044$) groups in comparison with the CTRL group (Table 1).

Table 2. Comparison of absolute values and mean percentage changes (in g/cm^2) of bone mineral density (BMD) of the total tibia among groups measured at baseline, 3 and 6 months post-ovariectomy

Group	Total Tibia BMD										
	Baseline		3 months*		6 months**		% change from baseline to 3 months*	% change from baseline to 6 months**	P*** Baseline vs 3 months	P*** Baseline vs 6 months	P*** 3 months vs 6 months
	Mean (SD) (g/cm^2)	Mean (SD) (g/cm^2)	Mean (SD) (g/cm^2)	Mean (SD)	Mean (SD)						
	0.213 (0.011)	0.215 (0.012)	0.217 (0.011)	1.04 (2.88)	1.68 (3.45)	0.816	0.471	0.975			
Control	0.213 (0.009)	0.200 (0.006)	0.190 (0.007)	-6.02 (2.65)	-10.73 (3.72)	0.000	0.000	0.000			
OVX	0.213 (0.012)	0.204 (0.013)	0.192 (0.009)	-4.06 (2.90)	-9.59 (4.97)	0.002	0.000	0.007			
OVX+RWP											

Groups: Control (CTRL), Ovariectomy (OVX), Ovariectomy treated with RWP extract (OVX+RWP).

* All pairwise comparisons between groups are statistically significant $P < 0.05$ except OVX vs. OVX+RWP group.

** All pairwise comparisons between groups are statistically significant $P < 0.05$ except OVX vs. OVX+RWP group.

*** Time effect comparisons after Bonferroni correction.

Table 3. Comparison of absolute values and mean percentage changes (in g/cm²) of bone mineral density (BMD) of the proximal tibia among groups measured at baseline, 3 and 6 months post-ovariectomy.

Proximal Tibia BMD								
Group	Baseline	3 months*	6 months**	% change from baseline to 3 months*	% change from baseline to 6 months**	P*** Baseline vs 3 months	P*** Baseline vs 6 months	P*** 3 months vs 6 months
	Mean (SD) (g/cm ²)	Mean (SD) (g/cm ²)	Mean (SD) (g/cm ²)	Mean (SD)	Mean (SD)			
Control	0.331 (0.022)	0.351 (0.018)	0.357 (0.017)	6.25 (3.39)	8.11 (3.13)	0.000	0.003	0.198
OVX	0.330 (0.023)	0.258 (0.031)	0.224 (0.024)	-21.82 (7.03)	-32.13 (5.91)	0.000	0.000	0.000
OVX+RWPs	0.351 (0.025)	0.318 (0.022)	0.285 (0.023)	-9.49 (4.91)	-18.57 (7.80)	0.000	0.000	0.001

Groups: Control (CTRL). Ovariectomy (OVX). Ovariectomy treated with RWPs extract (OVX+RWPs).

* All pairwise comparisons between groups are statistically significant P < 0.05.

** All pairwise comparisons between groups are statistically significant P < 0.05.

*** Time effect comparisons after Bonferroni correction.

Bone densitometry and biomechanical testing

Comparison of absolute values of BMD of the total tibia of the three groups, during the study, are shown in Table 2. Values are in grams per centimeters squared.

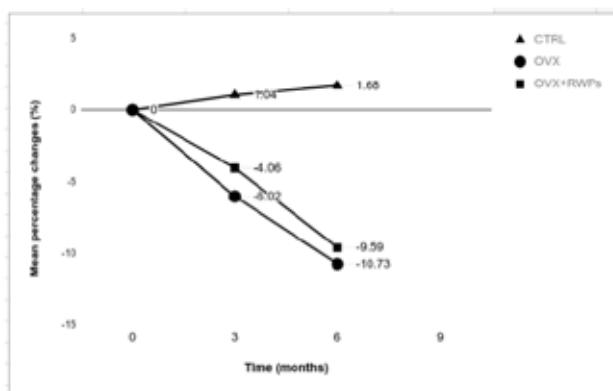
At baseline, statistical comparison of the absolute values of BMD of both sites examined within groups showed no statistical difference. At 3 and 6 months, the total tibia BMD of the CTRL rats had a significantly higher mean value than OVX and OVX+RWPs rats (P < 0.005).

Statistical comparison of the total tibia BMD within groups during the experimental period, showed a significant reduction in the OVX and OVX+RWPs groups both from baseline to 3 and 6 months and from 3 to 6 months, whereas in the CTRL group, there was no statistical significance. From the data in Table 3, it is apparent that the comparison of absolute values of BMD of the proximal tibia, during the experimental period, showed significant differences between the three groups. The BMD absolute values of the proximal tibial metaphysis within groups were slightly increased in the control group, whereas in both OVX and OVX+RWPs groups they decreased significantly from baseline to 3 months, from baseline to 6 months and from 3 to 6 months, respectively.

Treated rats (OVX+RWPs) had significantly higher BMD values than OVX rats with level of sig-

nificance P < 0.05 for the proximal tibia at 3 and 6 months. These findings suggest that that RWPs intake prevented ovariectomy-induced bone loss.

Comparison of the percentage changes of the total tibia at 3 and 6 months post-ovariectomy between CTRL and OVX and between CTRL and OVX+RWPs were significant, however between the OVX and OVX+RWPs groups they were nonsignificant (Fig. 3).

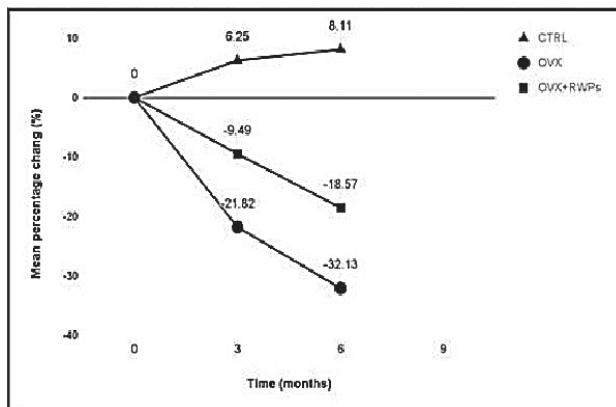


Groups: Control (CTRL), Ovariectomy (OVX), Ovariectomy treated with RWPs extract (OVX+RWPs).

Figure 3. Comparison of mean percentage changes (in g/cm²) of bone mineral density (BMD) of the total tibia among groups measured at baseline, 3 and 6 months post-ovariectomy.

For the proximal tibial metaphysis, pairwise comparison revealed highly statistical significant differences (Mann-Whitney test) between all groups at 3 and 6 months (Fig. 4). At 3 and 6 months, the dif-

ference between OVX and OVX+RWPs was highly significant ($P = 0.000$), indicating a highly protective effect on the treated animals.



Groups: Control (CTRL), Ovariectomy (OVX), Ovariectomy treated with RWPs extract (OVX+RWPs).

Figure 4. Comparison of mean percentage changes (in g/cm^2) of bone mineral density (BMD) of the proximal tibia among groups measured at baseline, 3 and 6 months post-ovariectomy.

Table 4 presents the results obtained from the biomechanical parameters of the three groups. Three-point-bending (3PB) revealed that the F_{\max} was significantly lower in the OVX group in contrast to CTRL ($P = 0.042$) and OVX+RWPs groups ($P = 0.048$). It is noteworthy that the F_{\max} in the OVX+RWPs animals was identical to CTRL rats. W_{abs} was significantly lower in the OVX group as compared with CTRL ($P = 0.004$) and OVX+RWPs group ($P = 0.002$). It is remarkable that the W_{abs} in the OVX+RWPs group was similar to CTRL group. σ_{max} was significantly greater in the treated group compared with OVX group ($P = 0.003$). Additionally, comparison of the σ_{max} mean values between OVX and CTRL was significant ($P = 0.048$), however between the CTRL and OVX+RWPs group, it was nonsignificant.

Table 4. Comparison of biomechanical parameters among three study groups.

Group	CTRL ^a Mean (SD)	OVX ^b Mean (SD)	OVX+RWPs ^c Mean (SD)	P Anova
F_{\max} (N)	118.90 (19.00) ^b	100.90 (16.60) ^{a,c}	118.50 (16.49) ^b	0.022
W_{abs} (mJ)	19.25 (2.91) ^b	12.95 (2.09) ^{a,c}	19.60 (6.58) ^b	0.001
σ_{max} (MPa)	184.29 (17.64) ^b	155.28 (12.71) ^{a,c}	198.01 (46.04) ^b	0.003

Note: superscript letters (a, b, c) indicates significant difference ($P < 0.05$) after Bonferroni correction.

Groups: a: Control (CTRL), b: Ovariectomy (OVX), c: Ovariectomy treated with RWPs extract (OVX+RWPs).

DISCUSSION

Hormone replacement therapy (HRT) has been used for prevention and treatment of postmenopausal osteoporosis but may cause serious side-effects (Ferguson, 2004). The results from the Women's Health Initiative Study that HRT significantly augments the risk of breast cancer, ovarian cancer, heart attack, stroke, thromboembolism and Alzheimer's disease (Canalis et al., 1988; Termine & Wong, 1998), increased women's non-compliance to HRT.

Polyphenols have been tested for their antiosteoporotic activity, because of their estrogenic and antioxidant effects. Some studies have displayed that grape

extracts have been promising in preserving or enhancing bone density (Hohman & Weaver, 2015; Yahara et al., 2005). We investigated the effect of RWPs extract administration as an alternative treatment to the long-term HRT commonly used by osteoporotic women.

The body weight of the ovariectomized rats increased steadily throughout the study even though all animals had the same daily food intake. These results are consistent with those of other studies which showed that OVX rats had significantly higher body weight compared to CTRL rats, due to fat deposition caused by estrogen deficiency (Patsaki et al., 2016). As also indicated by Dontas et al (2006; 2011), the

uteri of our OVX animals were macroscopically visibly atrophic, and their mean uterine weight was significantly lower in contrast to CTRL ($P = 0.002$).

Although the mechanism of beneficial health effects of RWPs remains unclear, several studies strongly suggest that they do not act via estrogen receptor binding (Agarwal et al., 2000; Eng et al., 2003; Sharma et al., 2004). Similarly, in this study RWPs extract administration was not able to modulate postmenopausal adiposity.

Cortical bone loss markedly increases activation of Haversian remodeling in humans (Lelovas, et al., 2008). However, the rat cortical compartment displays a low level of Haversian remodeling, in spite of increased endosteal osteoblasts and it is considered impractical to use cortical bones for bone loss studies (Jee & Yao, 2001). Data available from studies of our group revealed that the effects of OVX on BMD of cortical compartments of bone are lower than in trabecular bone (Patsaki et al., 2016; Dontas et al., 2011; Dontas et al., 2006). Statistical comparison of the total tibia BMD within groups during the study period, showed a significant reduction in the OVX and OVX+RWPs groups both from baseline to 3 and 6 months and from 3 to 6 months, whereas in the CTRL group, there was no statistical significance. Cortical bone, which has a longer remodelling period than trabecular bone (Jee & Yao, 2001), may need a longer experimental study to show potential beneficial effects of a treatment.

In the post-OVX period, a deterioration in trabecular bone density clearly shows rapid quantitative loss of bone, similar to the increased risk of fragility fractures in human postmenopausal osteoporosis, due to decline in endogenous estrogen production. The severe decrease of trabecular BMD in the metaphyseal sites after OVX were similar to the previous reports (Jee & Yao, 2001). Statistical comparison of the proximal tibia BMD absolute values, during the experimental period, showed a significant increase in the treated rats than OVX rats at 3 and 6 months.

Pairwise comparison of BMD percentage changes from baseline to 3 months and 6 months and from 3 to 6 months, revealed highly statistical differences between OVX and OVX+RWPs.

The bone density results of the present study, demonstrating bone quantity, are in accordance with the bone quality investigation regarding maximum load, energy absorption and maximum stress, conducted by *ex vivo* 3PB of the femur. OVX induced a significant reduction of biomechanical parameters as expected, whereas the OVX group treated with RWPs showed a significant increase in comparison with the nontreated OVX. It is noteworthy that the F_{max} , W_{abs} and σ_{max} in the OVX+RWPs animals was similar to CTRL rats. Similar studies with grape-enriched diet-fed rats, demonstrated improved bone strength of the femora (Hohman & Weaver, 2015; Yahara et al., 2005). Bone strength is the most important variable determined as it directly expresses the resistance of the whole bone to fracture, incorporating both its elastic and plastic behavior. Particularly in the clinical setting, bone strength is the ultimate goal of anti-osteoporotic treatments.

CONCLUSIONS

In conclusion, dietary intake of Red Wine extract for 6 months produced a remarkable effect on prevention of osteopenia and bone strength, in estrogen deficient ovariectomized rats, without hypertrophic effect on the uterus. RWPs could be considered as an attractive natural alternative anti-osteoporotic herbal treatment due to the additive beneficial action of its different substances, such as polyphenols. More experimental studies are needed to clarify the precise mechanism of action of RWPs, as well as a prospective clinical study in postmenopausal women with bone loss, before clinical recommendations can be made.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

Agarwal, C., Sharma, Y., Zhao, J., & Agarwal, R. (2000). A polyphenolic fraction from grape seeds causes irreversible growth inhibition of breast carcinoma MDA-MB468 cells by inhibiting mitogen-activated protein kinases activation and inducing G1 arrest and differentiation. *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research*, 6(7), 2921–2930.

Waynfirth, B. H., & Flecknell, P. A. (1992). *Experimental and Surgical Technique in the Rat*. London : Academic Press.

Canalis, E., McCarthy, T., & Centrella, M. (1988). Growth factors and the regulation of bone remodeling. *The Journal of Clinical Investigation*, 81(2), 277–281.

Cao, P.-C., Xiao, W.-X., Yan, Y.-B., Zhao, X., Liu, S., Feng, J., Lei, W. (2014). Preventive Effect of Crocin on Osteoporosis in an Ovariectomized Rat Model. *Evidence-Based Complementary and Alternative Medicine: eCAM*, 2014, 1–11.

De França, N. A. G., Camargo, M. B. R., Lazaretti-Castro, M., & Martini, L. A. (2013). Antioxidant intake and bone status in a cross-sectional study of Brazilian women with osteoporosis. *Nutrition and Health*, 22(2), 133–142.

Dontas, I. A., Lelovas, P. P., Kourkoulis, S. K., Aligiannis, N., Paliogianni, A., Mitakou, S., Galanos, A., Kassi, E., Mitousoudis, A., Xanthos, T., Papaioannou, N., Lyritis, G. P. (2011). Protective effect of Sideritis euboaea extract on bone mineral density and strength of ovariectomized rats. *Menopause*, 18(8), 915–922.

Dontas, I., Halabalaki, M., Moutsatsou, P., Mitakou, S., Papoutsi, Z., Khalidi, L., Galanos, A., Lyritis, G. P. (2006). Protective effect of plant extract from Onobrychis eboeoides on ovariectomy-induced bone loss in rats. *Maturitas*, 53(2), 234–242.

Eng, E. T., Ye, J., Williams, D., Phung, S., Moore, R. E., Young, M. K., ... Chen, S. (2003). Suppression of estrogen biosynthesis by procyandin dimers in red wine and grape seeds. *Cancer Research*, 63(23), 8516–8522.

Ferguson, N. (2004). *Osteoporosis in focus*. Pharmaceutical Press.

Hohman, E. E., & Weaver, C. M. (2015). A Grape-Enriched Diet Increases Bone Calcium Retention and Cortical Bone Properties in Ovariectomized Rats. *The Journal of Nutrition*, 145(2), 253–259.

Jee, W. S., & Yao, W. (2001). Overview: animal models of osteopenia and osteoporosis. *Journal of Musculoskeletal & Neuronal Interactions*, 1(3), 193–207.

Lelovas, P. P., Xanthos, T. T., Thoma, S. E., Lyritis, G. P., & Dontas, I. A. (2008). The laboratory rat as an animal model for osteoporosis research. *Comparative Medicine*, 58(5), 424–430.

L'Hermite, M. (2017). Custom-compounded bioidentical hormone therapy: why so popular despite potential harm? The case against routine use. *Climacteric: The Journal of the International Menopause Society*, 20(3), 205–211.

Lloret, A., Coiffier, G., Couchouron, T., Perdriger, A., & Guggenbuhl, P. (2016). Risk factors of mortality during the first year after low energy osteoporosis fracture: a retrospective case-control study. *Clinical Cases in Mineral and Bone Metabolism: The Official Journal of the Italian Society of Osteoporosis, Mineral Metabolism, and Skeletal Diseases*, 13(2), 123–126.

Miller, V. M., Lahr, B. D., Bailey, K. R., Heit, J. A., Harman, S. M., & Jayachandran, M. (2016). Longitudinal effects of menopausal hormone treatments on platelet characteristics and cell-derived microvesicles. *Platelets*, 27(1), 32–42.

Oster, P., Arab, L., Kohlmeier, M., Mordasini, R., Schellenberg, B., & Schlierf, G. (1982). Effects of estrogens and progestogens on lipid metabolism. *American Journal of Obstetrics and Gynecology*, 142(6 Pt 2), 773–775.

Patsaki, A., Tchoumtchoua, J., Passali, C., Lelovas, P., Kourkoulis, S., Papaioannou, N., Mbanya J-C., Njamen D., Mitakou S., Halabalaki M., Dontas, I. (2016). The Protective Effect of Amphimas pterocarpoides Plant Extract on Bone Mineral Density and Strength in Estrogen Deficient Ovariectomized Wistar Rats. *Medicinal & Aromatic Plants*, 5(5), 1–7.

Pawlowski, J. W., Martin, B. R., McCabe, G. P., Ferruzzi, M. G., & Weaver, C. M. (2014). Plum and soy aglycon extracts superior at increasing bone calcium retention in ovariectomized Sprague Dawley rats. *Journal of Agricultural and Food Chemistry*, 62(26), 6108–6117.

Ran, S. Y., Yu, Q., Chen, Y., & Lin, S. Q. (2017). Prevention of postmenopausal osteoporosis in Chinese women: a 5-year, double-blind, randomized, parallel placebo-controlled study. *Climacteric: The Journal of the International Menopause Society*, 20(4), 391–396.

Sharma, G., Tyagi, A. K., Singh, R. P., Chan, D. C. F., & Agarwal, R. (2004). Synergistic anti-cancer effects of grape seed extract and conventional cytotoxic agent doxorubicin against human breast carcinoma cells. *Breast Cancer Research and Treatment*, 85(1), 1–12.

Termine, J. D., & Wong, M. (1998). Post-menopausal women and osteoporosis: available choices for maintenance of skeletal health. *Maturitas*, 30(3), 241–245.

Thulkar, J., Singh, S., Sharma, S., & Thulkar, T. (2016). Preventable risk factors for osteoporosis in postmenopausal women: Systematic review and meta-analysis. *Journal of Mid-Life Health*, 7(3), 108–113.

Wang, T., Liu, Q., Tjhioe, W., Zhao, J., Lu, A., Zhang, G., Tan, R., Zhou, M., Feng, H. (2017). Therapeutic potential and outlook of alternative medicine for osteoporosis. *Current Drug Targets*.

Weisz, G. M. (2017). Osteoporosis: A Future Public Health Problem for Israel? Medical and Legal Obligations. *The Israel Medical Association Journal: IMAJ*, 19(4), 203–206.

Yahara, N., Tofani, I., Maki, K., Kojima, K., Kojima, Y., & Kimura, M. (2005). Mechanical assessment of effects of grape seed proanthocyanidins extract on tibial bone diaphysis in rats. *Journal of Musculoskeletal & Neuronal Interactions*, 5(2), 162–169.

Zhang, Z., Zheng, L., Zhao, Z., Shi, J., Wang, X., & Huang, J. (2014). Grape seed proanthocyanidins inhibit H2O2-induced osteoblastic MC3T3-E1 cell apoptosis via ameliorating H2O2-induced mitochondrial dysfunction. *The Journal of Toxicological Sciences*, 39(5), 803–813.