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PROTECTIVE EFFECT OF GRAPE SEED PROANTHOCYANIDINS ON DRUG-INDUCED OXIDATIVE STRESS IN RAT

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Abstract

Several drugs, including 13 cis-retinoic acid (13cRA) are producing reactive oxygen species that may be responsible for the toxicity and harmful adverse effects on bone and other tissues. The present study investigates the antiosteoporotic effects of grape seed proanthocyanidins (GSPs) in a 13cRA-induced osteoporosis model of rats and healthy rats.

Y59 rats were given GSPs (100 mg kg^{-1}) or alendronate (40 mg kg^{-1}, a positive control) concomitant with 13cRA (80 mg kg^{-1}) once daily for 14 days by a single intragastric application. Twenty four hours after the treatment, we analysed bone turnover serum biochemical markers, such as: osteocalcin (OC), C-terminal fragment of type I collagen (CTX), bone mineral density (BMD), calcium and phosphorus content, geometrical and physical parameters of bone as well as oxidative stress parameters, haematological and biochemical parameters in peripherial blood.

The treatment with GSPs increased BMD, OC level, femoral geometric characteristics, Ca and P content (P=0.025; P=0.025) in 13cRA-induced bone loss model. In addition, GSPs-treated rats had significantly lower serum ALP activity (P<0.001) and malondialdehyde (MDA) levels in both liver and kidney (P<0.05). Histological results showed its protective action as well, through promotion of bone formation.

According result GSPs could have similar protective action on bone health in humans

Introduction

Oxidative stress (OS) is a biochemical disequilibrium propitiated by an excessive production of free radicals (FR) and reactive oxygen species (ROS), that provoke oxidative damage to biomolecules and cannot be counteracted by antioxidative systems. It has has been linked with aging and more of 100 chronic degenerative diseases, including osteoporosis [1]. Other than OS, additional factors involved in osteoporosis include lifestyle, hormonal changes, body weight, and aging [1-3]. Recently, it has been demonstrated that FR intervene in bone resorption, promoting osteoclastic differentiation in such a manner that bone resorption is increased with OS [4-6]. Similarly, experimental studies have shown a diminution in antioxidant activity in patients with osteoporosis [3, 7]. In this regard, numerous authors have shown that administration of vitamins E and other antioxidants including polyphenols/flavonoids have a beneficial effect on bone quality and BMD in humans and animals [3-11].

The investigation group found a negative correlation between BMD and a total antioxidant status linked with low serum levels of glutathione peroxidase [9-11]; notwithstanding this, the influence of OS as an independent risk factor is unknown, considering the contribution of the additional risk factors linked with lifestyle, age, sex, diet and medicaments.

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Therefore, the objective of this study was to determine the relationship of OS induced with medicaments, as 13cRA, in rats and biological markers of osteoporosis and its relation to antioxidant diet. The vitamin A derivative 13-cis-retinoic acid (13-cis-RA, isotretinoin) is used clinically for the treatment of various skin conditions, such as severe recalcitrant acne. However, retinoids have long been known to cause pathologic effects on the bones. Wei et al. [10] and Fahmy et al. [11] demonstrated that application of high doses of vitamin A (13cRA) in a short period of 1-3 weeks causes the decrease in BMD, and histomorphometrical structural changes that correspond to osteoporosis. The main effects of 13cRA that contribute to bone loss are: a) increased oxidative stress (OS) and the formation of reactive radicals, decreased osteoblast and increased osteoclast activity; b) osteoblasts apoptosis; c) decreased estrogen levels and appearance of inflammatory cytokines, d) reduced activity of vitamin D receptor, decrease of the absorption of Ca^{2+} in the bowels, increased excretion of Ca through the kidneys; d) the effects on parathyroid hormone. From the afore mentioned facts, 13cRA-induced bone loss model of osteoporosis, caused by 13cRA, is the ideal model for studying antioxidative, anti-inflammatory, and regenerative capacity of flavonoid GSPs [10-12].
GSPs are widely found in fruits, vegetables, nuts, seeds, flowers and bark. They are a class of phenolic compounds that take the form of oligomers or polymers of polyhydroxy flavan-3-ol units, such as (+)-catechin and (−)-epicatechin [13]. The GSPs are mainly dimers, trimers and highly polymerized oligomers of monomeric catechins [13, 14]. GSPs have been shown to be potent antioxidants and FR scavengers, being more effective than either ascorbic acid or vitamin E [14, 15]. In addition to anti-oxidant activity, GSPs have been shown to have anti-carcinogenic activity in different tumour models [16, 17]. As there has been considerable interest in the usage of botanicals for the prevention of various diseases, phytochemicals might be of interest as protective agents for various diseases including osteoporosis. However, research on GSPs is limited and many questions still remain to be answered.

In the present study we examined the anti-osteoporotic effects of GSPs in 3cRA-induced osteoporosis model through analysis of molecular targets including biochemical markers of bone turnover such as OC, a marker of bone formation, and CTX, a marker of bone resorption, BMD, bone ash contents, and Ca and P analysis, geometrical and physical parameters of bone as well as histological analysis and oxidative stress parameters (glutathione levels and lipid peroxidation) in *in vivo* animal studies.

Material and methods

Animals
Present study was approved by the Ethics Committee (Faculty of Science, University of Zagreb, Croatia). Three months-old female rats from highly inbred Y59 strain (http://www.informatics.jax.org/external/festing/rat/docs/Y59.shtml), weighing 200 to 250 g, obtained from Department of Animal Physiology, Faculty of Science, University of Zagreb, were used in this study. The animals were kept in individual cages during the experiment and at 12 hours of light per day. They were fed a standard laboratory diet (4 RF 21, Mucedola, Settimo Milanese, Italy) and tap water ad libitum. Maintenance and care of all experimental animals were carried out according to the applicable guidelines in the Republic of Croatia (Law on the Welfare of Animals, Official Gazzette 19, 1999) and in accordance with EU Directive 2010/63/EU for animal experiments (reference: OJEU 2010) and carried out in compliance with the Guidance for the Care and Use of Laboratory Animals, DHHS Publ. # (NIH) 86-123.

Reagents
Isotretinoin (13cRA; 13 cis-Retinoic acid) (Accutane®, Hoffmann-La Roche Ltd, Basel, Switzerland) at 80 mg kg^{-1} dose was used for induction of osteoporosis in rats. AL (Alendor®70, Pliva, Croatia) at 40 mg kg^{-1} dose was used as positive control [10-12]. GSPs (Jianfeng Natural Products, Co. Ltd., Tianjing, China), was used in the study at dose of 100 mg kg^{-1} body weight.

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The GSPs content was 96.64% while analysed using High performance liquid chromatography (HPLC) with gas chromatography–mass spectrometry detection. They contained 6.1% catechin, 6.78% epicatechin, 55.59% dimeric forms, 11.91% trimeric forms, 6.55% tetrameric forms and small amounts of other polymeric forms [12]. GSPs was dissolved in saline before the use. GSPs was given to rats by intragastric application (*ig*) during 14 consecutive days at dose of 100 mg kg^{-1} body weight.

Experimental design: 13cRA-induced osteoporosis in rats and treatment

A total of 42 Y59 female rats were used in the study. Rats were divided into osteoporosis model group (A) and healty group (B), as follows:

(A) GSPs (n=7) at a dose of 100 mg kg^{-1} or AL (n=7) at a dose of 40 mg kg^{-1}, was administered orally via gastric tube to rats concomitant with 13cRA (80 mg kg^{-1}) once daily for 14 days. Seven rats were administered 13cRA intragastric only and served as osteoporotic model control group (13cRA group).

(B) The rest of the animals (21 animals) served as healthy control animals (control group), receiving *ig* saline or healthy animals treated with AL or GSPs.

All rats were fed and caged under consistent conditions during the study. Daily adjustment of dose was made according to the status of the rat's weight. Following the two weeks of treatement, the rats were anesthetized using a ketamine (Narketan®10, Vetoquinol AG, Belp Bern, Switzerland) at dose of 80 mg kg^{-1} and blood samples for haematological, biochemical and osteoporosis markers were collected after rats' exsanguination from axillary blood vessels. The blood was immediately placed on ice prior to isolation of the serum by centrifugation. The samples were then promptly frozen at − 80 °C until the analysis of markers related to bone turnover. The right and left femur were then harvested, carefully cleaned of soft tissue, weighed, and measured lengthwise using callipers. The right femurs were used for bone ash contents, and Ca and P analysis. The left femur was cut at the mid-diaphysis to assay bone density by a dual energy X-ray bone densitometer and was weighed and sectioned for histological analysis. The liver and kidney samples were collected 24 hours after the last dose of the study drugs and were used to measure the glutathione (GSH) level and lipid peroxidation of liver and kidney cells.

Bone harvesting and measurements

Immediately after sacrificing, the right hind leg of the rat was dissected. After removal of the flesh, femurs were soaked in the saline to prevent dehydration before the subsequent measurement of bone wet weight, length, mid shaft maximum and minimum diameters (physical dimensions). The femur wet weight, to the nearest 0.01 mg, was measured on an electrical balance (AA-160, Denver Instrument Company, U.S.A.) immediately after the bone harvesting. The femur length, to the nearest 0.01 mm, was measured with a digital sliding calliper (A.S.M., Germany), from the top of the femur head to the distal point of the femur. The calliper was then set at half the length of the measured femur, and the mid-point was marked with a marker pen on the femur. At that point, the bone mid shaft maximum diameter was measured from the anterior to posterior region of the bone, whereas the bone minimum diameter was measured from the medial to lateral region of the bone. The bones were then used for bone ash contents, Ca and P analysis.

Bone ash contents, and Ca and P analysis

For the Ca and P analysis, dissected right femurs were digested with an UltraCLAVE IV microwave digestion system (Milestone, Italy). Femurs were mixed with 5 mL 40% HNO_3 (v/v) in a quartz tube. The autoclave was pressurized with nitrogen gas (40 x 106 Pa) and heated at 260 ºC for 40 minutes. After digestion, all samples were diluted to 10 mL with deionized water and Ca and P content was performed as described in paper Oršolić et al. [12]. Results are expressed as μg Ca g^{-1} or P g^{-1} of wet tissue weight.

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Accuracy of the methods was evaluated using Animal bone reference material (H5, International Atomic Energy Agency, Austria). The results of our analysis were within ±10% of the reference values, i.e. 214.02±1.81 µg g^{-1} for Ca (reference value: 212 µg g^{-1} wet weight) and 102.29±0.31 µg g^{-1} for P (reference value: 102 µg g^{-1} wet weight).

Biochemical markers of bone turnover

Serum samples were used to measure two parameters of bone turnover: OC-a marker of bone formation and CTX-a marker of bone resorption. Enzyme immunoassay method (ELISA) was used to measure markers using commercially available ELISA kits for rats.

To determine the OC or CTX markers we used the kit for rat osteocalcin rat-MID™ Osteocalcin EIA (Immunodiagnostic Systems GmbH, Frankfurt am Main, Germany) and kit for rat CTX RatLaps™ EIA ELISA (Immunodiagnostic Systems GmbH, Frankfurt am Main, Germany). OC and CTX markers were measured according to the manufacturer's recommendations. The lowest measurable value of OC and CTXs were 1 ng mL^{-1} and 2 ng mL^{-1}, respectively.

Measurement of BMD

To determine BMD the left femur was stored after quarrying in test tubes with 10% buffered formalin.
The proximal and distal parts of the left neck of femur (thigh bone metaphysis) were subjected to measurement to obtain the values of bone area (cm^2-area) and bone mineral content (BMC, g). Data on BMD were calculated from the relationship of these parameters (g/cm^2).

To measure these parameters (area, BMC, BMD) dual energy x-ray densitometric absorptiometry method was used on Hologic apparatus QDR® 4000 (Hologic Inc.., Zaventem, Belgium) with appropriate software for small animals 1.0. high resolution.

The same technician performed all BMD measurements. In order to assess long-term reproducibility of the measured values, the analysis of these parameters followed the manufacturer's instructions. The coefficient of variation (QC) for BMD and femoral bone was 1.15% and 1.1% (0.61%), respectively.

Analysis of haematological and biochemical parameters

Blood samples were taken from the axillary plexus of blood vessels of experimental animals and placed in heparinized glass vacutainer with the addition of EDTA (Becton Dickinson, Plymouth, United Kingdom) and kept at the temperature from 4 °C in 2-4 hours to determine the haematological parameters.

For the evaluation of haematological parameters, we took 1 mL of blood in a test tube with added anticoagulant K_3EDTA. For the assessment of biochemical parameters, blood samples were collected in tubes without anticoagulant and the clotting of blood serum was used for the analysis of biochemical parameters. Blood samples for serum analysis were immediately placed in tubes without anticoagulant and the blood was centrifuged at 3000 rpm for 10 minutes. After centrifugation, serum was collected in 1.5 mL polypropylene tubes (Eppendorf AG, Hamburg, Germany) and stored at -20 °C until analysis of biochemical parameters. Haematological parameters included: number of erythrocytes (E), the average cellular volume of erythrocytes (MCV), haemoglobin (Hgb), haematocrit (Hct), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC), total leukocyte count (L), the total number of platelets (Plt), the percentage of the individual leucocytes in peripheral blood cells, neutrophils, lymphocytes, and basophils. Analysed biochemical parameters included aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), urea, creatinine, blood glucose levels (GLU), lactate dehydrogenase (LDH), and total protein.

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Blood was analysed using standard laboratory methods. We analysed hematologic parameters using blood cell counter Cell-Dyn ® 3200 (Abbott, USA), and the biochemical parameters with apparatus Alcylon 300 (Abbott, USA).

Tissue preparations
Portions of liver and kidney samples of 100 mg were homogenized in 1 mL of 50 mM cold phosphate buffer (pH 7.0) by ultrasonic homogenizer SONOPLUS Bandelin HD2070 (Bandelin, Germany) using probe MS73 (Bandelin, Germany) with a power of 10%. Homogenates were centrifuged by Micro 200R centrifuge (Hettich, Germany) for 15 minutes at a speed of 10 000 x g. The supernatant was used for the measurements of glutathione and level of lipid peroxidation.

Lipid peroxidation
The presence of lipid peroxidation was determined by measuring the concentration of malondialdehyde (MDA), the major product of lipid peroxidation as described by Oršolić et al. [12]. MDA reacts with thiobarbituric acid and produces chromogen that is measured spectrophotometrically at 532 nm and 600 nm with Libro S22 spectrophotometer (Biochrom, UK). The concentration of lipid peroxides was expressed as mg MDA / mL / mg protein measured by the method of Lowry [18].

Total glutathione (GSH) assay
Glutathione assay is described in paper [19]. The glutathione level was determined by 5-5'-dithiobis [2-nitrobenzoic acid] (DTNB, Ellman's Reagent) and measured on the spectrophotometer at 412 nm. The results were calculated from the standard curve of GSH measured by the same protocol.

Histological analysis
After the rats were sacrificed, the left femurs were removed, cleared from the attached muscle tissue and fixed in 10% neutral buffered formalin. The bone specimens were then washed thoroughly in tap water for 30 min and then placed in decalcifying fluid RDO (Apex Engineering Products; Plainfield, IL) for 24 hours. After decalcification, each of the bones were cut at the mid shaft of its diaphysis and then rinsed thoroughly in slowly running tap water, dehydrated in a graded alcohol series (70%, 80%, 96%, 100%) and after chloroform treatment embedded in paraplast. Deparaplasted 6-7 µm thick cross sections of the femur distal diaphysis were stained with haematoxylin and eosin (HE) following standard protocol. Stained sections taken from comparable areas were examined under a light microscope (Nikon Eclipse E600) at 40, 100 and 200x magnification. Photomicrographs were taken by digital camera (Nikon DMX1200) and Imaging Software Lucia G 4.80 (Laboratory Imaging Ltd., Prague, Czechoslovakia).

Statistical Analysis
All statistical analyses were performed using the Statistical Program for Social Sciences (SPSS 10.0 for windows; SPSS Inc., Chicago, Illinois, USA). The significance of differences between the groups was tested by the Mann-Whitney *U*-test with the significance level >95%. Continuous variables are expressed as median (interquartile range 25th–75th percentile (IQR)). The data were considered significant at $P<0.05$.

Results

(A) Results in osteoporosis model group

Retinoic acid is an effective drug for induction of osteoporosis in rats
The intragastric administration of 13cRA at dose of 80 mg kg^{-1} body weight daily for 2 weeks resulted in a significant increase of the bone markers values of OC and CTX-A (P = 0.021, P = 0.021, respectively) in comparison to the control group (Table 1).

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Table 1 Effects of grape seed proanthocyanidins or alendronate on the bone turnover markers against retinoic acid-induced osteoporosis in rats

Parameters	Treatment[a]	Median	Min	Max	25. percentile	75. percentile	*P**	*P***
OC (ng/mL)	**Control**	151.450	147.200	157.200	148.900	154.750	-	0.021
	13cRA	173.300	160.200	187.300	164.800	182.250	0.021	-
	GSPs+13cRA	121.300	112.900	135.400	112.900	135.400	0.034	0.034
	AL+13cRA	90.700	88.400	93.000	88.400	93.000	0.133	0.133
CTX (ng/mL)	**Control**	22.000	20.600	23.400	21.150	22.850	-	0.021
	13cRA	35.350	30.200	37.300	32.200	36.900	0.021	-
	GSPs+13cRA	24.600	21.800	34.100	21.800	34.100	0.157	0.077
	AL+13cRA	21.100	19.700	22.500	19.700	22.500	0.800	0.133

[a] Rats were given GSPs (100 mg kg^{-1}) or AL (40 mg kg^{-1}) concomitant with 13cRA treatment (80 mg kg^{-1}) once daily for 14 days by a single intragastric application. Results are presented as medians with interquartile ranges. Number of rats per group: 7.
P * *vs* control; ** *P vs* 13cRA
Legend: 13cRA -retinoic model of osteoporosis; GSPs - grape seed proanthocyanidins; AL- alendronate; OC – osteocalcin; CTX -C-terminal fragment of type I collagen.

The values of BMD in the proximal (*P* = 0.021) and distal (*P* = 0.021) metaphysis of the femoral neck were significantly lower in 13cRA group than in the control group (Table 2).

Table 2 Effects of grape seed proanthocyanidins or alendronate on bone mineral density against retinoic acid-induced osteoporosis in rats

Parameters	Treatment[a]	Median	Min	Max	25. percentile	75. percentile	*P**	*P***
BMD-prox g/cm^2	**Control**	0.273	0.264	0.283	0.268	0.279	-	0.021
	13cRA	0.240	0.236	0.243	0.237	0.242	0.021	-
	GSPs+13cRA	0.267	0.233	0.276	0.233	0.276	0.480	0.240
	AL+13cRA	0.263	0.249	0.277	0.249	0.277	0.267	0.800
BMD-dist g/cm^2	**Control**	0.280	0.265	0.283	0.272	0.282	-	0.021
	13cRA	0.239	0.237	0.241	0.238	0.240	0.021	-
	GSPs+13cRA	0.251	0.221	0.270	0.221	0.270	0.077	0.240
	AL+13cRA	0.254	0.239	0.268	0.239	0.268	0.133	0.267

[a] Rats were given GSPs (100 mg kg^{-1}) or AL (40 mg kg^{-1}) concomitant with 13cRA treatment (80 mg kg^{-1}) once daily for 14 days by a single intragastric application. Results are presented as medians with interquartile ranges. Number of rats per group: 7.
P * *vs* control; ** *P vs.* 13cRA
Legend: 13cRA - retinoic model of osteoporosis; GSPs - grape seed proanthocyanidins; AL- alendronate; BMD - bone mineral density

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The values of the total content of Ca and P in the femur were significantly lower as well (P = 0.025, P = 0.025) in 13cRA group compared to the control group (Table 3).

Table 3 Effects of grape seed proanthocyanidins or alendronate on the bone ash content and levels of calcium and phosphorus against retinoic acid-induced osteoporosis in rats

Parameters	Treatment[a]	Median	Min	Max	25. percentile	75. percentile	P*	P**
Bone ash /g	**Control**	0.61	0.61	0.65	0.61	0.62	-	0.655
	13cRA	0.65	0.60	0.68	0.60	0.68	0.655	-
	GSPs+3cRA	0.89	0.78	0.91	0.81	0.90	0.009	0.025
	AL+13cRA	ND	-	-	-	-	-	-
mg Ca/g	**Control**	167.65	162.51	179.62	163.99	169.60	-	0.025
	13cRA	156.19	150.00	159.90	150.00	159.90	0.025	-
	GSPs+13cRA	166.82	163.06	181.48	165.00	177.99	0.754	0.025
	AL+13cRA	ND	-	-	-	-	-	-
mg P/g	**Control**	74.18	71.67	77.72	72.25	74.46	-	0.025
	13cRA	67.00	60.00	68.00	60.00	68.00	0.025	-
	GSPs+3cRA	74.00	69.91	81.32	72.56	79.24	0.754	0.025
	AL+13cRA	ND	-	-	-	-	-	-

[a] Rats were given GSPs (100 mg kg^{-1}) or AL (40 mg kg^{-1}) concomitant with 13cRA treatment (80 mg kg^{-1}) once daily for 14 days by a single intragastric application. Results are presented as medians with interquartile ranges. Number of rats per group: 7.
P * *vs* control; ** *P vs.* 13Cra
Legend: 13cRA -retinoic model of osteoporosis; GSPs - grape seed proanthocyanidins; AL- alendronate; Ca – calcium; P - phosphorus ND –not determined

The median weight, length and both femur diameters (anterior-posterior and medial-lateral) were lower in 13cRA group but the difference was not statistically significant (Table 4 and 5).

Table 4 Effects of grape seed proanthocyanidins or alendronate on femoral bone weight and bone length against retinoic acid-induced osteoporosis in rats

Parameters	Treatment[a]	Median	Min	Max	25. percentile	75. percentile	P*	P**
Bone weight	**Control**	671.65	651.60	743.35	662.70	710.59	-	0.072
	13cRA	647.60	589.75	669.60	589.75	669.60	0.072	-
	GSPs+13cRA	878.50	782.30	912.40	809.55	880.75	0.040	0.018
	AL+13cRA	639.60	558.00	721.20	558.00	721.20	0.083	0.125
Bone length	**Control**	3.20	3.18	3.23	3.19	3.23	-	0.500
	13cRA	3.19	3.15	3.27	3.15	3.27	0.500	-
	GSPs+13cRA	3.46	3.40	3.56	3.41	3.52	0.004	0.018
	AL+13cRA	3.31	3.29	3.51	3.30	3.38	0.040	0.018

[a] Rats were given GSPs (100 mg kg^{-1}) or AL (40 mg kg^{-1}) concomitant with 13cRA treatment (80 mg kg^{-1}) once daily for 14 days by a single intragastric application. Results are presented as medians with interquartile ranges. Number of rats per group: 7.
P * *vs* control; ** *P vs.* 13cRA

Table 5 Effects of grape seed proanthocyanidins or alendronate on femoral bone diameter (medio-lateral diameter or anterior-posterior diameter) against retinoic acid-induced osteoporosis in rats

Parameters	Treatment[a]	Median	Min	Max	25. percentile	75. percentile	*P**	*P***
Diameter med/lat (mm)	**Control**	3.80	3.45	4.00	3.55	3.80	-	0.197
	13cRA	3.55	3.45	3.65	3.45	3.65	0.197	-
	GSPs+13cRA	3.95	3.85	4.10	3.95	4.05	0.028	0.018
	AL+13cRA	3.50	3.20	3.80	3.20	3.60	0.211	0.125
Diameter ant/post (mm)	**Control**	3.00	2.80	3.10	2.95	3.00	-	0.072
	13cRA	2.85	2.75	2.85	2.75	2.85	0.072	-
	GSPs+13cRA	3.00	2.95	3.10	3.00	3.00	0.345	0.018
	AL+13cRA	2.90	2.80	3.00	2.80	3.00	0.155	0.125

[a] Rats were given GSPs (100 mg kg^{-1}) or AL (40 mg kg^{-1}) concomitant with 13cRA treatment (80 mg kg^{-1}) once daily for 14 days by a single intragastric application. Results are presented as medians with interquartile ranges. Number of rats per group: 7.

P * *vs* control; ** *P vs.* 13cRA

Legend: 13cRA -retinoic model of osteoporosis; GSPs - grape seed proanthocyanidins; AL- alendronate; diameter med/lat and ant/post - the proximal epiphyseal diameters of the femur in medio-lateral and anterior-posterior directions

There were no differences in the initial mean body mass of the rats. After 2 weeks of experiment, intragastric administration of 13cRA in the rats caused an average weight reduction of -3.5%, whereas observed average weight gain in healthy control animals was 2.6% (Figure 1).

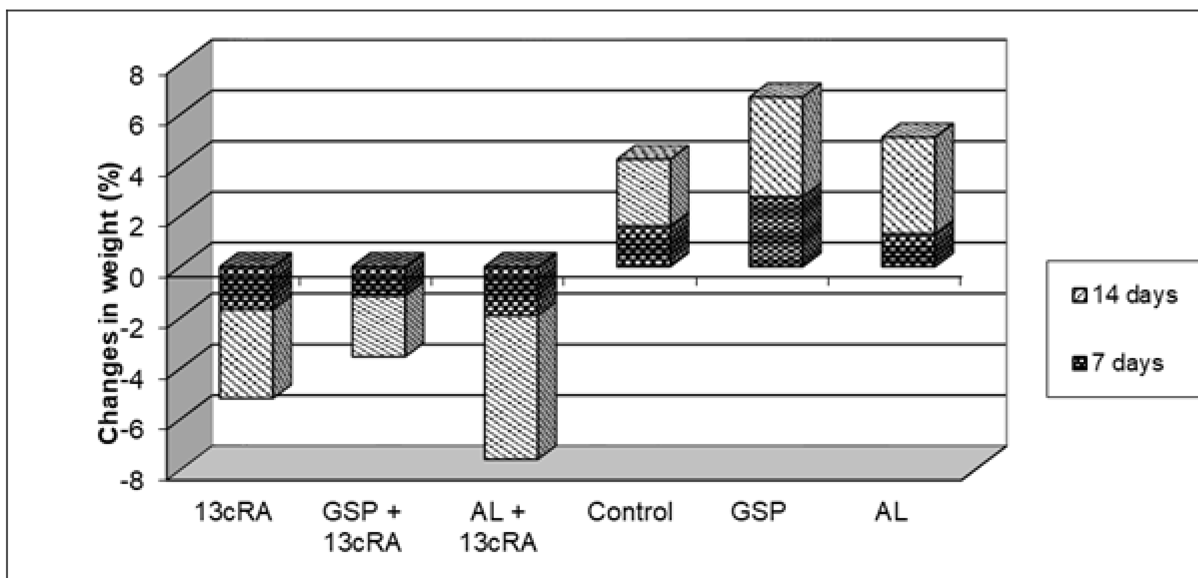

Figure 1 Effects of grape seed proanthocyanidins and alendronate on the changes in body weight in rats with retinoic acid-induced bone loss. Rats were given GSPs (100 mg kg^{-1}) or AL (40 mg kg^{-1}) concomitant with 13cRA treatment (80 mg kg^{-1}) once daily for 14 days by a single intragastric application. Number of rats per group: 7.
Legend: 13cRA -retinoic model of osteoporosis; GSPs - grape seed proanthocyanidins; AL- alendronate

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Treatment with 13cRA resulted in a significant increase of AST, ALT, and AP (P<0.003, P<0.037, P<0.001, respectively) with significant decrease of urea (P<0.001) (Table 6).
Treatment of rats with 13cRA significantly increased number of platelets (P<0.001) and decreased MCV levels in relation to the control group

Table 6 Effects of grape seed proanthocyanidins or alendronate on the biochemical parameters against retinoic acid-induced osteoporosis in rats

Parameters	Treatment[a]	Median	Min	Max	25. percentile	75. percentile	P*	P**
AST (U/L)	**Control**	207.00	162.00	218.00	180.00	214.00	-	0.003
	13cRA	234.00	206.00	280.00	226.00	272.00	0.003	-
	GSPs +13cRA	204.00	168.00	242.00	190.00	214.00	0.500	0.011
	AL + 13cRA	207.00	198.00	242.00	200.00	216.00	0.762	0.029
ALT (U/L)	**Control**	34.00	28.00	44.00	30.00	38.00	-	0.037
	13cRA	39.00	34.00	54.00	36.00	46.00	0.037	-
	GSPs +13cRA	31.00	0.00	42.00	18.00	40.00	0.197	0.037
	AL + 13cRA	38.00	2.00	44.00	18.00	41.00	0.348	0.173
ALP (U/L)	**Control**	140.00	130.00	160.00	136.00	148.00	-	<0.001
	13cRA	266.00	242.00	340.00	246.00	340.00	<0.001	-
	GSPs +13cRA	167.00	124.00	218.00	160.00	188.00	0.010	<0.001
	AL + 13cRA	190.00	184.00	250.00	187.00	220.00	<0.001	0.004
LDH (U/L)	**Control**	3097.00	2332.00	3458.00	2466.00	3258.00	-	0.202
	13cRA	3221.00	2936.00	3488.00	2984.00	3432.00	0.220	-
	GSPs +13cRA	2852.00	2262.00	3736.00	2460.00	3534.00	0.796	0.562
	AL + 13cRA	2922.00	2418.00	3874.00	2581.00	3470.00	0.897	0.755
GLU (mmol/L)	**Control**	5.90	5.00	7.60	5.40	7.00	-	0.357
	13cRA	6.30	5.40	7.40	6.00	6.40	0.357	-
	GSPs +13cRA	7.20	3.60	9.80	7.00	7.40	0.038	0.028
	AL + 13cRA	7.30	6.20	8.00	6.66	7.90	0.014	0.010
Urea (mmol/L)	**Control**	8.20	7.40	9.20	8.00	8.60	-	<0.001
	13cRA	6.10	5.00	6.80	5.40	6.80	<0.001	-
	GSPs +13cRA	5.60	5.00	6.00	5.20	5.80	<0.001	0.091
	AL + 13cRA	6.80	6.00	8.40	6.40	7.00	0.007	0.047
Creatinine (μmol/L)	**Control**	52.00	48.00	62.00	48.00	58.00	-	0.318
	13cRA	56.00	48.00	58.00	52.00	56.00	0.318	-
	GSPs +13cRA	57.00	54.00	64.00	56.00	58.00	0.026	0.091
	AL + 13cRA	55.00	48.00	58.00	51.00	57.00	0.317	0.475
TP (g/L)	**Control**	56.00	50.00	62.00	52.00	58.00	-	0.368
	13cRA	58.00	36.00	68.00	54.00	64.00	0.368	-

GSPs +13cRA	61.00	58.00	64.00	58.00	62.00	0.004	0.492
AL + 13cRA	59.00	54.00	64.00	54.00	62.00	0.173	0.345

[a] Rats were given GSPs (100 mg kg^{-1}) or AL (40 mg kg^{-1}) concomitant with 13cRA treatment (80 mg kg^{-1}) once daily for 14 days by a single intragastric application. Results are presented as medians with interquartile ranges. Number of rats per group: 7.
P * *vs* control; ** *P vs.* 13cRA
Legend: 13cRA -retinoic model of osteoporosis; GSPs - grape seed proanthocyanidins; AST - aspartate aminotransferase; ALT - alanine aminotransferase; ALP - alkaline phosphatase; LDH - lactate dehydrogenase; GLU – glucose; TP – total protein

Intrgastric administration of 13cRA in rats resulted in a significant increase of MDA in the liver ($P<0.05$) and a significant decrease in GSH level in liver and kidney ($P<0.05$) (Figure 2).

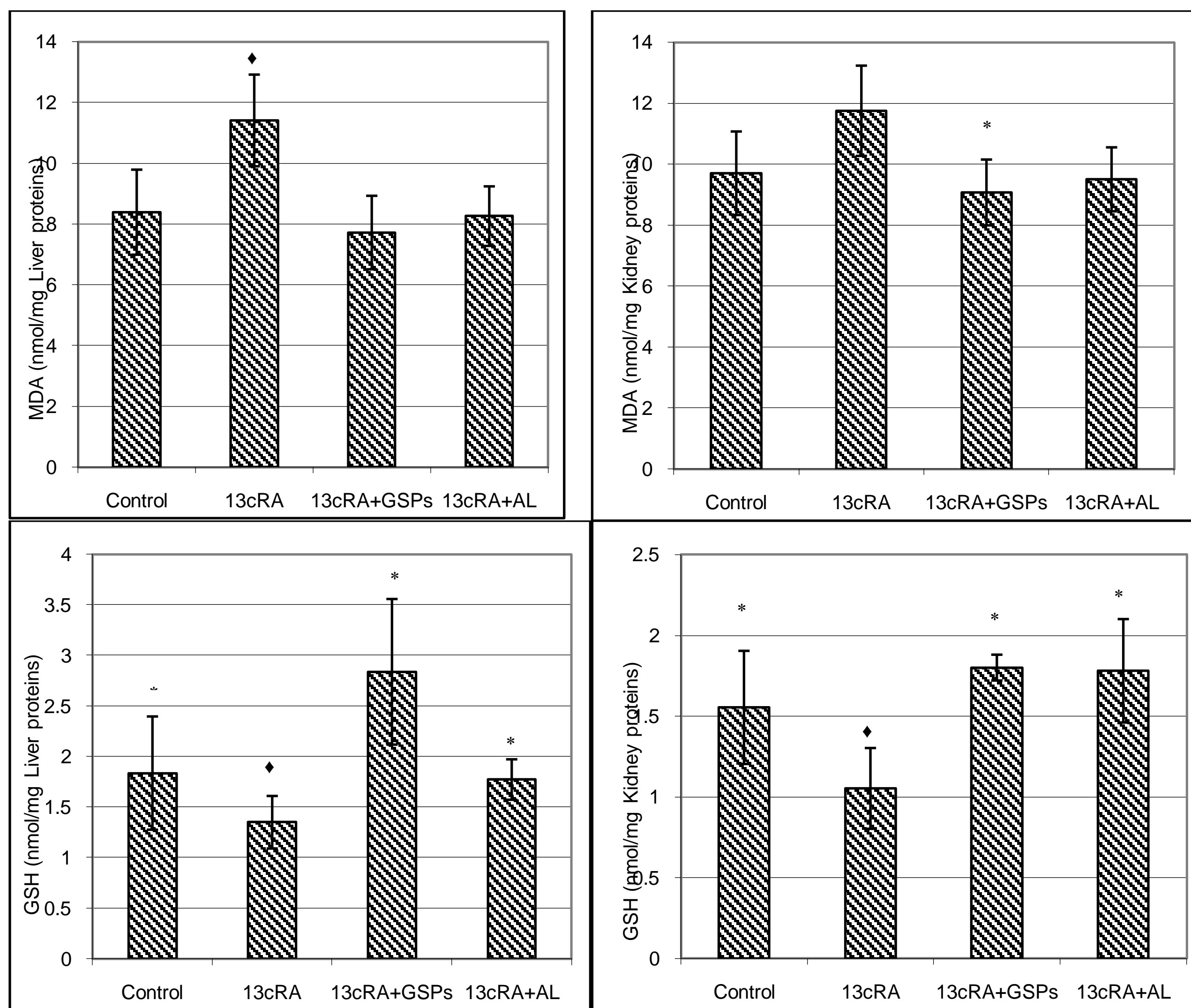

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Figure 2 Effects of grape seed proanthocyanidins and alendronate on MDA and GSH contents in liver and kidney in rats with retinoic acid-induced osteoporosis. Rats were given GSPs (100 mg kg^{-1}) or AL (40 mg kg^{-1}) concomitant with 13cRA treatment (80 mg kg^{-1}) once daily for 14 days by a single intragastric application. Number of rats per group: 5.
♦Statistically significantly different compared to control (♦P<0.05).
∗Statistically significantly different compared to 13cRA ($^{*}P$<0.05).
Legend: 13cRA - retinoic model of osteoporosis; GSPs - grape seed proanthocyanidins; AL- alendronate; MDA - malondialdehyde; GSH – glutathione.

Diaphyseal cortex of rats treated with 13cRA showed marked porosity caused by formation of multiple cavities of different sizes (Figure 3). Some cavities were bounded with osteoid or newly formed bone at the cortical surface and lined with osteoblasts and osteoclasts at the luminal surface. Endosteal surface appeared irregular with many notches (Figure 3).

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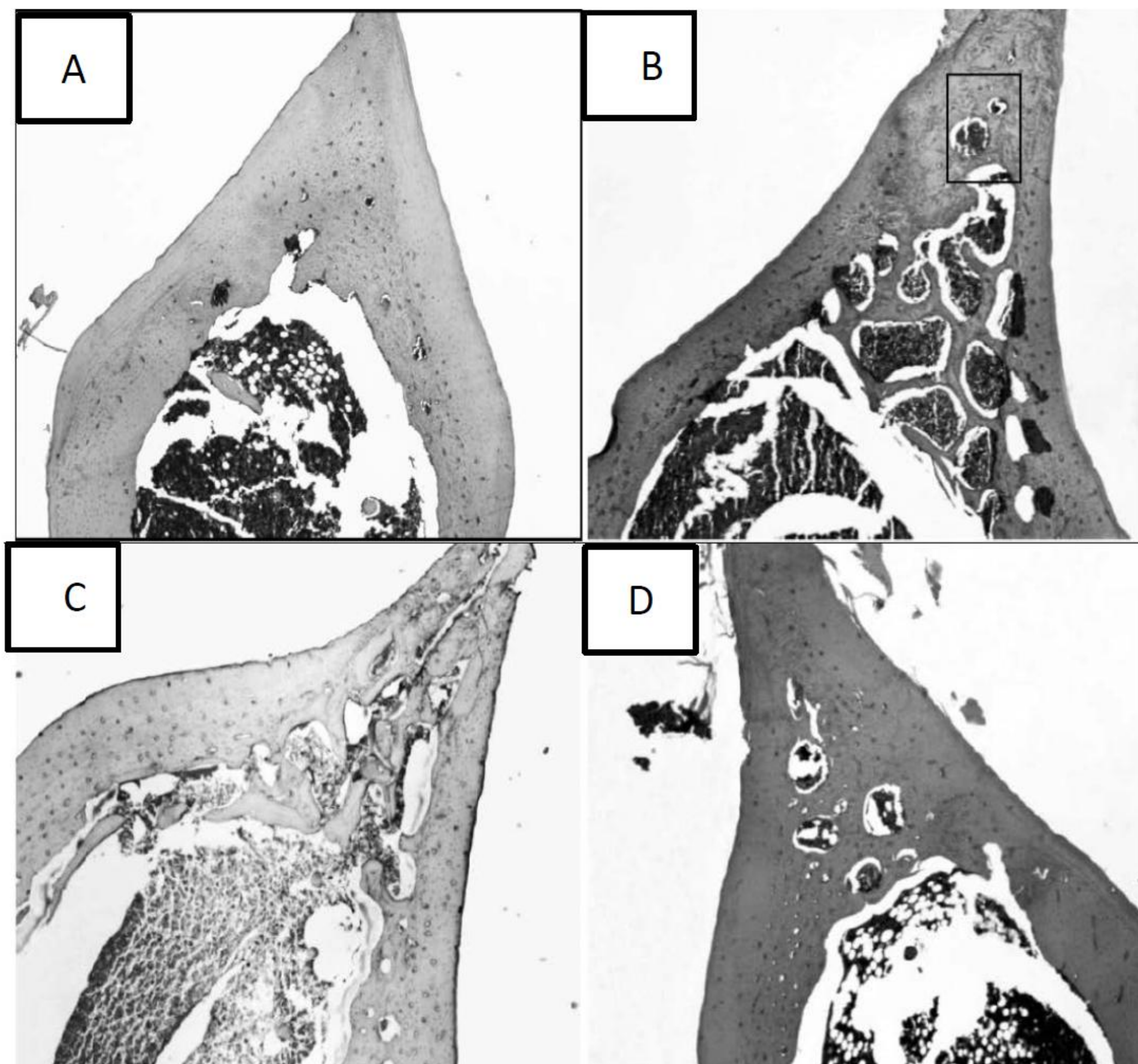

Figure 3 Cross sections of the distal femur diaphysis cortical bone of female rats stained with HE. A) In Control group cortical bone showing normal architecture of the bone tissue. **B)** Retinoic acid-treated group (osteoporotic group) showing formation of multiple large intracortical cavities and thinning of the cortex as well as intracortical cavities bounded with newly formed bone (marked) and osteoclasts lining the luminal surface of the cavities. C) In rats treated concurrently with alendronate and retinoic acid femoral cortex showing thinning, multiple cavities formation, widened Haversian canals and eroded endosteal surface. D) Diaphyseal cortex of rats treated concurrently with retinoic acid and proanthocyanidins showing more preserved bone tissue structure; osteoporotic cavities are reduced in their number, primarily their size while endosteal surface showing less irregularity.

Protective effect of GSPs or AL against the effect of 13cRA on osteoporotic markers of bone formation in rats

Application of GSPs concomitant with 13cRA has led to a significant decrease in OC (P = 0.034) compared to controls and 13cRA (P = 0.034) (Table 1). There were no differences in the value of CTX compared to controls (P =0,157) and 13cRA (P = 0,077) (Table 1). BMD in proximal and distal metaphysis of the femoral neck in the group

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treated with GSPs is equal to control BMD value (Table 2). The values of Ca and P in the group that received GSPs were significantly higher than in the 13cRA group (Table 3). Femoral bone weight was significantly higher in GSPs (P = 0.04) group compared to control and 13cRA (P = 0.018 (Table 4). Femoral bone length in GSPs group was significantly higher compared to control and 13cRA (Table 4).
Medio-lateral diameter of the bone was higher in the GSPs (P = 0.028) group, while the anterior-posterior diameter of the bone in either group did not significantly differ from the control (Table 5). Medio-lateral and anterioro-posterior diameters of GSPs group were significantly (P = 0.018; P = 0.018) higher in comparison to the 13cRA (Table 5). After 2 weeks of experiment, intragastric application of GSPs concomitant with 13cRA caused an average weight reduction of -2.38% (Figure 1).

The values of BMD in the proximal and in the distal metaphysis parts of the left femur at AL group were not significantly altered compared to the control (Prox, P = 0.267; dist P = 0.133) nor the 13cRA (Prox P = 0.800; dist P = 0.267) (Table 2). Other bone parameters, without bone length, OC, CTX and bone weight and diameter of bone in intragastric application of AL concomitant with 13cRA did not lead to significant changes.

Administration of AL with 13cRA resulted in significant reduction in body mass of -5.67% after two weeks. This combination was toxic for rats and half rats in the group died 2-5 days before the end of the experiment (Figure 1).
The result of MDA and GSH content in rats treated with 13cRA, GSPs or AL are depicted in Figure 2.

Figure 2 clearly shows that the 13cRA caused an increase in the MDA level and reduced GSH level (P<0.05) in liver and kidney. GSPs has reversed MDA and GSH levels to values within the control group. Treatment of rats with AL did not cause a significant change in MDA while GSH was increased.

Diaphyseal cortex of group treated with 13cRA and GSPs showed marked improvement of the structure of the femoral cortical bone (Figure 3) compared with the 13cRA group. One or two small intracortical cavities were found and endosteal surface became smooth with only few slight irregularities.

The histopathologic analysis of the femur in rats treated with 13cRA and AL, showed apparent decrease in the thickness of cortical, formation of multiple intracortical cavities, widened Haversian canals and eroded both endosteal and periosteal surface. In addition osteocytes appeared degenerated with piknotic nuclei but there were also some empty lacunae (Figure 3).

Protective effect of GSPs or AL against the effect of 13cRA on biochemical and haematological parameters
Concomitant application of GSPs with 13cRA has led to a significant increase in blood glucose values compared to the control and 13cRA. Also there was a significant decrease in the value of urea for GSPs group compared to control while the value of creatinine in GSPs group was significantly higher. No significant changes in the value of creatinine and urea in GSPs group compared to the 13cRA (Table 6).

The values of AST, ALT and AP in GSPs group were significantly lower compared to the 13cRA (P=0.011; P=0.037: P<0.001), whereas no significant change was observed for the values of ALT and AST in comparison to the control (Table 6).

The values of AP in AL group was significantly lower compared to the control and 13cRA (P <0.001, P=0.004) (Table 6).

The values of leukocytes and erythrocytes were not significantly changed in GSPs group compared to controls and 13cRA (Table 7). The values of platelets (Plt), MCH and MCHC were significantly higher in GSPs group compared to controls (Table 7).

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In GSPs treated group Hgb, Hct, and MCV levels were not significantly different from the control group (Table 7). Significantly higher values of MCV, MCH and MCHC were in GSPs treated group compared to 13cRA (Table 7). AL in combination with 13cRA showed a decrease in the number of red blood cells and MCV values in relation to 13cRA (*P*=0.014; *P*=0.004) while the number of PLT was higher compared to the control and 13cRA (*P*=0.001; *P*=0.022) (Table 7).

Table 7 Effects of grape seed proanthocyanidins on the haematological parameters against retinoic acid-induced osteoporosis in rats

Parameters	Treatment[a]	Median	Min	Max	25. percentile	75. percentile	*P**	*P***
L ($x10^9$/L)	**Control**	4.88	2.96	7.40	3.58	5.82	-	0.396
	13cRA	4.60	3.04	8.86	3.82	6.84	0.396	-
	GSPs +13cRA	7.11	2.60	8.50	4.64	8.24	0.095	0.281
	AL + 13cRA	4.69	4.26	7.94	4.37	6.30	0.348	0.331
E ($x10^{12}$/L)	**Control**	8.29	7.40	8.94	8.10	8.54	-	0.110
	13cRA	8.01	7.92	8.30	7.92	8.28	0.110	-
	GSPs +13cRA	8.29	5.10	8.76	7.54	8.70	0.385	0.281
	AL + 13cRA	7.88	7.54	8.78	7.81	8.11	0.237	0.014
Plt ($x10^9$/L)	**Control**	855.00	276.00	932.00	766.00	888.00	-	<0.001
	13cRA	1038.00	944.00	1190.00	948.00	1132.00	<0.001	-
	GSPs +13cRA	1063.00	916.00	1568.00	996.00	1162.00	0.001	0.318
	AL + 13cRA	930.00	876.00	1086.00	908.00	959.00	0.001	0.022
Hgb (g/L)	**Control**	140.60	123.60	154.60	127.00	146.60	-	0.281
	13cRA	135.50	134.00	142.40	135.00	138.40	0.281	-
	GSPs +13cRA	143.20	104.40	152.40	132.80	151.20	0.316	0.132
	AL + 13cRA	137.30	131.40	150.40	134.00	140.70	0.381	0.426
Hct (L/L)	**Control**	0.47	0.41	0.46	0.44	0.48	-	0.059
	13cRA	0.44	0.43	0.46	0.44	0.45	0.059	-
	GSPs +13cRA	0.46	0.34	0.49	0.43	0.48	0.427	0.157
	AL + 13cRA	0.44	0.42	0.49	0.43	0.45	0.119	0.378
MCV (fL)	**Control**	110.80	109.60	111.80	110.40	111.40	-	0.002
	13cRA	109.60	109.20	110.20	109.40	110.20	0.002	-
	GSPs +13cRA	111.20	109.40	134.40	110.60	112.80	0.158	0.004
	AL + 13cRA	110.40	110.00	112.60	110.20	111.80	0.348	0.004
MCH (pg)	**Control**	34.00	30.00	34.60	33.60	34.40	-	0.479
	13cRA	33.90	33.40	34.40	33.60	34.40	0.479	-
	GSPs +13cRA	35.10	34.00	42.00	34.80	35.80	<0.001	0.002
	AL + 13cRA	34.80	34.00	35.20	34.20	34.90	0.006	0.022
MCHC (g/L)	**Control**	618.00	538.00	622.00	604.00	622.00	-	0.246
	13cRA	617.00	606.00	628.00	612.00	628.00	0.246	-
	GSPs +13cRA	629.00	610.00	638.00	624.00	630.00	0.026	0.028
	AL + 13cRA	628.00	612.00	642.00	6.14.00	640.00	0.028	0.120

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[a] Rats were given GSPs (100 mg kg^{-1}) or AL (40 mg kg^{-1}) concomitant with 13cRA treatment (80 mg kg^{-1}) once daily for 14 days by a single intragastric application. Results are presented as medians with interquartile ranges. Number of rats per group: 7.
P * *vs* control; ** *P vs.* 13cRA
Legend: 13cRA -retinoic model of osteoporosis; GSPs - grape seed proanthocyanidins; L - leukocytes; E - erythrocytes; Plt – platelets; Hgb - hemoglobin; HCT - hematocrit; MCV- average volume of red blood cells; MCH - the average amount of hemoglobin in red blood cells; MCHC - mean concentration of hemoglobin in red blood cells

(A) Results in healty group

Effect of GSPs or AL on bone formation markers in healthy rats

Application of GSPs or AL in healthy rats did not lead to any changes in the value of bone markers: OC, CTX, BMD, Ca and P compared to controls (Table 8 and 9). Weight, length and diameter of bone did not significantly differ in GSPs or AL group in relation to control (data not shown). There were no statistically significant differences in body weight between groups at the beginning of the experiment and at the end of the experiment (Figure 1).

Table 8 Effects of grape seed proanthocyanidins or alendronate on the bone ash content and levels of calcium and phosphorus in healthy rats

Parameters	Treatment[a]	Median	Min	Max	25. percentile	75. percentile	*P**
Bone ash /g	**Control**	0.64	0.57	0.69	0.58	0.68	-
	GSPs	0.57	0.52	0.58	0.52	0.58	0.229
	AL	0.58	0.56	0.61	0.56	0.60	0.200
mg Ca/g	**Control**	164.48	154.56	175.00	154.62	174.64	-
	GSPs	158.76	151.82	161.32	151.82	161.32	0.629
	AL	162.25	149.71	176.41	152.77	172.53	0.999
mg P/g	**Control**	75.76	70.06	80.92	71.78	79.48	-
	GSPs	73.43	70.99	75.77	70.99	75.77	0.629
	AL	77.98	71.64	85.42	73.62	82.89	0.686

[a] Rats were given GSPs (100 mg kg^{-1}) or AL (40 mg kg^{-1}) concomitant with 13cRA treatment (80 mg kg^{-1}) once daily for 14 days by a single intragastric application. Results are presented as medians with interquartile ranges. Number of rats per group: 7.
P * *vs* control; ** *P vs.* 13cRA
Legend: 13cRA -retinoic model of osteoporosis; GSPs - grape seed proanthocyanidins, AL- alendronate, Ca - calcium, P – phosphorus

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Table 9 Effects of grape seed proanthocyanidins or alendronate on the osteoporotic markers of bone formation and bone mineral density in healthy rats

Parameters	Treatment[a]	Median	Min	Max	25. percentile	75. percentile	*P**
OC	**Control**	254.00	220.00	274.00	220.00	274.00	-
	GSPs	255.70	234.80	288.50	234.80	288.50	0.700
	AL	121.85	86.80	156.90	86.80	156.90	0.200
CTX	**Control**	53.00	48.00	55.00	48.00	55.00	-
	GSPs	37.80	36.90	40.10	36.90	40.10	0.100
	AL	26.28	24.80	27.75	24.80	27.75	0.200
BMD-prox	**Control**	0.23	0.23	0.23	0.23	0.23	-
g/cm^2	**GSPs**	0.23	0.23	0.24	0.23	0.24	0.999
	AL	0.22	0.22	0.22	0.22	0.22	0.333
BMD-dist	**Control**	0.23	0.23	0.23	0.23	0.23	-
g/cm^2	**GSPs**	0.23	0.22	0.23	0.22	0.23	0.800
	AL	0.26	0.25	0.26	0.25	0.26	0.333

[a] Rats were given GSPs (100 mg kg^{-1}) or AL (40 mg kg^{-1}) concomitant with 13cRA treatment (80 mg kg^{-1}) once daily for 14 days by a single intragastric application. Results are presented as medians with interquartile ranges. Number of rats per group: 7.
P * *vs* control; ** *P vs* 13cRA
Legend: 13cRA -retinoic model of osteoporosis; GSPs - grape seed proanthocyanidins. AL-alendronate; BMD - bone mineral density; OC – osteocalcin; CTX -C-terminal fragment of type I collagen

Effect of GSPs on biochemical and haematological parameters in healthy rats
Neither the use of GSPs nor AL lead to significant changes in biochemical values (glucose, urea, creatinine, AST, ALT, AP) in healthy rats compared to controls (data not shown)

In GSPs treated group there was a significant increase in the value of L and in Plt (data not shown) in relation to control. AL has led to a significant reduction in Hgb and Hct as well as significant increase in platelets compared to the control. Other haematological parameters were not changed compared to the control group (data not shown).
In healthy animals GSPs or AL treatment did not cause a significant change in MDA, GSH, in liver and kidney compared to control (data not shown).

Discussion
In this paper, we explored the preventive effects of GSPs on 13cRA-induced osteoporosis in rats. 13cRA successfully induced osteoporosis in rats in our study; osteoporosis was confirmed by a reduced content of Ca and P in bones, bone ash contents and geometrical, physical parameters of bone, BMD and the serum biochemical markers of bone turnover (OC, CTX, AP) as well as by histopathological analysis.

Our results of histological analysis of femoral cortical bone revealed that 13cRA exerted pathological changes characterized by the presence of multiple osteoporotic cavities of different sizes and thinning of the bone cortex which is consistent with the significant decrease in femur bone mineral density and both Ca and P content (Figure 3,

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Tables 2 and 3). We also noticed increase in osteoclast number on the luminal surface of cavities and irregularly eroded endosteal surface, and this bone loss may be due to increased osteoclastic activity and consequently increase in bone resorption. Receptors for 13cRA are found on both bone cells, osteoblasts and osteoclasts, which indicates that they are direct targets of 13cRA [20, 21]. Previous *in vitro* studies have reported that 13cRA stimulates osteoclast formation and suppresses osteoblast activity [22, 23], but recently Peng et al. [24] showed that bone resorption exceeded bone formation due to the promotion effect of 13cRA on osteoclasts instead of inhibition to osteoblasts. This was supported by the phenomena that 13cRA-treated group showed an active proliferation of osteoblasts as the control and a markedly increase in osteoclast number and activity. Our results from the qualitative histological analysis also showed that 13cRA stimulates both bone cells. We found that resorption cavities were bounded with osteoid or newly formed bone showing that bone formation followed bone resorption. This also showed that 13cRA has a more pronounced effect on osteoclasts, which results in an unbalance between bone resorption and formation, and leads to a bone loss.

Similar histopathological changes in bone tissue of osteoporotic rats were reported by other authors [24-28]. In addition to directly affecting bone cells by suppressing the activity of osteoblasts and increasing the activity of osteoclasts, 13cRA also reduces the levels of estrogen activity which is accompanied by an increased differentiation and activation of osteoclasts and increased production of ROS. It seems that 13cRA induced osteoporotic model is similar to human in many ways, such as symptoms, histomorphology and estrogenic response [20].

Our study indicates that decreased glutathione level and increased MDA level in liver and kidney in 13cRA-treated rats is related to increased ROS production (Figure 2). We clearly demonstrated a link between oxidative stress and osteoporosis since MDA is the measure of osteoclastic activity and its production of FR. It has been further suggested that the generation of ROS appears as an early event that precedes intracellular GSH depletion and cell damage in 13cRA hepatotoxicity. 13cRA-induced hepatocellular damage marked by increased plasma AST, ALT, AP and LDH activities (Table 6). These features might be attributed to the metabolic activation of 13cRA, which is considered a major mechanism of its toxicity and a rapid loss of GSH and lipid peroxidation in both liver and kidney. It is possible that toxic effect of 13cRA in combination with ROS may lead to extensive bone loss, decreased BMD and skeletal fragility, characteristic of osteoporosis as suggested by our results or by results in paper [3, 11, 17, 28, 31].

Our study clearly demonstrates the beneficial effects of GSPs on the prevention of bone loss that is associated with 13cRA. Preventive application of the GSPs successfully inhibits oxidative stress induced by 13cRA as a risk factor for low bone density and osteoporosis. High concentrations of ROS may cause oxidative stress, leading to the damage of cellular macromolecules, which may interfere with various cellular functions including bone resorption by osteoclasts [2-10]. Recent studies suggest that ROS may be required as a signal mediator for osteoclast differentiation and that antioxidants could limit bone resorption *in vivo* [10, 12]. So, number of research papers [10-12, 32] suggested that drugs with high antioxidant activity could be used as therapeutic modalities for osteoporosis. GSPs have higher antioxidant activity than other well-known antioxidants such as vitamin C, vitamin E and gallic acid [33]. Furthermore, the polyphenols in GSPs have already shown beneficial antioxidant effects on other oxidative stress-associated diseases [34] such as bone invasion in arthritis by simultaneously suppressing osteoclast differentiation and promoting osteoblast differentiation.

Our study revealed that GSPs inhibited osteoclastogenesis induced by 13cRA treatment. Intragastric administration of GSPs concomitant with 13cRA resulted in reduction of OC in comparison to control group and to 13cRA group while CTX did not change (Table 1). GSPs treatments significantly increased the BMD in distal and proximal metaphysis of the femoral neck and increased Ca and P content ($P<0.025$; $P<0.025$) compared to 13cRA group, respectively (Tables 2 and 3). Weight, length and diameter of the bone (medio-lateral and anteriorior-posterior) were significantly higher in GSPs group compared to the control ($P<$ 0.05; 0.01; 0.05) and to the 13cRA group. In addition, GSPs concomitant with 13cRA resulted in significant reduction of AST, ALT, and AP in comparison to

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13cRA group (Table 6). Previous enzymatic studies demonstrated that GSPs inhibited proteolytic enzymes that have an essential role in the initial process of bone resorption [35, 36]; this seems to support the suppressive effects of GSPs in osteoclasts.

Besides the FR scavenging and antioxidant activity, GSPs exhibits tissue regeneration properties, wound healing characteristic, vasodilatory, anticarcinogenic, anti-allergic, anti-inflammatory, antibacterial, cardioprotective, immune-stimulating, anti-viral and estrogenic activities, and they are also good inhibitors of the enzymes phospholipase A2, cyclooxygenase and lipoxygenase [37]. In addition, it is possible that more different co-operative and synergistic mechanisms of GSPs play a role in protecting the whole organism against 13cRA induced oxidative stress and its connection with osteoporosis.

GSPs in healthy rats did not induce significant changes in measured parameters.
However, no matter what the plant material is very available, inexpensive and easy to apply in humans, the bioavailability of polyphenolic constituents in different species is different and it is difficult to achieve the desired concentration of GSPs with a diet rich in polyphenols to suit the concentrations applied to animals. However, the metabolic fate of GSPs is still elusive as there is conflicting evidence on the absorption and metabolism of the oligomeric and polymeric flavan-3-ols in humans and animals [38]. In this experiment, GSPs was administered at 100 mg of GSPs/kg of body weight. This dose, using a translation of animal to human doses [39] and estimating the daily intake for a 70-kg human, corresponds to the intake of 1120 mg of GSPs/day. This GSPs intake cannot be achieved in humans with a polyphenol-rich diet but the desired amount could be taken via a capsule or pill. Although experimental data obtained in rats cannot be directly translatable to humans but the data obtained suggests that the use of GSPs-rich foods may have been a good strategy for preventing diseases associated with OS including osteoporosis.

The effectiveness of the GSPs for the prevention of bone loss induced by 13cRA was compared with the effectiveness of AL. AL is an aminobisphosphonate that inhibits bone resorption in osteoporotic humans and rats. Several bisphosphonates, including AL, also have direct positive actions on osteoblasts, bone formation, and mineralization [40]. In our study the values of BMD in the proximal and in the distal metaphysis of the left femur in AL group were not significantly altered compared to control and 13cRA groups (Table 3). Furthermore, AL did not cause changes in the value of bone markers: osteocalcin (OC) and C-terminal fragment of type I collagen (CTX) in healthy rats compared to controls (Table 6). On the other hand, concomitant treatment of rats with 13cRA and AL had toxic effect to animals and resulted in a significant decrease in body mass after two weeks. About half of rats in this group died 2-5 days before the end of the experiment. AL overdose is known to be associated with digestive disorders, heartburn, esophagitis and diarrhoea. The latest research shows that the application of bisphosphonates over a period longer than 5 years may increase the risk of atypical femur fractures [41, 42]. In addition, Deng et al., [43] showed that AL within osteoclasts and other cells (macrophages and some tumor cells), inhibits farnesyl pyrophosphate (FPP) synthase in the cholesterol biosynthesis pathway, and that inhibition of this pathway is involved in the AL-induced inflammatory reactions.

In present study, treatment of healthy rats with AL did not induce a significant reductions of glutathione in kidney while the combined treatment with AL and 13cRA led to a significant increase of GSH which may be characteristic of toxic substances. In the liver, however, AL alone led to a reduction in levels of glutathione, while the combined treatment with AL and 13cRA resulted in a range of control results. The results of this experiment suggest that AL may have toxic effects in combination with 13cRA, especially in the kidneys where bisphosphonates often cause renal insufficiency [44]. According to this statement, GSPs as anti-inflammatory component could be a good substitute for AL, which induces a strong inflammatory effect [43] which can be a risk factor for the development of tumours in post-menopausal women [42].

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Conclusion

13cRA has successfully induced osteoporosis in rats in our study; osteoporosis is confirmed by a decrease in BMD, Ca and P contents, weight, length and diameter of femur and increase of bone markers OC and CTX. Increased OS in the liver and kidney could be a risk factor for osteoporosis induced by 3cRA. GSPs can reduce OS and increase BMD, Ca and P contents, weight, length and diameter of femur in 13cRA-induced bone loss in rats. In addition, GSPs has the potential to promote new bone formation. Further research is essential to understand the mechanism of this bone formation. These data suggest that natural compounds, such as GSPs, may provide an adequate and safe alternative or complimentary treatment to the traditional drugs for the treatment of bone destruction and osteoporosis in conditions such as increased level of ROS. Furthermore, this study contributes to a better understanding of the relationship between diet and health and development of the concept of functional foods with the fundamental aim of achieving better health status, prevention and treatment of disease.

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