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Effects of caffeic and chlorogenic acids on the rat skeletal system

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Abstract. – OBJECTIVE: Caffeic acid, predominantly as esters linked to quinic acid (chlorogenic acids), is a phenolic acid present at high levels in coffee. The aim of the study was to investigate effects of caffeic and chlorogenic acids on the skeletal system of female rats with normal estrogen levels and estrogen-deficient.

MATERIALS AND METHODS: Caffeic acid (5 and 50 mg/kg p.o. daily) and chlorogenic acid (100 mg/kg p.o. daily) were administered for 4 weeks to non-ovariectomized and bilaterally ovariectomized mature Wistar rats, and their effects were compared with appropriate controls. Moreover, estradiol (0.2 mg/kg p.o. daily) was administered to ovariectomized rats. Bone turnover markers, mass, mineralization and mechanical properties were examined.

RESULTS: Although caffeic acid at a low dose exerted some unfavorable effects on the skeletal system, at high doses, caffeic and chlorogenic acids slightly increased mineralization in the tibia and improved mechanical properties of the femoral diaphysis (compact bone). Unlike estradiol, they did not counteract the worsening of the tibial metaphysis bone strength (cancellous bone) and increases in osteocalcin concentration induced by estrogen deficiency.

CONCLUSIONS: High doses of the phenolic acids slightly favorably affected the rat skeletal system independently of the estrogen status.

Key Words:
Caffeic acid, Chlorogenic acid, Skeletal system, Osteoporosis, Rats.

Introduction

Hydroxycinnamic acids are natural phenolic acids, commonly present in foods of plant origin (fruits, vegetables, grains, coffee, spices, etc.)[1,2]. Some natural phenolic acids have been recently reported to affect the skeletal system in experimental conditions[3-11].

A major source of hydroxycinnamic acids is coffee; it contains mainly chlorogenic acids. The term "chlorogenic acids" includes a family of esters of *trans* hydroxycinnamic acids with quinic acid. The most commonly occurring chlorogenic acid is 5-*O*-caffeoylquinic acid, often called "chlorogenic acid"[12]. In an example of brewed coffee portion, approximately 86% of chlorogenic acids were caffeoylquinic acids (3-, 4- and 5), while the rest - feruoylquinic and dicaffeoylquinic acids[13]. The chlorogenic acid contents in various commercial coffees vary within wide limits[14]. A 200 ml serving of coffee provides 70-350 mg of chlorogenic acids, i.e. about 35-175 mg of caffeic acid[12]. Regular coffee consumers can easily have an intake of chlorogenic acids in excess of 1 g per day[15].

There are big differences in the amounts of hydroxycinnamic acids consumed by humans. Coffee and related products are their most significant and constant sources due to regular consumption in Western countries[2]. Some phenolic acids are available in dietary supplements and used in self-medication. Extracts of green coffee beans and extracts of whole coffee fruit are marketed as functional ingredients or health-supporting dietary supplements[16]. For example, supplements containing chlorogenic acid are proposed as body weight reducing agents[17]. Coffee drinking is considered a potential risk factor for bone fractures; however, the data on coffee and caffeine skeletal effects are inconsistent[12,18-25]. Phenolic acids may contribute to the coffee effects on bones.

We have previously observed that caffeic and chlorogenic acids at a moderate dose of 10

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mg/kg p.o. counteracted some skeletal changes caused by estrogen deficiency, but caffeic acid also unfavorably affected the skeletal system[9,10]. The aim of the present study was to further investigate effects of coffee phenolic acids (chlorogenic and caffeic) on the skeletal system of mature female rats with normal estrogen levels and estrogen-deficient.

Materials and Methods

Phenolic acids used: caffeic acid (purity ≥ 98%, Sigma-Aldrich Co., St. Louis, Mo, USA); chlorogenic acid (1,3,4,5-tetrahydroxycyclohexanecarboxylic acid 3-(3,4-dihydroxycinnamate), purity ≥ 95%, Sigma-Aldrich).

Other drugs used: estradiol hemihydrate (Estrofem, Novo Nordisk A/S, Bagsværd, Denmark), ketamine – Bioketan (Vetoquinol Biowet, Gorzów Wlkp., Poland), xylazine – Xylapan (Vetoquinol Biowet), tetracycline hydrochloride (Sigma-Aldrich), calcein (Sigma-Aldrich).

The study was carried out with the permission of the Local Ethics Commission in Katowice, on 15-17-week-old female Wistar rats obtained from the Center of Experimental Medicine, Medical University of Silesia in Katowice.

The animals were divided into 9 groups (n = 9-10). Caffeic acid (5 and 50 mg/kg p.o. daily) and chlorogenic acid (100 mg/kg p.o. daily) were administered for 4 weeks to non-ovariectomized and ovariectomized (estrogen-deficient) rats, and their effects were compared with appropriate controls. Moreover, estradiol (0.2 mg/kg p.o. daily; positive control) was administered to ovariectomized rats.

Bilateral ovariectomy was performed under ketamine-xylazine anesthesia, 7-8 days before the start of the administration of the investigated compounds. Control rats were administered the vehicle – tap water. The phenolic acids, estradiol or vehicle were administered by a gastric tube (2 ml/kg). The rats were fed a soy-free diet with decreased content of phenolic acids *ad libitum* (Table I), starting from the day before the beginning of the administration of the investigated compounds. To mark the calcification front, tetracycline hydrochloride (20 mg/kg) on the day before the start of the treatment, and calcein (10 mg/kg) after 3 weeks, were injected intraperitoneally.

The rats were fasted for 24 h after the last drug administration. The next day, the animals were killed by cardiac exsanguination, in full ketamine-xylazine anesthesia. The femurs, tibias, and L-4 vertebra were isolated, weighed and measured. The uterus and thymus were weighed.

Bone Mechanical Properties Studies

Mechanical properties of the femoral diaphysis, proximal tibial metaphysis and the femoral neck were investigated using Instron 3342 500N apparatus with Bluehill 2 version 2.14 software. The bones were stored below -20°C (wrapped in gauze soaked in 0.9% NaCl solution) and thawed before testing.

Mechanical properties of the left femoral diaphysis and left tibial metaphysis were studied us-

Table I. Composition of the soy-free diet with decreased content of phenolic acids.

Raw material components		Nutrient components	
Vitacel (cellulose)	10.0%	Energy	11.5 MJ/kg
Corn	8.0%	Crude protein	201.0 g/kg
Oat	15.0%	Crude fat	46.1 g/kg
Wheat	15.0%	Fiber	115.0 g/kg
Fodder wheat flour	30.5%	Starch	298.0 g/kg
Fish meal	6.0%	Calcium	9.3 g/kg
Fodder yeast	3.0%	Phosphorus	7.5 g/kg
Potato protein	5.0%		
Dried whey	2.0%		
Flax meal	2.0%		
$Ca(H_2PO_4)_2*H_2O$	0.5%		
Fodder chalk	2.0%		
Fodder salt (NaCl)	0.2%		
Mix of microelements and vitamins	0.8%		

The composition of the diet was designed in cooperation with the producer of the fodder, Wytwórnia Pasz "Morawski", Poland. The rats were switched from the standard laboratory diet (Labofeed B) to the experimental diet on the day before the beginning of the phenolic acid or estradiol administration.

ing three-point bending tests, as previously described[25-30]. The load was applied perpendicularly to the long axis of the femur in the mid-length of the bone (distance between the points supporting the femur: 16 mm) or to the proximal metaphysis of the tibia deprived of the proximal epiphysis. The displacement rate was 0.01 mm/s. Maximum load and displacement, energy and stress for the maximum load were assessed. The same parameters were determined for the yield point (0.05% offset) and fracture point. Young's modulus was also determined.

Mechanical properties of the neck of the right femur were investigated using a compression test[28-30]. The load was applied to the head of the femur along the long axis of the femur with a displacement rate of 0.01 mm/s, and the maximum load was measured.

Bone Histomorphometric Studies

To prepare histological specimens, the right femurs were used, as previously described[25,29,31]. Histomorphometric measurements were made using Lucia G 4.51 software (Laboratory Imaging, Praha, Czech Republic) or OsteoMeasure XP v1.3.0.1 (OsteoMetrics, Inc., Decatur, GA, USA) software. In the longitudinal preparation from the femur, the width of trabeculae in the distal epiphysis and metaphysis were measured. In transverse cross-sections of the femoral diaphysis in the mid-length of the femur, the area of the cortical bone and marrow cavity were determined. The periosteal and endosteal transverse growth of the femur were also measured.

Bone Mineralization Studies

The left femur, left tibia and L-4 vertebra were lyophilized for 9 days to determine the dehydrated bone mass, and mineralized (640°C for 48 h in the muffle furnace) to determine the mass of bone mineral (ash). The ratios of mass of bone mineral to bone mass and to lyophilized bone mass were determined as substitutes for bone mineral density measurements. Calcium and phosphorus content in the bone mineral (dissolved in 6 M HCl and then diluted in distilled water) was determined spectrophotometrically, using a calcium reagent and a phosphorus reagent sets (Pointe Scientific, Inc., Canton, MI, USA).

Biochemical Studies

Serum estradiol levels were determined by an ELISA method (Mouse/Rat Estradiol ELISA, Calbiotech, Inc., Spring Valley, CA, USA). Serum concentrations of osteocalcin and type I collagen fragments released from bone during bone resorption were studied using enzyme immunoassay methods (Rat-MID Osteocalcin EIA and RatLaps EIA, respectively, Immunodiagnostic Systems Ltd., Boldon, Tyne and Wear, UK). Serum calcium, inorganic phosphorus and total cholesterol levels were determined spectrophotometrically (Pointe Scientific, Inc.).

Statistical Analysis

The results are presented as arithmetical means ± SEM. Statistical evaluation was performed using one-way ANOVA, followed by Duncan's *post-hoc* test. In case of a lack of normality (Shapiro-Wilk's test) or of homogeneity of variance (Levene's test), non-parametric tests were used: Kruskal-Wallis ANOVA and Mann-Whitney U test. The results obtained in each experimental group were compared with those of the non-ovariectomized control rats, and results of the treated ovariectomized rats were compared with those of the ovariectomized control rats. Moreover, two-way ANOVA performed on 6 groups (non-ovariectomized and ovariectomized control rats, and rats treated with the higher doses of phenolic acids) was used. The main effects were: estrogen status (effect of ovariectomy) and the treatment with the phenolic acids.

Results

Effects of Phenolic Acid Administration on Body Mass Gain, Mass of Estrogen-Dependent Organs and Serum Levels of Estradiol and Total Cholesterol

The ovariectomized control rats had significantly increased body mass gain and serum total cholesterol level in comparison with the non-ovariectomized control rats (Table II). Serum estradiol strongly tended to decrease, and the mass of estrogen-dependent organs, uterus and thymus, was significantly decreased and increased, respectively. Caffeic and chlorogenic acids did not affect those parameters, both in non-ovariectomized and ovariectomized rats. Estradiol counteracted the changes induced by estradiol deficiency, with the exception of total cholesterol level.

Effects of Phenolic Acid Administration on Bone Mass and Mineralization

Bone mass and mass of bone mineral were not significantly affected in the estrogen-deficient

Table II. Effects of caffeic acid (CA) and chlorogenic acid (CGA) on the body mass gain, mass of estrogen-dependent organs and serum levels of estradiol and total cholesterol in non-ovariectomized (NOVX) and ovariectomized (OVX) rats.

	NOVX				OVX					Two-way ANOVA#		
										Main effects		Interaction
Parameters	Control	CA5	CA50	CGA100	Control	CA5	CA50	CGA100	Estradiol	ES	PA	ES x PA
Body mass at the start of drug administration [g]	215.5±3.3	216.6±3.3	215.8±3.9	218.3±2.7	224.0±3.1	222.3±2.7	223.7±2.8	224.5±2.7	224.7±2.6	$p < 0.01$	NS	NS
Body mass gain after 4 weeks [g]	13.0±2.4	16.2±2.6	14.0±1.8	14.8±1.9	$39.2±2.4^{***}$	$39.1±3.2^{***}$	$37.6±3.5^{***}$	$38.5 ± 3.3^{***}$	$26.5±3.0^{**§§}$	$p < 0.001$	NS	NS
Total cholesterol [mg/100 ml]	49.5±3.7	53.3±3.2	48.3±4.9	40.6±3.7	$65.7±2.9^{**}$	$67.9±4.5^{**}$	$67.8±3.6^{**}$	$64.8 ± 3.7^{*}$	$62.5±4.9^{*}$	$p < 0.001$	NS	NS
Estradiol [pg/ml]	22.13±7.36	26.84±6.73	28.96±8.00	15.60±1.92	10.68±0.78	$10.29±0.75^{*}$	$8.14±0.79^{**}$	12.25±2.16	12.58±1.68	$p < 0.01$	NS	NS
Uterus mass [g]	0.587±0.080	0.596±0.054	0.589±0.110	0.471±0.034	$0.093±0.004^{***}$	$0.097±0.007^{***}$	$0.096±0.003^{***}$	$0.088±0.006^{***}$	$0.164±0.015^{***§§§}$	$p < 0.001$	NS	NS
Thymus mass [g]	0.285±0.010	0.329±0.021	0.297±0.018	0.303±0.015	$0.515±0.019^{***}$	$0.532±0.050^{***}$	$0.482±0.015^{***}$	$0.549±0.053^{***}$	$0.446±0.035^{***§}$	$p < 0.001$	NS	NS

Caffeic acid at doses of 5 mg/kg (CA5) and 50 mg/kg (CA50), chlorogenic acid at a dose of 100 mg/kg (CGA100) or estradiol (0.2 mg/kg), were administered orally to rats once daily for 4 weeks. Results are presented as the mean ± SEM (n=9–10). One-way ANOVA followed by Duncan's test, or, when appropriate, Kruskal-Wallis ANOVA followed by Mann-Whitney U test were used for evaluation of the significance of the results. $^{*}p \le 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ – significantly different from NOVX control rats. $^{§}p \le 0.05$, $^{§§}p < 0.01$, $^{§§§}p < 0.001$ – significantly different from OVX control rats. #Two-way ANOVA was conducted on 6 groups of rats: NOVX and OVX control groups, and groups treated with CA50 and CGA100. The evaluated main effects were: estrogen status (ES) and the treatment with high dose phenolic acids (PA). NS – not significant in two-way ANOVA

control rats in comparison with the non-ovariectomized controls, and the tibia and femur were significantly longer (not shown). The ratios of bone mineral mass to bone mass and to bone mass after lyophilization were decreased in the long bones, and calcium and phosphorus contents in the bone mineral were not significantly affected (Table III). Administration of caffeic and chlorogenic acids did not affect bone mass and bone mineral mass. A decrease in the calcium content in the mineral of the tibia in non-ovariectomized rats receiving the lower dose of caffeic acid was noted in relation to the non-ovariectomized controls. However, at the higher dose, caffeic acid significantly increased bone mineralization (ratio of bone mineral mass to bone mass) in the tibia of non-ovariectomized rats. There were significant main effects of the high-dose phenolic acids concerning mineralization of the tibia (increased ratios of bone mineral mass to bone mass and to lyophilized bone mass), indicating effects independent of the estrogen status. There was also a significant increase in the calcium content in the mineral of the tibia in ovariectomized rats treated with the high dose of caffeic acid, and a significant phenolic acid main effect on the calcium content in the mineral of the femur (increasing).

Effects of Phenolic Acid Administration on Mechanical Properties of the Tibial Metaphysis

Estrogen deficiency strongly worsened mechanical properties of cancellous bone of the tibial metaphysis (Young's modulus, the load and stress for the maximum load and fracture points, and the energy for the fracture point, Table IV). Supplementation with estradiol partially counteracted the changes. Administration of the phenolic acids in high doses did not significantly affect the mechanical properties of cancellous bone. There were only tendencies for main effects of the high-dose acids, indicating slight increases in the maximum load and stress ($p < 0.1$), for which mainly the effects in non-ovariectomized rats were responsible. However, in non-ovariectomized rats receiving the low dose of caffeic acid, significant decreases in the yield point parameters (load and energy) were noted (not shown).

Table III. Effects of caffeic acid (CA) and chlorogenic acid (CGA) on bone mineralization in non-ovariectomized (NOVX) and ovariectomized (OVX) rats.

		NOVX				OVX					Two-way ANOVA#		
											Main effects		Interaction
Parameters		Control	CA5	CA50	CGA100	Control	CA5	CA50	CGA100	Estradiol	ES	PA	ES x PA
Bone mineral mass/bone mass ratio	Tibia	0.466±0.004	0.463±0.005	0.479±0.004*	0.473±0.004	0.450±0.004*	0.449±0.004*	0.452±0.003*	0.454±0.002	0.457±0.007	$p < 0.001$	$p < 0.05$	NS
	Femur	0.452±0.003	0.446±0.003	0.463±0.007	0.459±0.003	0.439±0.004	0.432±0.005**	0.431±0.003**	0.435±0.004*	0.445±0.004	$p < 0.001$	NS	$p < 0.05$
	L-4 vertebra	0.432±0.005	0.421±0.008	0.430±0.013	0.434±0.008	0.424±0.007	0.419±0.006	0.418±0.005	0.423±0.007	0.428±0.006	NS	NS	NS
Bone mineral mass/ liophylized bone mass ratio	Tibia	0.640±0.003	0.638±0.004	0.645±0.002	0.643±0.002	0.630±0.002**	0.627±0.003**	0.632±0.002*	0.634±0.002*	0.627±0.008	$p < 0.001$	$p < 0.05$	NS
	Femur	0.649±0.001	0.648±0.002	0.652±0.002	0.650±0.002	0.642±0.002*	0.637±0.004**	0.638±0.002***	0.644±0.002*	0.645±0.003	$p < 0.001$	NS	NS
	L-4 vertebra	0.624±0.003	0.610±0.008	0.622±0.005	0.621±0.004	0.616±0.005	0.611±0.005	0.613±0.003	0.616±0.003	0.621±0.004	$p < 0.05$	NS	NS
Calcium content [g/g of bone mineral]	Tibia	0.430±0.009	0.409±0.006*	0.444±0.011	0.421±0.012	0.411±0.010	0.398±0.005**	0.455±0.015§	0.415±0.009	0.416±0.008	NS	NS	NS
	Femur	0.376±0.005	0.388±0.004	0.392±0.005	0.390±0.005	0.384±0.005	0.391±0.006	0.388±0.003	0.390±0.005	0.387±0.003	NS	$p < 0.05$	NS
	L-4 vertebra	0.418±0.008	0.409±0.009	0.419±0.008	0.421±0.011	0.411±0.005	0.414±0.008	0.419±0.008	0.423±0.008	0.428±0.012	NS	NS	NS
Phosphorus content [g/g of bone mineral]	Tibia	0.160±0.003	0.154±0.002	0.163±0.003	0.154±0.003	0.156±0.003	0.150±0.001	0.165±0.005	0.158±0.004	0.155±0.002	NS	NS	NS
	Femur	0.163±0.002	0.162±0.001	0.162±0.002	0.163±0.002	0.162±0.001	0.163±0.002	0.164±0.002	0.163±0.002	0.160±0.002	NS	NS	NS
	L-4 vertebra	0.168±0.002	0.162±0.004	0.165±0.003	0.165±0.003	0.163±0.002	0.164±0.003	0.164±0.002	0.168±0.003	0.168±0.003	NS	NS	NS

Caffeic acid at doses of 5 mg/kg (CA5) and 50 mg/kg (CA50), chlorogenic acid at a dose of 100 mg/kg (CGA100) or estradiol (0.2 mg/kg), were administered orally to rats once daily for 4 weeks. Results are presented as the mean ± SEM (n=9–10). One-way ANOVA followed by Duncan's test, or, when appropriate, Kruskal-Wallis ANOVA followed by Mann-Whitney U test were used for evaluation of the significance of the results. *$p \leq 0.05$, **$p < 0.01$, ***$p < 0.001$ – significantly different from NOVX control rats. §$p \leq 0.05$ – significantly different from OVX control rats. #Two-way ANOVA was conducted on 6 groups of rats: NOVX and OVX control groups, and groups treated with CA50 and CGA100. The evaluated main effects were: estrogen status (ES) and the treatment with high dose phenolic acids (PA). NS – not significant in two-way ANOVA

Table IV. Effects of caffeic acid (CA) and chlorogenic acid (CGA) on mechanical properties of the tibial metaphysis in non-ovariectomized (NOVX) and ovariectomized (OVX) rats.

	NOVX				OVX					Two-way ANOVA#		
										Main effects		Interaction
Parameters	Control	CA5	CA50	CGA100	Control	CA5	CA50	CGA100	Estradiol	ES	PA	ES x PA
Maximum load [N]	112.6±8.2	111.6±6.7	126.0±5.9	124.3±4.8	66.8±3.6***	69.9±3.3***	68.0±3.5***	71.8±1.6***	87.9±3.7**§§§	$p < 0.001$	NS	NS
Displacement for maximum load [mm]	0.860±0.060	0.826±0.046	0.888±0.045	0.839±0.036	0.906±0.066	0.895±0.043	0.868±0.053	0.964±0.051	0.808±0.048	NS	NS	NS
Energy for maximum load [mJ]	51.80±5.10	51.10±5.56	58.65±4.10	53.27±4.79	38.64±4.16	37.52±2.05	35.71±2.92*	42.61±2.78	38.25±3.10	$p < 0.001$	NS	NS
Maximum stress [MPa]	92.0±6.9	97.1±8.2	102.4±4.6	103.7±5.2	53.0±4.3***	56.4±3.9***	53.7±3.7***	58.3±3.6***	71.2±4.4**§	$p < 0.001$	NS	NS
Fracture load [N]	80.2±6.8	80.5±5.5	91.8±7.8	89.6±5.1	46.9±2.7***	47.6±2.9***	46.7±1.9***	48.7±2.1**	60.9±3.7*§§	$p < 0.001$	NS	NS
Displacement for fracture load [mm]	1.258±0.106	1.167±0.048	1.131±0.039	1.181±0.041	1.437±0.069	1.498±0.061*	1.402±0.058	1.441±0.091	1.239±0.068	$p < 0.001$	NS	NS
Energy for fracture load [mJ]	87.81±7.71	82.67±6.34	85.38±4.35	89.10±5.12	68.72±4.48*	72.89±3.39*	66.40±3.33**	71.06±4.53*	68.54±4.75*	$p < 0.001$	NS	NS
Stress for fracture load [MPa]	66.9±7.9	69.6±5.5	74.5±5.8	74.6±4.7	36.8±2.4***	38.4±3.0**	36.7±2.0***	39.6±2.9**	49.5±3.9§	$p < 0.001$	NS	NS
Young's modulus [MPa]	3052±201	3523±314	2886±215	3063±171	2218±191*	2275±209**	2239±152*	2326±244	2684±271	$p < 0.001$	NS	NS

Caffeic acid at doses of 5 mg/kg (CA5) and 50 mg/kg (CA50), chlorogenic acid at a dose of 100 mg/kg (CGA100) or estradiol (0.2 mg/kg), were administered orally to rats once daily for 4 weeks. Results are presented as the mean ± SEM (n=9-10). One-way ANOVA followed by Duncan's test, or, when appropriate, Kruskal-Wallis ANOVA followed by Mann-Whitney U test were used for evaluation of the significance of the results. $^{*}p \leq 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ – significantly different from NOVX control rats. $^{\S}p \leq 0.05$, $^{\S\S}p < 0.01$, $^{\S\S\S}p < 0.001$ – significantly different from OVX control rats. $^{\#}$Two-way ANOVA was conducted on 6 groups of rats: NOVX and OVX control groups, and groups treated with CA50 and CGA100. The evaluated main effects were: estrogen status (ES) and the treatment with high dose phenolic acids (PA). NS – not significant in two-way ANOVA.

Effects of Phenolic Acid Administration on Mechanical Properties of the Femur

Estrogen deficiency did not significantly affect mechanical properties of compact bone of the femoral diaphysis, and the femoral neck strength (Table V). The treatments did not significantly affect the mechanical parameters of the femur in relation to appropriate controls. However, two-way ANOVA demonstrated significant main effects concerning the values of load registered in the yield point (not shown), the maximum load point and the fracture point, indicating increases in the strength of the femoral diaphysis induced by administration of the high-dose phenolic acids. Also the stress for the maximum load and fracture points, and energy accumulated to the fracture point tended to increase ($p \leq 0.1$ for phenolic acid main effects).

Effects of Phenolic Acid Administration on Histomorphometric Parameters of the Femur

In the ovariectomized control rats, the width of trabeculae in the femoral epiphysis and metaphysis decreased, and the periosteal and endosteal transverse growth of the femoral diaphysis increased in comparison with the non-ovariectomized control rats (Table VI). Those effects were counteracted by administration of estradiol. There were no effects of estrogen deficiency on the transverse cross-section areas of the marrow cavity and the cortical bone (not shown). Caffeic and chlorogenic acids did not affect the histomorphometric parameters in non-ovariectomized rats. However, in ovariectomized rats, caffeic acid at the high dose and chlorogenic acid counteracted the increase in the endosteal transverse growth, and the acids counteracted the decreases in the trabeculae width induced by estrogen deficiency.

Effects of Phenolic Acid Administration on Serum Biochemical Bone Turnover Markers

In the ovariectomized control rats, serum bone resorption marker, RatLaps, and bone formation marker, osteocalcin, markedly increased in relation to the non-ovariectomized control rats (Table VII). The latter effect was counteracted by estradiol administration. Administration of the phenolic acids did not affect the levels of bone turnover markers. No effects on serum calcium and inorganic phosphorus levels were noted (not shown).

Discussion

In recent years, several experimental reports indicating beneficial effects of some phenolic acids on the skeletal system have been published[4-11]. Results of our screening studies were inconclusive: while phenolic acids (ferulic, p-coumaric, caffeic, and chlorogenic; 10 mg/kg p.o. daily) counteracted some changes in the skeletal system developing due to estrogen deficiency, caffeic acid decreased bone mass[9], and worsened mechanical properties of the femur in non-ovariectomized rats[10]. Moreover, caffeic acid raised the serum estradiol level in estrogen-deficient rats[32].

To elucidate the effects of coffee phenolic acids on the rat skeletal system, higher oral doses were used in the present study: 100 mg/kg/day of chlorogenic acid and 50 mg/kg/day of caffeic acid, for 4 weeks, a period long enough to observe skeletal effects in rats[9,10,28-31]. The caffeic acid dose of 50 mg/kg corresponded to the amount of caffeic acid received by the rats administered 100 mg/kg dose of chlorogenic acid.

The experiments were carried out in two models: rats with normal estrogen levels and estrogen-deficient (bilaterally ovariectomized) rats. Estrogen deficiency induces the increased bone remodeling rate, with the balance between bone resorption and formation moved in favor of the former[35]. Consistently, in the ovariectomized control rats, serum markers of bone formation and bone resorption were increased, and bone mineralization and cancellous bone strength were decreased. Mechanical properties of compact bone were not significantly affected, consistently with our previous studies[9,29,30,36]. Supplementation with estradiol counteracted the effects of estrogen deficiency.

Results of the present study with the use of high doses of the phenolic acids did not confirm the unfavorable effects on the skeletal system from our previous studies[9,10]. However, after administration of caffeic acid at the low dose (5 mg/kg p.o. daily) to non-ovariectomized rats, some unfavorable effects were demonstrated: worsening of mechanical properties of cancellous bone of the tibial metaphysis in the yield point and decrease of the calcium content in the tibia mineral. Most of other investigated skeletal parameters were not affected by the low-dose caffeic acid, and both acids at the high doses slightly improved some of them. In estrogen-deficient rats, they counteracted the changes in bone histomor-

Table V. Effects of caffeic acid (CA) and chlorogenic acid (CGA) on mechanical properties of the femoral diaphysis and neck in non-ovariectomized (NOVX) and ovariectomized (OVX) rats.

	NOVX				OVX					Two-way ANOVA#		
										Main effects		Interaction
Parameters	Control	CA5	CA50	CGA100	Control	CA5	CA50	CGA100	Estradiol	ES	PA	ES x PA
Maximum load [N]	112.1±4.7	116.3±3.1	121.7±6.1	119.3±3.3	116.0±4.0	125.1±3.7	120.1±4.7	127.6±3.2	121.1±3.6	NS	$p < 0.05$	NS
Displacement for maximum load [mm]	0.482±0.013	0.516±0.031	0.508±0.025	0.517±0.011	0.532±0.024	0.516±0.023	0.483±0.016	0.541±0.017	0.516±0.022	NS	NS	NS
Energy for maximum load [mJ]	31.87±1.51	36.50±3.38	36.42±3.14	36.14±1.96	36.77±3.06	38.72±2.96	34.41±2.39	41.53±2.62	37.75±2.78	NS	NS	NS
Maximum stress [MPa]	173.5±5.7	185.1±5.3	179.1±5.3	187.4±8.9	174.6±6.7	186.9±2.4	178.7±5.7	189.2±5.7	189.3±6.7	NS	NS	NS
Fracture load [N]	111.3±4.7	115.5±3.2	120.6±6.3	118.8±3.4	115.5±4.1	123.8±3.3	119.7±4.8	126.1±3.1	120.3±3.4	NS	$p < 0.05$	NS
Displacement for fracture load [mm]	0.486±0.013	0.524±0.032	0.537±0.025	0.529±0.012	0.538±0.025	0.531±0.024	0.489±0.016	0.558±0.025	0.531±0.024	NS	NS	NS
Energy for fracture load [mJ]	32.38±1.55	37.38±3.57	39.78±3.12	37.56±2.09	37.50±3.12	40.74±3.26	35.05±2.33	43.81±3.66	39.71±3.31	NS	NS	NS
Stress for fracture load [MPa]	172.3±5.5	183.9±5.3	177.4±5.5	186.6±8.9	173.8±6.6	185.0±2.4	178.1±5.8	186.9±5.5	188.2±6.4	NS	NS	NS
Young's modulus [MPa]	9845±645	9868±551	8657±380	9847±750	8665±617	9555±450	9559±450	9414±444	9960±646	NS	NS	NS
Maximum load of the femoral neck [N]	76.1±3.8	73.4±5.3	85.6±3.8	68.0±4.8	69.5±3.2	75.3±3.8	66.6±4.6	76.1±4.5	75.0±4.7	NS	NS	NS

Caffeic acid at doses of 5 mg/kg (CA5) and 50 mg/kg (CA50), chlorogenic acid at a dose of 100 mg/kg (CGA100) or estradiol (0.2 mg/kg), were administered orally to rats once daily for 4 weeks. Results are presented as the mean ± SEM (n=9-10). One-way ANOVA followed by Duncan's test, or, when appropriate, Kruskal-Wallis ANOVA followed by Mann-Whitney U test were used for evaluation of the significance of the results. #Two-way ANOVA was conducted on 6 groups of rats: NOVX and OVX control groups, and groups treated with CA50 and CGA100. The evaluated main effects were: estrogen status (ES) and the treatment with high dose phenolic acids (PA). NS – not significant in two-way ANOVA.

Table VI. Effects of caffeic acid (CA) and chlorogenic acid (CGA) on bone histomorphometric parameters in non-ovariectomized (NOVX) and ovariectomized (OVX) rats.

Parameters		NOVX				OVX				Two-way ANOVA#		
		Control	CA5	CA50	CGA100	Control	CA5	CA50	CGA100	Estradiol	Main effects ES	Main effects PA	Interaction ES x PA
Transverse growth of the femur [μm]	Periosteal	28.86±2.03	30.05±1.55	31.13±3.14	30.59±1.48	41.31±1.99***	41.74±2.09***	39.62±2.50***	39.27±1.76***	33.85±0.62§	$p < 0.001$	NS	NS
	Endosteal	25.43±1.70	25.58±1.28	27.34±0.85	25.33±1.04	31.24±0.52**	29.73±1.67*	26.27±0.90§	26.47±1.00§	24.66±1.24§§	$p < 0.01$	$p < 0.05$	$p < 0.01$
Width of trabeculae in the femur [μm]	Epiphysis	60.08±0.92	58.83±1.18	58.84±1.10	57.23±1.26	53.41±0.82***	56.12±0.95*	56.07±0.67*	59.51±1.32§§§	60.18±0.79§§§	$p < 0.001$	NS	$p < 0.01$
	Metaphysis	37.12±0.61	36.83±0.71	37.77±0.56	36.66±0.42	34.52±0.38**	37.01±0.47§§§	36.33±0.80	37.75±0.84§§§	36.57±0.54§§	$p < 0.05$	$p < 0.05$	$p < 0.05$
Width of epiphyseal cartilage in the femur[μm]		59.23±1.47	57.40±1.73	58.99±2.20	57.59±1.39	55.92±1.19	59.63±1.84	58.64±1.60	57.77±1.21	59.52±1.78	NS	NS	NS

Caffeic acid at doses of 5 mg/kg (CA5) and 50 mg/kg (CA50), chlorogenic acid at a dose of 100 mg/kg (CGA100) or estradiol (0.2 mg/kg), were administered orally to rats once daily for 4 weeks. Results are presented as the mean ± SEM (n=9-10). One-way ANOVA followed by Duncan's test, or, when appropriate, Kruskal-Wallis ANOVA followed by Mann-Whitney U test were used for evaluation of the significance of the results. $^{*}p \leq 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ – significantly different from NOVX control rats. $^{§}p \leq 0.05$, $^{§§}p < 0.01$, $^{§§§}p < 0.001$ – significantly different from OVX control rats. #Two-way ANOVA was conducted on 6 groups of rats: NOVX and OVX control groups, and groups treated with CA50 and CGA100. The evaluated main effects were: estrogen status (ES) and the treatment with high dose phenolic acids (PA). NS – not significant in two-way ANOVA.

phometric parameters, however, the effect was not strong enough to improve mechanical properties of the tibial metaphysis.

Phenolic acids in the body are hydrolyzed by bacteria in the intestines or absorbed in the unchanged form, and then metabolized in the liver. It is still unresolved how much absorption occurs of intact chlorogenic acids[37]. In blood they are present in the form of various conjugates[2,15]. In this regard, there may be some species differences between humans and rats. In human serum considerable amounts of various chlorogenic acids have been reported[13], whereas chlorogenic acids in the unchanged form are poorly absorbed in rats[38]. It has been proposed that some effects of chlorogenic acid can be attributed to its metabolite: caffeic acid[39,40]. In fact, in this study high doses of the phenolic acids tended to exert similar favorable effects on the rat skeletal system. The results were subjected to two-way ANOVA statistical analysis, in which the main effect of the high-dose caffeic and chlorogenic acids combined was evaluated. Statistically significant main effects of the phenolic acids combined (and lack of interaction between main effects) indicate their potential to affect the parameters regardless of estrogen level.

With the above mentioned approach, we observed significant main effects of the high-dose phenolic acids concerning increases in bone mineralization and the femoral diaphysis strength. There was a significant main effect concerning increases in the width of metaphyseal trabeculae (for which the effect in the ovariectomized rats was responsible) and only a tendency to increase strength of the tibial metaphysis. Taken together, the phenolic acids at high doses favorably affected both compact and cancellous bone, independently of the estrogen status.

It may be speculated that the differences in the effects of caffeic acid between the present and our previous studies may result not only from different doses used, but also from the different laboratory diets. To avoid interference of the dietary phenolic acids and soy phytoestrogens, rats were fed a soy-free diet with decreased content of phenolic acids during this experiment. Although standard laboratory diet should not contain meaningful amounts of chlorogenic and caffeic acids, which are present mainly in coffee and some fruits, larger amounts of ferulic and p-coumaric acids may be present, since they are found in cereal cell wall[2,33]. Standard rodent laboratory diet usually contains soy, which may be responsible

Table VII. Effects of caffeic acid (CA) and chlorogenic acid (CGA) on the serum bone turnover markers in non-ovariectomized (NOVX) and ovariectomized (OVX) rats.

Parameters	NOVX				OVX					Two-way ANOVA#		
										Main effects		Interaction
	Control	CA5	CA50	CGA100	Control	CA5	CA50	CGA100	Estradiol	ES	PA	ES x PA
Osteocalcin [ng/ml]	204.0±15.1	206.5±18.1	234.2±22.6	229.7±29.1	345.4±28.4**	323.6±22.9**	326.9±26.0***	317.7±20.2**	245.4±20.3§	$p < 0.001$	NS	NS
RatLaps [ng/ml]	15.67±1.95	15.28±0.90	17.35±1.22	16.21±1.06	30.23±1.45***	31.76±3.19***	28.10±1.57**	28.25±1.64***	25.75±3.41*	$p < 0.001$	NS	NS

Caffeic acid at doses of 5 mg/kg (CA5) and 50 mg/kg (CA50), chlorogenic acid at a dose of 100 mg/kg (CGA100) or estradiol (0.2 mg/kg), were administered orally to rats once daily for 4 weeks. Results are presented as the mean ± SEM (n=9-10). Kruskal-Wallis ANOVA followed by Mann-Whitney U test were used for evaluation of the significance of the results. $^{*}p \leq 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ – significantly different from NOVX control rats. $^{\S}p \leq 0.05$ – significantly different from OVX control rats. #Two-way ANOVA was conducted on 6 groups of rats: NOVX and OVX control groups, and groups treated with CA50 and CGA100. The evaluated main effects were: estrogen status (ES) and the treatment with high dose phenolic acids (PA). NS – not significant in two-way ANOVA.

for high exposure to phytoestrogens (soy isoflavones)[34]. Food matrix (i.e. presence of positive and negative effectors of absorption, like fat or fiber) and possibility of interaction with other dietary compounds may significantly affect the bioavailability of phenolic compounds[41]. Previously, we also observed that caffeic acid decreased body mass gain and total cholesterol level in ovariectomized rats, which was accompanied by an increase in the serum estradiol level[9,32]. Consistently, in the present work, administration of estradiol decreased the body mass gain, increased due to estrogen deficiency, but no effects of the phenolic acids on the body mass gain, total cholesterol level and estradiol level were demonstrated. Relatively high content of soy isoflavones in the standard diet may lead to decreases in serum estradiol levels[42], since phytoestrogens may decrease activity of enzymes taking part in steroidogenesis[43] and affect gonadotropin secretion[44]. It is possible that the caffeic acid effect on the estradiol levels and body mass gain in our previous study resulted from the interaction between them and soy isoflavones, and could not be observed in the absence of the phytoestrogens.

Mechanism of action of the phenolic acids on the skeletal system may be only speculated. They targeted bone locations different from those of estradiol, indicating that their effect was not estrogenic one. Recently, it has been reported that dietary-induced serum phenolic acids from blueberries promote bone growth *via* p38 MAPK/β-catenin canonical Wnt signaling[11]. However we did not observe increases in bone formation. The mechanism of action of the phenolic acids may be connected with their antioxidant activity, characteristic of polyphenolic compounds. Oxidative stress may take part in the development of osteoporosis, and loss of estrogen leads to the decrease of the defense against oxidative stress in bone, which in turn contributes to the increased bone resorption associated with estrogen deficiency[35].

Phenolic acids probably act on the rat skeletal system through different mechanisms, their effect being a resultant of action on diverse cellular targets. That would explain differential effects of low doses (potentially unfavorable) and high doses (rather favorable) observed in the present study.

It should be noted that the high doses of the phenolic acids used in our research seem to be comparable with the intake achievable by coffee drinkers. It has been estimated that a heavy coffee drinker may ingest up to 2000 mg of chlorogenic acids (and about 250-500 mg of caffeic acid) dai-

ly, whereas a non-coffee drinker – less than 25 mg per day[2]. Although the high doses we used were about 3 times bigger than those taken by heavy coffee drinkers (assuming that human body mass is 65-70 kg), in rat studies, due to the faster metabolism, a conversion factor of 10 in relation to human doses is often used.

Results of this work should be taken with caution. High intake of chlorogenic or caffeic acids may not exert similar effects on the skeletal system in humans. Although the phenolic acids rather beneficially affected the rat skeletal system in the present study, we have previously demonstrated unfavorable effect of caffeic acid on bone strength in rats fed a different laboratory diet[10]. Moreover, despite the findings of epidemiological reports indicating that high dietary intake of polyphenols is associated with decreased risk of many diseases, there is growing concern that dietary polyphenol antioxidants may act as a double edge sword, eliciting adverse effects through pro-oxidative action in higher doses[45].

Conclusions

High doses of the phenolic acids slightly favorably affected the rat skeletal system independently of the estrogen status.

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Conflict of Interest

The Authors declare that they have no conflict of interests..

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