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Regular paper

Effect of diosgenin, a steroidal sapogenin, on the rat skeletal system

Joanna Folwarczna[1], Maria Zych[2], Barbara Nowińska[1], Maria Pytlik[1], Magdalena Bialik[1], Anna Jagusiak[1], Maria Lipecka-Karcz[1] and Michał Matysiak[1]

[1]Department of Pharmacology, School of Pharmacy with the Division of Laboratory Medicine, Medical University of Silesia, Katowice, Poland; [2]Department of Pharmacognosy and Phytochemistry, School of Pharmacy with the Division of Laboratory Medicine, Medical University of Silesia, Katowice, Poland

Diosgenin is a steroidal sapogenin present in fenugreek and *Dioscorea* spp. as glycosides (saponins). Diosgenin has already been reported to inhibit osteoclastogenesis and to stimulate osteogenic activity of osteoblastic cells *in vitro*, and to exert some antiosteoporotic effects in rats *in vivo*. The aim of the present study was to investigate the effects of diosgenin administration on the skeletal system of rats with normal estrogen level and with estrogen deficiency induced by bilateral ovariectomy. The experiments were carried out on 3-month-old non-ovariectomized and ovariectomized Wistar rats, divided into control rats and rats receiving diosgenin (50 mg/kg p.o. daily) for 4 weeks. Serum bone turnover markers, bone mass and mineralization, histomorphometric parameters and mechanical properties were studied. Diosgenin improved some investigated parameters in both non-ovariectomized and ovariectomized rats, in which estrogen deficiency induced osteoporotic changes. Diosgenin increased compact bone formation and probably inhibited cancellous bone resorption, which led to improvement of mechanical properties of compact and cancellous bone. In conclusion, this *in vivo* study demonstrated that diosgenin may be one of sparse compounds increasing bone formation.

Key words: diosgenin, skeletal system, osteoporosis, estrogen deficiency, rats

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INTRODUCTION

Prevention and treatment of osteoporosis become more and more important, since the prevalence of osteoporosis increases due to population aging. In the treatment of osteoporosis, drugs inhibiting bone resorption or inducing bone formation are used; the majority of patients are treated with antiresorptive drugs (mainly bisphosphonates), whereas bone anabolic therapies (parathyroid hormone, strontium ranelate) are much less common (Rachner *et al*., 2011; Tella & Gallagher, 2014). There is an interest in natural compounds present in dietary and medicinal plants that could be useful in the prevention and treatment of osteoporosis.

Diosgenin is a steroidal sapogenin of fenugreek (*Trigonella foenum-graecum*), different yams (*Dioscorea* spp., including *D. villosa, D. spongiosa, D. esculenta, D. zingiberensis, D. opposita*) and some other edible or medicinal plants, where it is present as glycosides (saponins) (Raju & Mehta, 2009; Patel *et al*., 2012; Chiang & Pan, 2013; Sato *et al*., 2014; Zhang *et al*., 2014b). Various health-promoting activities have been demonstrated for diosgenin, including anti-diabetic, anti-hyperlipidemic, cancer-chemopreventive, anti-inflammatory, immunomodulatory (Raju & Mehta, 2009; Jung *et al*., 2010; Uemura *et al*., 2010; Chen *et al*., 2011; Patel *et al*., 2012; Rajput & Mandal, 2012; Chiang & Pan, 2013; Sato *et al*., 2014).

Diosgenin has been reported to exert some antiosteoporotic effect in preliminary studies on estrogen-deficient rats treated with its sustained delivery, as well as to inhibit osteoclastogenesis and to stimulate osteogenic activity of osteoblastic cells *in vitro* (Higdon *et al*., 2001; Scott *et al*., 2001; Shishodia & Aggarwal, 2006; Alcantara *et al*., 2011). Very recently, a report on favorable effects of high-dose diosgenin in ovariectomized rats has been published (Zhang *et al*., 2014a). Moreover, diosgenin has been observed to counteract the development of some changes in rats with retinoic acid-induced osteoporosis (Zhao *et al*., 2015) and in OXYS rats with accelerated senescence (Tikhonova *et al*., 2015). Also, favorable effects of diosgenin-containing plant organs, their extracts or isolated saponins on the skeletal system have been demonstrated in experimental conditions (Yin *et al*., 2004; Yin *et al*., 2010; Chiang *et al*., 2011; Folwarczna *et al*., 2014a; Qu *et al*, 2014; Zhang *et al*., 2014b). The aim of the present study was to investigate the effects of diosgenin administration on the skeletal system of female rats with normal estrogen levels and with estrogen deficiency (bilaterally ovariectomized).

MATERIALS AND METHODS

Experiments were performed on mature (3-month-old) female Wistar rats obtained from the Center of Experimental Medicine, Medical University of Silesia, Katowice. The rats were fed a soy-free diet with decreased content of phenolic acids, *ad libitum*. The composition of the diet was presented in our previous reports (Folwarczna *et al*., 2013; 2015). The animals were switched from the standard laboratory diet (Labofeed B) to the experimental diet the day before the beginning of diosgenin administration. Both diets were produced by Wytwórnia Pasz "Morawski", Poland. The protocol for the experiments

✉e-mail: jfolwarczna@sum.edu.pl
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on animals was approved by the Local Ethics Commission, Katowice, Poland (permission no 33/2010).

Chemicals and drugs used: diosgenin (Sigma-Aldrich) at a dose of 50 mg/kg p.o. daily for 4 weeks, ketamine - Bioketan (Vetoquinol Biowet), xylazine – Xylapan (Vetoquinol Biowet).

The animals were divided into 4 groups (n=8–10):

I. Non-ovariectomized control rats,

II. Non-ovariectomized rats receiving diosgenin (50 mg/kg p.o. daily),

III. Ovariectomized control rats,

IV. Ovariectomized rats receiving diosgenin (50 mg/kg p.o. daily).

Bilateral ovariectomy was performed 7–8 days before the start of diosgenin administration, under intraperitoneal ketamine-xylazine anesthesia. Diosgenin was administered once daily for 4 weeks, by a stomach tube (p.o.). Control rats were given the vehicle – tap water at the same volume of 2 ml/kg p.o. Moreover, to mark the calcification front, the animals were administered tetracycline hydrochloride (20 mg/kg i.p.) twice: one day before the start of diosgenin or vehicle administration, and after 4 weeks.

The next day after the last diosgenin or vehicle administration, after overnight fasting, the animals were anesthetized with ketamine and xylazine, and sacrificed by cardiac exsanguination. The tibias, femurs and L-4 vertebra, as well as estrogen-dependent organs (uterus and thymus) were isolated. The length and diameter in the mid-length of left femur were measured. The left femur, L-4 vertebra, uterus and thymus were weighed. The left femurs and tibias, and proximal part of the right femurs were kept below –20°C (wrapped in gauze soaked in 0.9% NaCl solution) until the mechanical tests were performed on thawed bones (Turner & Burr, 1993).

Biochemical studies. Serum concentrations of osteocalcin and type I collagen fragments released from bone during bone resorption were studied using enzyme immunoassays (Rat-MID Osteocalcin EIA and RatLaps EIA, respectively, Immunodiagnostic Systems Ltd). Serum estradiol concentrations were determined by an ELISA method (Mouse/Rat Estradiol ELISA, Calbiotech, Inc.). Moreover, serum levels of calcium were assayed colorimetrically (Pointe Scientific, Inc., reagent set).

Bone mineralization studies. To determine the mass of bone mineral, the bones were mineralized (ashed) at 640°C for 48 h in a muffle furnace and weighed. The ratio of bone mineral mass to bone mass was calculated and treated as a substitute for bone mineral density measurements. Calcium and phosphorus content in the mineralized bones were assayed spectrophotometrically, using kits manufactured by Pointe Scientific, Inc., as previously described (Folwarczna *et al*., 2013).

Histomorphometric studies. The specimens for histomorphometric measurements were prepared from the right tibias and femurs, as previously described (Folwarczna *et al*., 2004; 2013). The measurements were performed using Optiphot-2 microscope (Nikon) equipped with an RGB camera (Cohu) and connected to a personal computer, using Lucia G 4.51 software (Laboratory Imaging), with final magnifications of 200 and 500 times, or using Axio Imager.A1 microscope (Zeiss), Olympus DP 71 camera and OsteoMeasure XP v1.3.0.1 (OsteoMetrics) software (final magnification 70 times).

In transverse cross-sections made from the tibial diaphysis close to the point where the fibula grows into it, the area of the transverse cross-section of the cortical bone, the area of the transverse cross-section of the marrow cavity, the endosteal osteoid width, as well as the periosteal and endosteal transverse growth of the tibia were determined. The width of trabeculae in the distal epiphysis and metaphysis of the femur were measured in the longitudinal preparations.

Table 1. Effects of diosgenin (50 mg/kg p.o. daily for 4 weeks) on the body mass gain, mass of estrogen-dependent organs and serum biochemical parameters in non-ovariectomized and ovariectomized rats

Parameter/Group	Non-ovariectomized rats		Ovariectomized rats		Two-way ANOVA		
					Main effects		Interaction
	Control	Diosgenin	Control	Diosgenin	Ovariectomy	Diosgenin	
Body mass at the start of diosgenin administration [g]	235.6 ±3.3	235.9±5.0	226.8±4.7	229.4±3.4	NS	NS	NS
Body mass gain after 4 weeks [g]	21.3±2.5	14.2±3.7	33.5±3.8*	31.5±3.4*	$p<0.001$	NS	NS
Estradiol [pg/ml]	24.57±2.48	18.61±1.71	16.82±2.12*	17.94±0.63*	$p<0.05$	NS	NS
Uterus mass [g]	0.385±0.045	0.487±0.065	0.080±0.004***	0.081±0.002***	$p<0.001$	NS	NS
Thymus mass [g]	0.305±0.014	0.295±0.014	0.536±0.034***	0.489±0.036***	$p<0.001$	NS	NS
Osteocalcin [ng/ml]	195.2±18.6	171.3±12.0	327.1±27.5**	329.1±38.8**	$p<0.001$	NS	NS
RatLaps [ng/ml]	24.23±2.64	22.90±1.98	42.21±2.73***	40.71±2.72***	$p<0.001$	NS	NS
Calcium [mg/100 ml]	10.01±0.21	10.17±0.16	9.65±0.18	9.49±0.24	$p<0.05$	NS	NS

Results are presented as means ± SEM. 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. *$p<0.05$, **$p<0.01$, ***$p<0.001$, significantly different from non-ovariectomized control rats. NS, not significant.

Bone mechanical properties studies. Mechanical properties of bones were studied using an Instron 3342 500N apparatus and Bluehill 2 version 2.14 software (Instron).

Mechanical properties of the left femoral diaphysis (compact bone) and tibial metaphysis (cancellous bone) were assessed using bending tests with three-point loading, as previously described (Turner & Burr, 1993; Stürmer *et al*., 2006; Folwarczna *et al*., 2013). The load was applied perpendicularly to the long axis of the femur in the mid-length of the bone, or to the proximal tibial metaphysis (3 mm from the proximal edge of the bone deprived of the proximal epiphysis), also perpendicularly to the long axis of the bone. 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. For computation of the moment of inertia, necessary to calculate the intrinsic parameters (stress and Young's modulus), it was assumed that the femoral diaphysis was an elliptical pipe, and the tibial metaphysis – a circular beam (Folwarczna *et al*., 2013).

The femoral neck strength was studied using the compression test (Folwarczna *et al*., 2013). The load was applied to the head of the right femur along the long axis of the femur and the maximum load was measured.

Statistical analysis. Results are presented as means ±SEM. One-way ANOVA followed by Duncan's test or, in case of the lack of normality or of homogeneity of variance, Kruskal-Wallis ANOVA followed by Mann-Whitney *U* test, were used to evaluate the statistical significance of the results. Moreover, two-way ANOVA was also conducted in order to evaluate whether the effect of diosgenin depended on estrogen status. Interaction between the main effects of estrogen deficiency and diosgenin treatment was assessed.

RESULTS

Effects of diosgenin on the body mass gain, mass of estrogen-dependent organs and serum biochemical parameters

The ovariectomized rats were estrogen-deficient, as demonstrated by significant decrease in the serum estradiol level and uterine mass, as well as the increase in the thymus mass and body mass gain (Table 1). Administration of diosgenin (50 mg/kg p.o. daily for 4 weeks) did not affect the body mass gain, serum estradiol level and the mass of estrogen-dependent organs, both in non-ovariectomized and ovariectomized rats. After administration of diosgenin, no effects on levels of biochemical markers of bone formation (osteocalcin) and bone resorption (RatLaps) were observed, both in non-ovariectomized and ovariectomized rats, in which they were significantly increased in relation to the non-ovariectomized

Table 2. Effects of diosgenin (50 mg/kg p.o. daily for 4 weeks) on bone mass, mineralization and macrometric parameters in non-ovariectomized and ovariectomized rats

Parameter/Group		Non-ovariectomized rats		Ovariectomized rats		Two-way ANOVA		
		Control	Diosgenin	Control	Diosgenin	Main effects: Ovariectomy	Main effects: Diosgenin	Interaction
Bone mass [g]	Femur	0.710±0.014	0.715±0.017	0.709±0.019	0.728±0.012	NS	NS	NS
	L-4 vertebra	0.200±0.004	0.200±0.006	0.203±0.009	0.207±0.007	NS	NS	NS
Bone mineral mass [g]	Femur	0.314±0.004	0.320±0.007	0.290±0.007*	0.305±0.004	$p<0.01$	NS	NS
	L-4 vertebra	0.083±0.001	0.083±0.002	0.075±0.003*	0.080±0.002	$p<0.05$	NS	NS
Mass of bone mineral/bone mass ratio	Femur	0.443±0.006	0.448±0.003	0.410±0.004***	0.419±0.004**	$p<0.001$	NS	NS
	L-4 vertebra	0.418±0.007	0.414±0.005	0.371±0.010***	0.387±0.009*	$p<0.001$	NS	NS
Calcium content [g/g of bone mineral]	Femur	0.360±0.004	0.363±0.004	0.358±0.006	0.362±0.004	NS	NS	NS
	L-4 vertebra	0.362±0.007	0.360±0.005	0.339±0.003*	0.353±0.003•	$p<0.01$	NS	NS
Phosphorus content [g/g of bone mineral]	Femur	0.162±0.002	0.165±0.002	0.162±0.002	0.164±0.001	NS	NS	NS
	L-4 vertebra	0.176±0.003	0.174±0.002	0.168±0.002	0.176±0.001	NS	NS	NS
Femur length [mm]		34.5±0.4	34.0±0.4	33.9±0.6	34.4±0.4	NS	NS	NS
Femur diameter [mm]		2.99±0.03	3.10±0.05	3.00±0.03	3.06±0.03	NS	$p<0.05$	NS

Results are presented as means ±SEM. 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. *$p<0.05$, **$p<0.01$, ***$p<0.001$, significantly different from non-ovariectomized control rats. •$p<0.05$, significantly different from ovariectomized control rats. NS, not significant.

Table 3. Effects of diosgenin (50 mg/kg p.o. daily for 4 weeks) on bone histomorphometric parameters in non-ovariectomized and ovariectomized rats

Parameter/Group		Non-ovariectomized rats		Ovariectomized rats		Two-way ANOVA		
		Control	Diosgenin	Control	Diosgenin	Main effects		Interaction
						Ovariectomy	Diosgenin	
Transverse growth of the tibia [μm]	Periosteal	41.72±2.20	57.53±4.96*	47.57±1.59	62.99±6.32**	NS	$p<0.001$	NS
	Endosteal	25.53±1.25	28.17±2.61	31.62±2.36	33.94±2.88	$p<0.05$	NS	NS
Width of endosteal osteoid in the tibia [μm]		10.80±0.17	10.50±0.16	11.56±0.10*	11.89±0.14**	$p<0.001$	NS	$p<0.05$
Transverse cross-section area of the cortical bone in the tibial diaphysis [mm^2]		3.490±0.079	3.754±0.059*	3.419±0.089	3.745±0.083*•	NS	$p<0.001$	NS
Transverse cross-section area of the tibial marrow cavity [mm^2]		0.870±0.058	0.822±0.044	0.840±0.030	0.901±0.040	NS	NS	NS
Transverse cross-section area of the tibial marrow cavity / transverse cross-section area of the tibial diaphysis ratio		0.199±0.012	0.179±0.008	0.198±0.008	0.194±0.009	NS	NS	NS
Width of trabeculae in the femur [μm]	Epiphysis	63.44±0.35	65.16±0.40**	60.68±0.50***	62.08±1.23	$p<0.001$	$p<0.05$	NS
	Metaphysis	45.22±0.24	47.33±0.44***	42.59±0.41***	46.45±1.28••	$p<0.05$	$p<0.001$	NS

Results are presented as means ± SEM. 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. *$p<0.05$, **$p<0.01$, ***$p<0.001$, significantly different from non-ovariectomized control rats. • $p<0.05$, •• $p<0.01$, significantly different from ovariectomized control rats. NS, not significant.

controls. There was also no diosgenin effect on the serum calcium concentration.

Effects of diosgenin on bone mass, macrometric parameters and mineralization

Administration of diosgenin did not significantly affect bone mass, bone mineral mass and mineralization (the ratio of bone mineral mass to bone mass), both in non-ovariectomized and ovariectomized rats, although mass and mineral mass of the femur tended to increase (Table 2). Diosgenin increased the calcium content in the mineral of the vertebra in ovariectomized rats. The diameter of the femur increased in the diosgenin-treated rats (as shown in two-way ANOVA). It should be noted that estrogen deficiency decreased the mass of bone mineral and strongly reduced bone mineralization both in the femur and vertebra, and decreased calcium content in the vertebra mineral. There were no effects of estrogen deficiency on the femoral length and diameter.

Effects of diosgenin on bone histomorphometric parameters

Histomorphometric measurements in the ovariectomized control rats demonstrated a tendency to increase compact bone formation (there was a tendency to increase the transverse growth in the tibial diaphysis and a significant increase in the width of endosteal osteoid) and no effect on compact bone resorption (the transverse cross-section area of tibial marrow cavity and its ratio to the transverse cross-section area of the whole diaphysis, Table 3). Administration of diosgenin increased the periosteal transverse growth of the tibia, both in non-ovariectomized and ovariectomized rats in relation to appropriate controls. Also, an increase in the area of the cortical bone in the transverse cross-sections of the tibial diaphysis was observed. Diosgenin did not affect the width of endosteal osteoid in the tibial diaphysis.

In cancellous bone of the femoral epiphysis and metaphysis, diosgenin increased the width of trabeculae in comparison with appropriate controls, regardless of the estrogen status. Those parameters were significantly decreased in the ovariectomized control rats, indicating increased bone resorption.

Effects of diosgenin on bone mechanical properties

In ovariectomized rats, diosgenin significantly increased the maximum load sustained by the tibial metaphysis and the energy accumulated to the fracture point in relation to the ovariectomized controls, slightly counteracting the effects of estrogen deficiency (Table 4), since in the ovariectomized control rats, Young's modulus, yield load and the load, stress and energy accumulated for both the points of maximum load and fracture, were significantly decreased in relation to the non-ovariectomized control rats. Diosgenin did not significantly affect the strength of the tibial metaphysis in non-ovariectomized rats in comparison to the non-ovariectomized control rats, except the increase in the yield load and stress. However, two-way ANOVA revealed significant diosgenin main effects (increasing) for the yield

Table 4. Effects of diosgenin (50 mg/kg p.o. daily for 4 weeks) on mechanical properties of the tibial metaphysis in non-ovariectomized and ovariectomized rats

Parameter/Group	Non-ovariectomized rats		Ovariectomized rats		Two-way ANOVA		
	Control	Diosgenin	Control	Diosgenin	Main effects		Interaction
					Ovariectomy	Diosgenin	
Young's modulus [MPa]	2382±237	2561±343	1722±266*	1406±246**	$p<0.01$	NS	NS
Yield load (0.05% offset) [N]	44.1±2.7	67.2±9.8*	35.3±3.1*	38.7±4.1	$p<0.01$	$p<0.05$	NS
Displacement for yield load [mm]	0.264±0.026	0.395±0.059	0.274±0.024	0.350±0.070	NS	$p<0.05$	NS
Energy for yield load [mJ]	5.49±0.77	14.37±4.04	4.90±0.89	6.92±2.17	NS	$p<0.05$	NS
Stress for yield load [MPa]	30.1±3.4	45.2±7.0*	23.9±2.7	23.0±2.4	$p<0.01$	NS	NS
Maximum load [N]	101.9±5.5	111.9±6.7	64.6±2.5***	76.9±4.4** •	$p<0.001$	$p<0.05$	NS
Displacement for maximum load [mm]	0.898±0.067	0.837±0.062	0.738±0.058	0.905±0.043	NS	NS	NS
Energy for maximum load [mJ]	54.94±4.82	52.51±6.01	29.34±2.96**	41.86±4.30	$p<0.001$	NS	NS
Maximum stress [MPa]	69.2±7.2	76.5±7.2	43.6±3.4**	46.2±4.0*	$p<0.001$	NS	NS
Fracture load [N]	77.8±5.6	72.4±3.1	43.2±1.9***	48.3±2.1***	$p<0.001$	NS	NS
Displacement for fracture load [mm]	1.175±0.055	1.194±0.070	1.197±0.058	1.393±0.069	NS	NS	NS
Energy for fracture load [mJ]	79.57±4.71	87.21±7.94	54.26±2.32**	73.16±5.44•	$p<0.01$	$p<0.05$	NS
Stress for fracture load [MPa]	52.5±5.8	50.3±5.4	29.4±2.6***	28.9±2.1**	$p<0.001$	NS	NS

Results are presented as means ± SEM. 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. *$p<0.05$, **$p<0.01$, ***$p<0.001$, significantly different from non-ovariectomized control rats. • $p<0.05$, significantly different from ovariectomized control rats. NS, not significant

load, displacement and energy, as well as for the maximum load and the energy accumulated to the fracture point, indicating that the improvement of the mechanical properties in the tibia did not depend on the estrogen status.

In the femoral diaphysis, diosgenin slightly tended to increase the maximum load sustained by the bone, both in non-ovariectomized and ovariectomized rats (Table 5). Diosgenin significantly increased the load value registered at fracture point in non-ovariectomized rats. There was also a significant diosgenin main effect concerning the fracture load in two-way ANOVA (increasing). What is more, diosgenin counteracted the increase in the yield point displacement in ovariectomized rats. Diosgenin did not significantly affect the strength of the femoral neck, regardless of estrogen status. The mechanical parameters of the femoral neck and diaphysis (with the exception of the yield point displacement) were not affected by estrogen deficiency.

DISCUSSION

The first reports on the effects of diosgenin on the skeletal system in experimental conditions were published by Hidgon, Scott and coworkers in 2001. Diosgenin in sustained delivery from subcutaneous implants (for 33 days) was reported to exert some beneficial effects on histomorphometric parameters and mechanical strength of the femoral diaphysis in ovariectomized rats (Higdon *et al.*, 2001; Scott *et al.*, 2001). However, the small number of rats per experimental group (n=4) and the lack of data on the daily dose of diosgenin released from the implant (containing 500 mg of diosgenin) allow to treat those reports as preliminary observations. The studies on effects of diosgenin on osteoclastogenesis and osteoblast activity confirmed the possibility of its beneficial influence on the skeletal system (Shishodia & Aggarwal, 2006; Alcantara *et al.*, 2011).

There also exist several reports concerning favorable effects of glycosides of diosgenin, as well as their plant sources, on the skeletal system of ovariectomized rats (Yin *et al.*, 2004; Chiang *et al.*, 2011; Folwarczna *et al.*, 2014a; Zhang *et al.*, 2014b). However, their effects may differ from those of diosgenin. Plant extracts contain diosgenin mainly as glycosides (saponins); they also include other, various components (like flavonoids or alkaloids) which may exert their own effects on the skeletal system. For example, in studies performed parallelly to the study reported here, we observed that fenugreek seed (1% in the diet) tended to favorably affect the rat skeletal system, whereas at a higher dose (administered as an

Table 5. Effects of diosgenin (50 mg/kg p.o. daily for 4 weeks) on mechanical properties of the femoral diaphysis and femoral neck in non-ovariectomized and ovariectomized rats

Parameter/Group	Non-ovariectomized rats		Ovariectomized rats		Two-way ANOVA		
					Main effects		Interaction
	Control	Diosgenin	Control	Diosgenin	Ovariectomy	Diosgenin	
Young's modulus [MPa]	8263±422	8590±453	7800±444	8062±408	NS	NS	NS
Yield load (0.05% offset) [N]	74.5±3.0	79.1±4.2	73.7±2.4	75.3±2.5	NS	NS	NS
Displacement for yield load [mm]	0.242±0.010	0.239±0.008	0.299±0.013**	0.260±0.014•	$p<0.01$	NS	NS
Energy for yield load [mJ]	8.84±0.60	9.04±0.73	9.60±0.52	8.89±0.46	NS	NS	NS
Stress for yield load [MPa]	115.9±4.8	115.8±5.9	117.4±5.4	115.3±4.4	NS	NS	NS
Maximum load [N]	111.4±3.0	118.5±4.3	108.3±3.1	113.8±3.5	NS	NS	NS
Displacement for maximum load [mm]	0.563±0.022	0.528±0.024	0.590±0.021	0.576±0.028	NS	NS	NS
Energy for maximum load [mJ]	39.61±2.16	38.16±2.48	36.21±1.75	39.71±3.30	NS	NS	NS
Maximum stress [MPa]	173.7±5.7	173.5±5.6	175.3±9.2	174.2±6.1	NS	NS	NS
Fracture load [N]	98.9±4.2	115.8±4.0*	103.0±4.1	108.4±4.1	NS	$p<0.05$	NS
Displacement for fracture load [mm]	0.682 ±0.049	0.568±0.044	0.633±0.027	0.685±0.060	NS	NS	NS
Energy for fracture load [mJ]	51.87±5.13	43.13±5.57	40.87±2.58	51.59±5.45	NS	NS	NS
Stress for fracture load [MPa]	153.6±6.0	169.6±5.7	165.3±9.0	166.1±7.2	NS	NS	NS
Femoral neck – maximum load [N]	88.3±3.1	88.6±5.3	79.9±2.2	89.0±8.3	NS	NS	NS

Results are presented as means ± SEM. 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. *$p<0.05$, **$p<0.01$, significantly different from non-ovariectomized control rats. • $p<0.05$, significantly different from ovariectomized control rats. NS, not significant.

extract) it induced some unfavorable effects in estrogen-deficient rats (Folwarczna *et al.*, 2014a), to which trigonelline could contribute (Folwarczna *et al.*, 2014b). Effects of diosgenyl saponins may also differ from those of diosgenin, due to, among others, different bioavailability. In fact, the bioavailability of dioscin seemed to be better than that of diosgenin (Manda *et al.*, 2013).

The diosgenin dose, used in the present study (50 mg/kg p.o. daily), was rather high, however diosgenin is poorly absorbed (Cayen & Dvornik, 1979). The low oral bioavailability of diosgenin (about 4.3% in rats) (Okawara *et al.*, 2013) is probably due to the fact that it is a substrate for P-glycoprotein (Manda *et al.*, 2013). Taking the above into account, the oral dose used was of similar range to the intraperitoneal dose effective in hyperglycemic rats (Sato *et al.*, 2014). The duration of treatment (4 weeks) was long enough to evaluate the skeletal effects of different drugs and natural compounds in rats in our previous studies (Folwarczna *et al.*, 2004; 2013; 2015).

Results of the present study indicate that diosgenin at a dose of 50 mg/kg p.o. daily, administered for 4 weeks, favorably affected the female rat skeletal system, slightly improving bone structure and strength. We investigated diosgenin effects in rats with normal estrogen levels and in estrogen-deficient (bilaterally ovariectomized) rats, in which characteristic osteoporotic changes developed: increase in bone resorption and formation, disordered mineralization and worsened mechanical properties of cancellous bone (tibial metaphysis) (Folwarczna *et al.*, 2014a; 2014b).

Administration of diosgenin counteracted some of the skeletal changes developing due to estrogen deficiency in ovariectomized rats. Although diosgenin did not improve bone mineralization (the ratio of mass of bone mineral to bone mass), it alleviated the effect of estrogen deficiency on the calcium content in the vertebra mineral and on cancellous bone structure (width of femoral trabeculae), which induced improvement in the mechanical properties of the tibial metaphysis. Those effects might have resulted from the decreased cancellous bone resorption and/or increased bone formation. Diosgenin not only did not counteract the increased compact bone formation in ovariectomized rats, but further intensified it. The minor increase in the compact bone growth (periosteal transverse growth of the tibial diaphysis and femoral diaphysis diameter) probably led to slight improvement in strength of the femoral diaphysis. In fact, diosgenin affected rather the extrinsic, i.e. depending on the bone size, mechanical parameters (like the values of

load and of energy absorbed), than the intrinsic parameters (stress, Young's modulus).

Interestingly, the same effects or tendencies were demonstrated for the skeletal parameters of normal, non-ovariectomized, female rats. Two-way ANOVA revealed that diosgenin exerted its effects regardless of the estrogen status (significant diosgenin main effects and the lack of interactions between the main effects of diosgenin and ovariectomy).

The decreasing of bone resorption by diosgenin is consistent with its effects on osteoclast formation *in vitro*. It was demonstrated that diosgenin inhibited receptor activator of nuclear factor κB ligand (RANKL)-stimulated osteoclastogenesis in the culture of Raw 264.7 (mouse macrophage) cells (Shishodia & Aggarwal, 2006). Moreover, the long-term (12-week) treatment with diosgenin at a high dose of 96 mg/kg p.o. daily in 6-month-old ovariectomized rats counteracted the effects of estrogen-deficiency on bone mineral density and trabecular bone histomorphometry, decreasing the RANKL/osteoprotegerin ratio (assessed by immunohistochemistry and *in situ* hybridization; Zhang *et al*., 2014a).

One may speculate that inhibition of bone resorption by diosgenin may be connected with its ability to induce activation of high conductance Ca^{2+}-activated K^+ (BK) channels, through which it exerts the vasodilatory action (Au *et al*., 2004; Dias *et al*., 2007). Since targeted deletion of BK channel resulted in increased cathepsin K release in osteoclasts and development of osteopenia in juvenile mice (Sausbier *et al*., 2011), it is possible that diosgenin, by activation of BK channels, may decrease cathepsin K release and in consequence inhibit bone resorption.

Also, the increase in bone formation observed in the present study is consistent with the previous *in vitro* studies (Yen *et al*., 2005; Alcantara *et al*., 2011). Low concentrations of diosgenin increased proliferation and stimulated the synthesis and secretion of type 1 collagen, alkaline phosphatase and osteopontin in murine osteoblastic MC3T3-E1 cells, although at a higher concentration (10 μM) diosgenin was cytotoxic (Alcantara *et al*., 2011). Consistently, Zhang *et al*. (2014a) demonstrated the decrease in bone formation indices after a larger diosgenin dose *in vivo*. It is possible that diosgenin may stimulate bone formation through activation of angiogenesis in bone. In MC3T3-E1 cells, diosgenin upregulated vascular endothelial growth factor-A, playing an important role during fracture repair and bone formation, usually accompanied by the formation of new capillary vessels (Yen *et al*., 2005). However, the diosgenin effects on bone resorption and formation were not strong enough to affect the serum levels of bone turnover markers in the present *in vivo* study.

Diosgenin has been widely used in the pharmaceutical industry as a precursor in the synthesis of steroids, including sex hormones. It is believed that diosgenin is not metabolized to progesterone and further to androgens and estrogens in mammalian organisms (Raju & Mehta, 2009; Depypere & Comhaire, 2014). It also does not bind to human estrogen or progesterone receptors *in vitro* (Depypere & Comhaire, 2014). Recently, diosgenin was reported to not affect uterine weight and histomorphometric parameters, as well as expression of estrogen receptor α and progesterone receptor in the immature rat assay (Medigović *et al*., 2014), and estradiol level in both ovariectomized and non-ovariectomized adult rats (Chang *et al*., 2011). Consistently, diosgenin did not affect the serum estradiol level, and the mass of estrogen-dependent organs, the uterus and thymus, in the present study. It should be pointed out that the diosgenin effects on bone formation in the present study were different from those of estradiol (0.2 mg/kg p.o.) which counteracted the increasing effect of estrogen-deficiency on compact bone formation in our previous report (Folwarczna *et al*., 2015). Moreover, estradiol did not affect the mechanical parameters of compact bone (Folwarczna *et al*., 2015). Taken together, the diosgenin effects on the skeletal system seemed to be not estrogenic.

However, diosgenin has been reported to exert its angiogenesis promoting effects on murine osteoblastic cells through estrogen receptor-related pathways (Yen *et al*., 2005), and to increase uterine weight in older rats after a longer treatment than that used in the present study (Zhang *et al*., 2014a). Also, Zhao *et al*. (2015) observed that diosgenin at a high dose (90 mg/kg p.o.) increased serum estradiol level, which was decreased due the treatment with retinoic acid. A recent report by Sato *et al*. (2014) indicated that diosgenin increased levels of dehydroepiandrosterone and 5α-dihydrotestosterone in the serum and muscle of male rats with streptozotocin-induced diabetes. It is possible that diosgenin may affect the skeletal system by acting through androgen receptors in bones. It is known that androgens induce periosteal growth, at least in male organisms (Manolagas, 2013). It was also proposed that there may be a specific receptor for dehydroepiandrosterone on osteoblasts, since dehydroepiandrosterone promoted proliferation and inhibited apoptosis of osteoblasts *via* pathways independent of androgen or estrogen receptors (Wang *et al*., 2007).

The slight, positive skeletal effects of diosgenin demonstrated in the present study confirmed that diosgenin or its derivates of better bioavailability may favorably affect bones, especially in disorders with decreased bone formation. It seems important that diosgenin effects were observed also in rats with normal bone turnover. However, there are some safety problems concerning the potential prophylactic use of diosgenin for osteoporosis. Although diosgenin probably did not affect estrogenic pathways in the present study, its possible androgenic effects should be taken into account, especially in women. Another problem is that saponins are known to induce hemolysis (Francis *et al*., 2002). However, diosgenin, a sapogenin, was reported to exert very low hemolytic activity, even at a high concentration of 100 μg/ml (Liu *et al*., 2013).

Diosgenin exerts its actions through various molecular targets (Shishodia & Aggarwal, 2006; Raju & Mehta, 2009; Jung *et al*., 2010; Alcantara *et al*., 2011; Kim *et al*., 2012; Patel *et al*., 2012). The resultant of the actions may be not favorable to bone. For example, it has been reported that anti-lipidemic effects of diosgenin are mediated by peroxisome proliferator-activated receptor γ (PPARγ) (Sangeetha *et al*., 2013). Deleterious effects of thiazolidinediones, PPARγ agonists, on the skeletal system were demonstrated (Lecka-Czernik, 2010). There is also a problem of the therapeutic window of diosgenin. Diosgenin exerted positive effects on osteoblastic cells *in vitro* at lower, but not at a high concentration, in which it became cytotoxic (Alcantara *et al*., 2011).

In conclusion, this study confirmed the previous reports on favorable skeletal effects of diosgenin in different experimental models and demonstrated *in vivo* that diosgenin may be one of sparse compounds increasing bone formation.

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