European **Rev**iew for **Med**ical and **Pharmacol**ogical **Sci**ences 2014; 18: 1937-1947

Effects of fenugreek (*Trigonella foenum-graecum* L.) seed on bone mechanical properties in rats

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Abstract. – BACKGROUND AND AIM: An example of a medicinal plant with numerous potential activities is fenugreek (*Trigonella foenum-graecum* L.). The aim of the present study was to investigate the effects of fenugreek seed on bone mechanical properties in rats with normal and decreased estrogen level (developing osteoporosis).

MATERIALS AND METHODS: The experiments were carried out on 3-month-old non-ovariectomized (NOVX) and ovariectomized (OVX) Wistar rats, divided into control rats, rats receiving pulverized fenugreek seed (1% in the diet) and rats receiving fenugreek seed extract standardized for 4-hydroxy-L-isoleucine (50 mg of 4-hydroxy-L-isoleucine/kg p.o. daily) for 4 weeks. Serum bone turnover markers, bone mineralization and mechanical properties were examined.

RESULTS: Fenugreek seed added to food did not significantly affect bone mineralization and serum turnover markers, independently of the estrogen status. It tended to increase the strength of the tibial metaphysis (cancellous bone) in NOVX rats, and increased the strength of the femoral diaphysis (compact bone) in OVX rats. The fenugreek seed extract did not affect the skeletal system of NOVX rats, and significantly worsened mineralization of the vertebra in OVX rats, decreased due to estrogen deficiency.

CONCLUSIONS: Low dietary intake of fenugreek seed may exert slight favorable skeletal effects, whereas at high doses it may damage the skeletal system.

Key Words:
Fenugreek seed, Fenugreek seed extract, Bone mechanical properties, Osteoporosis, Rats.

Introduction

An example of a medicinal plant with potential activites in the skeletal system is fenugreek (*Trigonella foenum-graecum* L.). Fenugreek, native to southeastern Europe, northern Africa, and western Asia, but also cultivated in other parts of the world, is an annual herb belonging to the Leguminosae family[1,2]. It is one of the oldest Ayurvedic medicinal plants, used in traditional Chinese medicine and as dietary spice[3]. It has been used for numerous indications, like lactation stimulation, aiding digestion and to generally improve metabolism and health[3,4]. It is also used in dietary supplements because of presumed antidiabetic, anabolic and other activities.

Fenugreek seed contains at least three major constituents, which could be expected to affect the skeletal system. Diosgenin, a steroidal sapogenin, present in fenugreek seed as glycosides (saponins), has already been reported to favorably affect the osseous tissue *in vitro* and *in vivo*[5-8]. Trigonelline, an alkaloid, has been demonstrated to possess phytoestrogenic properties[9]. 4-Hydroxyisoleucine, an amino acid characteristic of fenugreek, is considered responsible for antidiabetic and lipid-lowering actions[10], together with galactomannan, trigonelline and diosgenin[11-14]; antidiabetic drugs may exert differential effects on bones[15]. Moreover, some anabolic properties of fenugreek have been reported, which probably can be attributed to its different components[16]. The skeletal effects of the use of fenugreek seed have never been reported. The aim of the present study was to investigate the effects of fenugreek seed, added to the diet at a moderate dose, and administration of fenugreek seed extract, standardized for 4-hydroxyisoleucine, at a high dose, on the skeletal system of rats with normal and decreased estrogen level (a model of postmenopausal osteoporosis). Moreover, estradiol was administered to ovariectomized rats as a positive control.

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Materials and Methods

Materials

Botanical supplements used: pulverized fenugreek (*Trigonella foenum-graecum* L.) seed, Zakład Zielarski "Kawon-Hurt", Poland, added to the laboratory diet in an amount of 1%; fenugreek (*Trigonella foenum-graecum* L.) seed dry extract, standardized for 4-hydroxy-L-isoleucine, obtained from ASA Sp. z o.o., Poland (50 mg of 4-hydroxy-L-isoleucine/kg p.o. daily, i.e. 390.6 mg of the extract/kg p.o. daily).

Other drugs used: estradiol hemihydrate (Estrofem, Novo Nordisk A/S, 0.2 mg of estradiol/kg/day, p.o.), ketamine, Bioketan (Vetoquinol Biowet), xylazine, Xylapan (Vetoquinol Biowet).

Animals

The study was carried out with permission of the Local Ethics Commission in Katowice, Poland, on 3-month-old female Wistar rats obtained from the Center of Experimental Medicine, Medical University of Silesia, Katowice.

Bilateral ovariectomy with the access to the ovaries from the dorsal side was performed in general anesthesia induced by intraperitoneal injections of ketamine with xylazine. The intervention took place 7-8 days before the start of fenugreek administration.

During the experiment, the rats were fed a soy-free diet with decreased content of phenolic acids, *ad libitum*. Details of the soy-free diet composition were presented elsewhere[17]. The standard laboratory diet and the experimental diets were produced by Wytwórnia Pasz "Morawski", Poland. The animals were switched from the standard laboratory diet (Labofeed B) to the soy-free diet on the day before the beginning of pulverized fenugreek seed diet, fenugreek seed extract or estradiol administration.

The rats were divided into following groups (n = 8-10 per group, with an exception of group VII, where n = 6):

I – non-ovariectomized (NOVX) control rats,
II – ovariectomized (OVX) control rats
III – NOVX rats receiving pulverized fenugreek seed (1% in the diet)
IV – OVX rats receiving pulverized fenugreek seed (1% in the diet)
V – NOVX rats receiving fenugreek seed extract (50 mg of 4-hydroxy-L-isoleucine/kg p.o. daily)
VI – OVX rats receiving fenugreek seed extract (50 mg of 4-hydroxy-L-isoleucine/kg p.o. daily)
VII – OVX rats receiving estradiol (0.2 mg/kg p.o. daily).

Fenugreek seed extract or estradiol were administered by a gastric tube (p.o.) for 28 days. Control rats and rats fed the diet with 1% fenugreek seed were administered the vehicle – tap water at the same volume of 2 ml/kg p.o.

The next day after the last drug administration, after 24-h fasting, the animals were killed by cardiac exsanguination, in full ketamine-xylazine anesthesia. The estrogen-dependent organs (the uterus and thymus) and bones: the left and right femur, left tibia, and L-4 vertebra were isolated. The uterus, thymus, left femur and L-4 vertebra were weighed immediately after isolation. The left femur, left tibia, and proximal part of the right femur from each rat were wrapped in gauze soaked in 0.9% NaCl solution and kept below -20°C until the mechanical tests were performed on thawed bones[18].

Biochemical Studies

Serum estradiol concentrations were studied by an ELISA method (Mouse/Rat Estradiol ELISA, Calbiotech, Inc., Spring Valley, CA, USA). To determine serum levels of type I collagen fragments released from bone during bone resorption an enzyme immunoassay RatLaps EIA, and to determine serum osteocalcin levels, a marker of bone formation, an enzyme immunoassay Rat-MID Osteocalcin EIA (both produced by Immunodiagnostic Systems Ltd., Boldon, Tyne and Wear, UK) were used. Serum concentrations of calcium and inorganic phosphorus were assayed colorimetrically, using Pointe Scientific (Canton, MI, USA) reagent sets.

Bone Mineralization Studies

The left femur and L-4 vertebra were lyophilized for 9 days and weighed to determine the dehydrated bone mass, and then mineralized at the temperature of 640°C for 48 h in the muffle furnace and weighed to determine the mass of bone mineral (ash). The ratio of mass of bone mineral to bone mass was determined as a substitute for bone mineral density measurements. Moreover, the ratios of bone organic substances and water mass to bone mass were calculated.

Calcium and phosphorus content in the mineralized bones (dissolved in 6 M HCl and then di-

luted in distilled water) was determined colorimetrically, using a calcium reagent set and a phosphorus reagent set, both produced by Pointe Scientific (Canton, MI, USA).

Bone Mechanical Properties Studies

Mechanical properties of bones (the left femoral diaphysis, the neck of the right femur and the left proximal tibial metaphysis) were assessed using Instron 3342 500N apparatus with Bluehill 2 version 2.14 software (Norwood, MA, USA).

Mechanical properties of the diaphysis of the left femurs were studied using a bending test with three-point loading as previously described[17-21]. The distance between the points supporting the femur was 16 mm. The load was applied perpendicularly to the long axis of the femur at the mid-length of the bone. After pre-conditioning to obtain steady positioning, the mechanical test was started, with displacement rate of 0.01 mm/s and sampling rate of 100 Hz. From the load-displacement curves obtained for each bone, maximum load, displacement for the maximum load and energy for the maximum load were assessed. The same parameters were determined for the yield point (0.05% offset) and fracture point. The intrinsic parameters: stress and Young's modulus were also determined, assuming that the femoral diaphysis was an elliptical pipe. In order to determine moment of inertia in the break-section, necessary for the calculation, the transverse cross-sections of the right femoral diaphysis were made in the mid-length, and the inside and outside diameters were measured, according to Kiebzak et al[22], using OsteoMeasure XP v1.3.0.1 (OsteoMetrics) software for histomorphometric measurements.

The mechanical strength of the femoral neck was studied in a compression test. The bone was prepared for measurement by fixing the diaphysis, cut at the mid-length of the femur, in a methacrylate plate[17,19-21,23]. The load was applied to the head of the femur along the long axis of the femur (with a preload of 1 N, displacement rate of 0.01 mm/s). The load causing the fracture of the femoral neck (maximum load) was determined.

Mechanical properties of the proximal metaphysis of the left tibia were assessed in a bending test with three-point loading according to Stürmer et al[24], as previously described[17,19-21], after a preload of 1 N, with a displacement rate of 0.01 mm/s. The same parameters as for the femoral diaphysis were determined. To estimate moment of inertia in the break-section, the mean diameter of the metaphysis was measured using a caliper, assuming that the tibial metaphysis was a circular beam.

Statistical Analysis

The results are presented as arithmetical means ± SEM. Statistical estimation was carried out based on the analysis of variance. After confirmation of statistically significant differences in one-way ANOVA ($p < 0.05$), further analysis was carried out by means of Fisher's LSD *post-hoc* test. When appropriate (i.e. in case of a lack of normality in Shapiro-Wilk's test or a lack of homogeneity of variance in Levene's test), nonparametric tests were used: Kruskal-Wallis ANOVA followed by Mann-Whitney U test. The results obtained in each experimental group were compared with those of the NOVX control rats, and the results of treated OVX rats were compared with the OVX control rats.

Results

Effects of Estrogen Deficiency on the Skeletal System

In the OVX control rats, an increased body mass gain in comparison with the NOVX control rats was observed. Serum estradiol level was significantly decreased (Table I), and estrogen-dependent organs, the uterus and thymus, were significantly decreased and increased, respectively (not shown). Serum bone resorption marker, RatLaps, and bone formation marker, osteocalcin, significantly increased.

Bone mass (not shown) and mass of bone mineral in the L-4 vertebra were not significantly affected in the estrogen-deficient control rats (Table II). Mass of bone mineral in the femur, and the ratios of bone mineral mass to bone mass in the femur and L-4 vertebra were significantly decreased, the ratios of mass of organic substances to bone mass were unaffected, and the ratios of water mass to bone mass were increased in comparison with the NOVX controls. Moreover, calcium content in the bone mineral of the L-4 vertebra was significantly decreased (Table II). Phosphorus content in the bone mineral was not affected.

Estrogen deficiency strongly worsened mechanical properties of cancellous bone of the tibial metaphysis, decreasing Young's modulus, as well as the load, stress and accumulated energy

Table I. Effects of fenugreek seed in the diet (1%), fenugreek seed extract (50 mg of 4-hydroxy-L-isoleucine/kg p.o. daily) and estradiol (0.2 mg/kg p.o. daily) for 4 weeks on the body mass gain and serum levels of estradiol and bone turnover markers in rats.

Parameter/group	NOVX rats			OVX rats			
	Control	Fenugreek seed in the diet	Fenugreek seed extract	Control	Fenugreek seed in the diet	Fenugreek seed extract	Estradiol
Body mass at the start of drug administration (g)	235.6 ± 3.3	235.0 ± 4.6	233.0 ± 6.1	226.8 ± 4.7	225.4 ± 7.0	225.0 ± 2.0	226.0 ± 6.1
Body mass gain after 4 weeks (g)	21.3 ± 2.5	18.5±2.9	10.9 ± 1.9*	33.5 ± 3.8*	37.8 ± 3.6***	24.0 ± 5.6	30.7 ± 3.1
Estradiol (pg/mL)	24.57 ± 2.48	22.16 ± 1.65	20.71 ± 1.31	16.82 ± 2.12*	16.26 ± 1.30**	18.79 ± 1.03	19.53 ± 0.83
Osteocalcin (ng/mL)	195.2 ± 18.6	188.4 ± 26.9	160.7 ± 18.8	327.1 ± 27.5**	339.1 ± 38.0**	310.3 ± 20.1**	340.8 ± 41.3**
RatLaps (ng/mL)	24.23 ± 2.64	27.90 ± 2.02	20.69 ± 2.31	42.21 ± 2.73***	40.56 ± 4.61***	36.70 ± 4.08**	46.68 ± 4.14***

Results are presented as means ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ – significantly different from NOVX control rats.

for the yield point, maximum load and the fracture points (Table III), and did not affect mechanical properties of compact bone of the femoral diaphysis (Table IV). The decreasing effect on the femoral neck strength was not statistically significant.

Supplementation of the OVX rats with estradiol (0.2 mg/kg p.o.) significantly counteracted the decrease in the uterus mass (not shown), the decrease in the calcium content in the mineral of the vertebra (Table II) and worsening of the strength of the tibial metaphysis (Table III).

Effects of Fenugreek on Body Mass Gain and Serum Levels of Estradiol and Bone Turnover Markers

Administration of fenugreek seed in the diet did not affect the body mass gain, whereas administration of the fenugreek seed extract decreased it in NOVX rats (Table I). Fenugreek seed and fenugreek seed extract did not affect serum estradiol level, nor the mass of estrogen-dependent organs (the uterus and thymus, not shown) in NOVX and OVX rats. The treatments also did not significantly affect the bone resorption and formation markers (RatLaps and osteocalcin, respectively). There was no effect of fenugreek on serum calcium and phosphorus levels (not shown).

Effects of Fenugreek on Bone Mass and Mineralization

Bone mass (not shown) and mass of bone mineral were not significantly affected, both in NOVX and ovariecomized rats, after administration of pulverized fenugreek seed in the diet and the fenugreek seed extract in relation to appropriate controls.

Administration of pulverized fenugreek seed in the diet to NOVX and OVX rats did not affect the ratios of mass of bone mineral, organic substances and water to bone mass, as well as calcium and phosphorus content in the bone mineral, in comparison with the appropriate controls (Table II). The fenugreek seed extract administration also did not affect those parameters in NOVX rats, as well as it did not change them in the femur of OVX rats. However, in the L-4 vertebra of estrogen-deficient rats, the extract administration caused further, significant, decrease in the bone mineral mass/bone mass ratio, and increase in the bone water mass/bone mass ratio in relation to the OVX controls (with no effect on the bone organic substance mass/bone mass ra-

Table II. Effects of fenugreek seed in the diet (1%), fenugreek seed extract (50 mg of 4-hydroxy-L-isoleucine/kg p.o. daily) and estradiol (0.2 mg/kg p.o. daily) for 4 weeks on bone composition in rats.

Parameter/group		NOVX rats			OVX rats			
		Control	Fenugreek seed in the diet	Fenugreek seed extract	Control	Fenugreek seed in the diet	Fenugreek seed extract	Estradiol
Mass of bone mineral (mg)	Femur	0.314 ± 0.004	0.323 ± 0.006	0.313 ± 0.008	0.290 ± 0.007*	0.304 ± 0.007	0.293 ± 0.007*	0.307 ± 0.011
	L-4 vertebra	0.083 ± 0.001	0.084 ± 0.002	0.083 ± 0.002	0.075 ± 0.003	0.081 ± 0.003	0.080 ± 0.002	0.079 ± 0.003
Mass of bone mineral/bone mass ratio	Femur	0.443 ± 0.007	0.449 ± 0.004	0.442 ± 0.003	0.410 ± 0.004***	0.423 ± 0.007*	0.417 ± 0.006**	0.419 ± 0.006*
	L-4 vertebra	0.418 ± 0.007	0.417 ± 0.007	0.417 ± 0.006	0.371 ± 0.010***	0.383 ± 0.011**	0.344 ± 0.008***,•	0.371 ± 0.014***
Mass of bone water/bone mass ratio	Femur	0.337 ± 0.007	0.337 ± 0.004	0.344 ± 0.004	0.379 ± 0.006***	0.367 ± 0.008*	0.369 ± 0.008**	0.366 ± 0.008*
	L-4 vertebra	0.314 ± 0.007	0.313 ± 0.007	0.315 ± 0.007	0.359 ± 0.007***	0.346 ± 0.010*	0.386 ± 0.007***,•	0.360 ± 0.013**
Mass of bone organic substances/ bone mass ratio	Femur	0.220 ± 0.003	0.214 ± 0.002	0.214 ± 0.002	0.211 ± 0.003	0.210 ± 0.003	0.214 ± 0.003	0.215 ± 0.004
	L-4 vertebra	0.268 ± 0.002	0.270 ± 0.001	0.268 ± 0.002	0.270 ± 0.003	0.271 ± 0.002	0.269 ± 0.002	0.269 ± 0.003
Calcium content (g/g of bone mineral)	Femur	0.360 ± 0.004	0.370 ± 0.007	0.370 ± 0.005	0.358 ± 0.006	0.358 ± 0.005	0.355 ± 0.004	0.361 ± 0.004
	L-4 vertebra	0.362 ± 0.007	0.362 ± 0.005	0.357 ± 0.007	0.339 ± 0.003*	0.340 ± 0.005*	0.359 ± 0.011•	0.370 ± 0.009••
Phosphorus content (g/g of bone mineral)	Femur	0.162 ± 0.002	0.165 ± 0.002	0.165 ± 0.002	0.162 ± 0.002	0.164 ± 0.002	0.162 ± 0.001	0.161 ± 0.001
	L-4 vertebra	0.176 ± 0.003	0.174 ± 0.002	0.172 ± 0.001	0.168 ± 0.002	0.169 ± 0.003	0.169 ± 0.005	0.174 ± 0.002

Results are presented as means ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ – significantly different from NOVX control rats. •$p < 0.05$, ••$p < 0.01$ – significantly different from OVX control rats.

Table III. Effects of fenugreek seed in the diet (1%), fenugreek seed extract (50 mg of 4-hydroxy-L-isoleucine/kg p.o. daily) and estradiol (0.2 mg/kg p.o. daily) for 4 weeks on mechanical properties of the tibial metaphysis in rats.

Parameter/group	NOVX rats			OVX rats			
	Control	Fenugreek seed in the diet	Fenugreek seed extract	Control	Fenugreek seed in the diet	Fenugreek seed extract	Estradiol
Young's modulus (MPa)	2382 ± 237	2529 ± 250	2277 ± 256	1722 ± 266*	1723 ± 178*	2020 ± 375	1620 ± 222*
Yield load (offset 0.05%) (N)	44.1 ± 2.7	63.61 ± 14.22	45.4 ± 3.7	35.3 ± 3.1*	35.5 ± 5.0	27.2 ± 2.0***	34.0 ± 5.5*
Displacement for yield load (mm)	0.264 ± 0.026	0.380 ± 0.083	0.306 ± 0.054	0.274 ± 0.024	0.301 ± 0.062	0.195 ± 0.028	0.265 ± 0.061
Energy for yield load (mJ)	5.49 ± 0.77	15.36 ± 7.04	7.08 ± 1.49	4.90 ± 0.89	6.07 ± 2.20	2.62 ± 0.38	5.05 ± 2.15
Stress for yield load (MPa)	30.1 ± 3.4	47.0 ± 12.4	30.7 ± 2.9	23.95 ± 2.7	23.0 ± 3.1	17.5 ± 1.3***$^{\bullet}$	22.56 ± 4.3
Maximum load (N)	101.9 ± 5.5	120.5 ± 9.9	102.1 ± 5.6	64.6 ± 2.5***	65.5 ± 3.3***	67.7 ± 2.7***	78.0 ± 6.3*
Displacement for maximum load (mm)	0.898 ± 0.067	0.909 ± 0.057	0.841 ± 0.061	0.738 ± 0.058	0.759 ± 0.062	0.777 ± 0.059	0.933 ± 0.092
Energy for maximum load (mJ)	54.94 ± 4.82	60.95 ± 5.32	47.72 ± 3.59	29.34 ± 2.96***	29.71 ± 3.30***	32.20 ± 2.80***	43.96 ± 5.69$^{\bullet}$
Stress for maximum load (MPa)	69.2 ± 7.2	87.2 ± 9.0	68.9 ± 5.3	43.6 ± 3.4***	42.9 ± 2.8***	43.9 ± 2.6**	51.3 ± 5.8
Fracture load (N)	77.8 ± 5.6	85.6 ± 8.2	67.5 ± 3.3	43.2 ± 1.9***	43.6 ± 2.8***	47.1 ± 3.2***	50.6 ± 2.6**
Displacement for fracture load (mm)	1.175 ± 0.055	1.241 ± 0.060	1.214 ± 0.086	1.197 ± 0.058	1.308 ± 0.094	1.222 ± 0.073	1.416 ± 0.107
Energy for fracture load (mJ)	79.57 ± 4.71	94.17 ± 6.37*	79.53 ± 5.56	54.26 ± 2.32***	58.98 ± 5.21**	58.36 ± 3.62**	75.47 ± 8.50$^{\bullet}$
Stress for fracture load (MPa)	52.5 ± 5.8	63.5 ± 8.9	45.7 ± 3.6	29.4 ± 2.6***	28.8 ± 2.5***	30.3 ± 2.0**	33.1 ± 2.8**

Results are presented as means ± SEM. * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$ – significantly different from NOVX control rats. $^{\bullet}p < 0.05$ – significantly different from OVX control rats.

Table IV. Effects of fenugreek seed in the diet (1%), fenugreek seed extract (50 mg of 4-hydroxy-L-isoleucine/kg p.o. daily) and estradiol (0.2 mg/kg p.o. daily) for 4 weeks on mechanical properties of the femoral diaphysis and femoral neck in rats,

Parameter/group	NOVX rats			OVX rats			
	Control	Fenugreek seed in the diet	Fenugreek seed extract	Control	Fenugreek seed in the diet	Fenugreek seed extract	Estradiol
Young's modulus (MPa)	8263 ± 422	8602 ± 418	8746 ± 777	7800 ± 444	8252 ± 729	8692 ± 535	8157 ± 612
Yield load (offset 0.05%) (N)	74.5 ± 3.0	81.2 ± 3.6	70.5 ± 8.8	73.7 ± 2.4	81.5 ± 2.3	75.8 ± 3.27	73.7 ± 1.2
Displacement for yield load (mm)	0.242 ± 0.010	0.257 ± 0.015	0.234 ± 0.034	0.299 ± 0.013	0.283 ± 0.010	0.261 ± 0.013	0.278 ± 0.027
Energy for yield load (mJ)	8.84 ± 0.60	10.06 ± 0.68	8.51 ± 1.4	9.60 ± 0.52	10.19 ± 0.58	9.05 ± 0.58	9.56 ± 0.74
Stress for yield load (MPa)	115.9 ± 4.8	122.3 ± 6.2	108.9 ± 13.8	117.4 ± 5.4	117.7 ± 6.5	120.5 ± 6.8	117.2 ± 6.8
Maximum load (N)	111.5 ± 3.0	116.2 ± 3.8	112.9 ± 3.2	108.3 ± 3.1	117.1 ± 2.6	111.4 ± 3.3	108.4 ± 4.5
Displacement for maximum load (mm)	0.563 ± 0.022	0.571 ± 0.037	0.505 ± 0.028	0.590 ± 0.022	0.555 ± 0.020	0.561 ± 0.026	0.611 ± 0.019
Energy for maximum load (mJ)	39.61 ± 2.16	42.05 ± 4.47	32.99 ± 2.25	36.21 ± 1.75	37.62 ± 2.25	37.75 ± 2.82	41.42 ± 3.13
Stress for maximum load (MPa)	173.7 ± 5.7	174.9 ± 6.9	171.8 ± 6.7	175.3 ± 9.2	168.9 ± 8.0	176.9 ± 8.1	171.8 ± 10.7
Fracture load (N)	98.9 ± 4.2	108.1 ± 4.2	108.8 ± 2.6	103.0 ± 4.1	114.8 ± 3.5**,•	96.9 ± 4.1	103.3 ± 4.3
Displacement for fracture load (mm)	0.682 ± 0.049	0.647 ± 0.046	0.566 ± 0.037	0.633 ± 0.027	0.606 ± 0.038	0.773 ± 0.065	0.675 ± 0.047
Energy for fracture load (mJ)	51.87 ± 5.13	50.22 ± 5.89	39.95 ± 4.39	40.87 ± 2.58	43.09 ± 3.75	59.97 ± 6.86	48.19 ± 5.27
Stress for fracture load (MPa)	153.6 ± 6.0	162.0 ± 5.3	165.9 ± 6.8	165.3 ± 9.0	164.9 ± 7.0	154.5 ± 10.4	162.9 ± 6.5
Femoral neck–maximum load (N)	88.3 ± 3.1	89.8 ± 3.6	82.7 ± 2.8	79.9 ± 2.2	79.0 ± 2.9	79.7 ± 3.6	87.5 ± 4.0

Results are presented as means ± SEM. **$p < 0.01$ – significantly different from NOVX control rats. •$p < 0.05$ – significantly different from OVX control rats.

tio). The extract administration also increased the content of calcium in the bone mineral in the L-4 vertebra in OVX rats.

Effects of Fenugreek on Mechanical Properties of the Tibial Metaphysis

Administration of pulverized fenugreek seed in the diet tended to improve the mechanical properties of cancellous bone of the tibial metaphysis in rats with normal estrogen levels (Table III). The energy accumulated to the fracture point significantly increased in relation to the control rats. Administration of fenugreek seed extract did not significantly affect the mechanical properties of the tibial metaphysis in NOVX rats.

There was a complete lack of any favorable effect of both the seed and the extract in OVX rats. Moreover, OVX rats receiving the extract had significantly decreased the stress in the yield point (offset 0.05%) in relation to the OVX controls.

Effects of Fenugreek on Mechanical Properties of the Femur

Administration of pulverized fenugreek seed in the diet slightly tended to increase the strength of the femoral diaphysis, both in NOVX and OVX rats (Table IV). There was a significant increase in the fracture load in OVX rats receiving fenugreek seed in the diet in relation to the OVX controls. Administration of the fenugreek seed extract did not favorably affect the investigated mechanical parameters of the femoral diaphysis.

Fenugreek administration did not affect the strength of the femoral neck, both in NOVX and OVX rats.

Discussion

Functional foods or food supplements may be of value in prophylaxis of different diseases, including osteoporosis. However, the excessive intake of some functional foods may induce adverse effects. Although numerous preparations are available at the market, their safety in many cases has not been confirmed[25]. Fenugreek is available commercially in seed powder capsules, teas, and pulverized seeds that can be mixed in water[3]. There are also fenugreek extracts marketed[16].

In the present study, effects of fenugreek at two levels of exposure were investigated: the pulverized fenugreek seed was added to the diet in an amount of 1% and the fenugreek seed extract was administered at a dose of 50 mg of 4-hydroxy-L-isoleucine/kg p.o. daily. Although fenugreek dose of 1.25% in the laboratory diet has been regarded as low[25], and 50 mg/kg and bigger doses of particular plant compounds are often used in pharmacological studies, in our opinion, the doses used in the present study should be considered as moderate and high, respectively. The extract was administered by a gastric tube to secure exact dosing. The same approach could not be applied in case of the pulverized fenugreek seed, which formed gel-like suspensions in water, impossible to be administered to the rats in a reasonable volume. The fenugreek diet or extract were administered to the rats for 4 weeks, starting a week after the ovariectomy. Such a period of administration was long enough to induce skeletal effects of substances of plant origin in our previous studies[17,19,26,27].

We have not data concerning the content of diosgenin, trigonelline and 4-hydroxyisoleucine in the fenugreek seed batch used in the present study, and the fenugreek seed extract was standardized for 4-hydroxy-L-isoleucine only. However, according to literature data, the average content of diosgenin, trigonelline and 4-hydroxyisoleucine in dried fenugreek seed is of similar order. For example, Thomas et al[28] reported that the content of 4-hydroxyisoleucine was 0.68-1.26% and that of diosgenin - 0.57-0.67% in fenugreek grown in different environments. Trigonelline content in commercial samples was 0.24%-0.73%[29]. Taking into account the average daily food consumption by rats (about 20-30 g) and the active substance content in the fenugreek seed, it may be roughly estimated that the rats administered the extract received even about 10-times bigger doses of the active compounds than the rats fed *ad libitum* with the diet containing 1% of pulverized fenugreek seed.

During life, bone undergoes growth, modeling and remodeling to enable it preserve the skeletal functions. Bone strength depends on their mass, geometry and composition, material properties, and microstructure[30]. Bone contains specialized cells (osteoclasts, osteoblasts and osteocytes) and mineralized extracellular matrix. There are also spaces including the bone marrow cavity, bone vasculature as well as lacunae and canaliculi surrounding the osteocytes and their cytoplasmic processes[31]. In simple, bone is composed of the mineral (mostly hydroxyapatite), proteins (mainly type I collagen), water (residing mostly in abovementioned spaces) and small amount of lipids[30].

Estrogen deficiency induced characteristic increases in bone turnover (both bone formation and resorption, as indicated by the levels of serum bone turnover markers), leading to the changes in bone composition. Estrogen deficiency worsened bone mineralization, decreasing the ratio of mass of bone mineral to bone mass in the femur and vertebra and calcium content in the mineral of the vertebra. There was no effect on the content of the organic substances, and the water content in the bones increased. Mechanical properties of cancellous bone of the tibial metaphysis were significantly worsened, and there was no effect on the compact bone of the femoral diaphysis.

Although addition of 1% of pulverized fenugreek seed to the laboratory diet did not affect bone composition, it resulted in slight improvement in mechanical properties of the rat bones. In rats with normal estrogen levels, a tendency to increase the strength of cancellous bone of the tibial metaphysis was observed, however, in estrogen-deficient rats, there was a complete lack of any favorable effect in relation to the OVX controls. On the other hand, fenugreek seed in the diet increased strength of compact bone of the femoral diaphysis in OVX rats.

The mechanism of favorable effects of the fenugreek seed added to the diet may be speculated. Trigonelline was reported to exert phytoestrogenic activity[9]. However, as far as potential estrogenic effects of fenugreek seed as the whole are concerned, its extracts were reported to exert rather opposing effects on MCF-7 cells (human estrogen receptor positive breast cancer cell line): estrogenic, proliferative[32], and inducing apoptosis[33]. Based on comparison of the effects of fenugreek seed added to the diet with those of supplementation of estradiol in OVX rats, it may be concluded that the fenugreek seed effects on the skeletal system were not estrogenic. In contrast to the rats fed with fenugreek diet, estradiol administration significantly increased the mass of uterus, counteracted the worsening of the mechanical properties of cancellous bone and did not affect those of compact bone.

Diosgenin, present in fenugreek seed as glycosides, has been reported to inhibit osteoclastogenesis[8] and to stimulate osteogenic activity of osteoblastic cells[5] *in vitro*, as well as to exert some antiosteoporotic effect in rats treated with its sustained delivery *in vivo*[6,7]. However, in our study diosgenin (50 mg/kg p.o.) did not affect the compact bone, and counteracted the worsening of cancellous bone strength in estrogen-deficient rats[34], as opposed to the results presented here for the fenugreek seed and extract.

In previous studies we observed significant increases in the strength of compact bone after administration of large doses of *Trifolium pratense* and *Trifolium medium* extracts[19] and after administration of high doses of phenolic acids[35], which also could not be attributed to estrogenic activity. It may be speculated that those effects might be connected with antioxidant activity of different polyphenolic compounds, which are also present in fenugreek seed[4,36]. In fact, fenugreek seed has been shown to possess antioxidant potential[37-39].

Administration of the higher dose of fenugreek (the fenugreek seed extract) did not significantly affect the investigated parameters in rats with normal estrogen levels, with an exception of the decreased body mass gain. The decreased body mass gain after administration of big doses of fenugreek seed has already been reported[25].

In OVX rats, the fenugreek seed extract not only did not exert a bigger effect on the strength of the femoral diaphysis (compact bone) than that of the fenugreek seed, but it induced unfavorable changes in cancellous bone. In the tibial metaphysis, the mechanical parameters assessed in the yield point (offset 0.05%) further decreased in relation to the OVX controls, indicating some worsening of material properties. Also the ratio of mass of bone mineral to bone mass in the vertebra further decreased, despite the normalization of calcium content in the bone mineral. Water content in the bone increased in comparison with the OVX control rats. Approximately 90% of the total volume of bone water resides in the bone marrow cavity and other spaces mentioned above, and the rest is incorporated within collagen fibrils, hydroxyapatite crystals, and in submicroscopic channels[31]. It seems likely that the increase in the water content in bones of the OVX rats was the result of the increase in bone resorption and widening of the spaces filled with fluids, and that administration of the extract intensified the changes in cancellous bone. Summing up, the slight favorable effects observed in rats receiving pulverized fenugreek seed at a moderate (1%) level in the diet, completely disappeared in rats receiving much bigger dose of the fenugreek seed in a form of extract. It may be supposed that the mechanism of fenugreek action on the skeletal system is probably complex, due to effects of its different constituents.

Results of this study are consistent with the results of Muraki et al[25,40], who observed some favorable effects of lower intake of fenugreek or fenugreek with reduced bitterness in the diet-induced metabolic disorders, whereas after very high fenugreek intake (12.3% in the diet) also adverse effects were observed[25].

Results of the present study may be of some practical value. Although the favorable dietary fenugreek effects were rather weak, the unfavorable effects of the extract standardized for 4-hydroxyisoleucine should raise concern. Even cursory survey of Internet websites reveals that fenugreek supplements containing 4-hydroxyisoleucine have been used by bodybuilders. Similarly to anabolic steroids, in consequence, they may turn out dangerous to the skeletal system[41].

Conclusions

Low dietary intake of fenugreek seed may exert slight favorable skeletal effects, whereas at high doses fenugreek seed may damage the skeletal system.

Acknowledgements

We thank Justyna Kozłowska, Maria Lipecka-Karcz and Michał Matysiak for their help during the experiments. The study was supported by grant No KNW-1-076/P/2/0, Medical University of Silesia, Katowice, Poland.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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