Acta Poloniae Pharmaceutica – Drug Research, Vol. 72 No. 3 pp. 587–596, 2015 ISSN 0001-6837
Polish Pharmaceutical Society

BIOCHANIN A SHOWS NO EFFECT ON SKELETAL SYSTEM IN OVARIECTOMIZED RATS, WHEN ADMINISTERED IN MODERATE DOSE

ILONA KACZMARCZYK-SEDLAK*, MARIA ZYCH, WERONIKA WOJNAR, EWA OZIMINA-KAMIŃSKA, SŁAWOMIR DUDEK, NATALIA CHADAŁA and AGNIESZKA KACHEL

Medical University of Silesia in Katowice, School of Pharmacy with the Division of Laboratory Medicine, Department of Pharmacognosy and Phytochemistry, Jagiellońska 4, 41-200 Sosnowiec, Poland

Abstract: Biochanin A is a naturally occurring isoflavone. Its main sources are clover species such as *Trifolium pretense*, *Trifolium subterraneum* or *Trifolium incarnatum*. Phytoestrogens, including isoflavones, are plant-derived substances, which exhibit estrogen-like properties, thus they may be used as an alternative for hormonal replacement therapies and prevent postmenopausal osteoporosis. Therefore, the aim of the presented study, was to investigate the effect of biochanin A on chemistry and mechanical properties of skeletal system in rats with ovariectomy-induced osteoporosis. The animals were divided into 4 groups – (I) sham-operated rats, (II) ovariectomized rats, (III) ovariectomized rats receiving estradiol at a dose of 0.2 mg/kg *p.o.*, which were a positive control, and (IV) ovariectomized rats receiving biochanin A at a dose of 5 mg/kg *p.o.* for four weeks. The administered dose of biochanin A is considered as moderate for human, which can be received in the dietary supplements, and was established using ten-fold conversion rate resulting from faster metabolism in rats. Obtained results showed that ovariectomy induced harmful changes in bone tissue, causing worsening in both chemistry and mechanical parameters in bones. Administration of biochanin A to ovariectomized rats did not affect any changes in bone tissue in comparison to the bones of untreated ovariectomized rats. There was neither improvement nor deterioration noted in chemical composition and mechanical properties in all analyzed bones. Basing on the results, it could be concluded, that biochanin A administered in a moderate dose shows no influence on bone tissue of rats with ovariectomy-induced osteoporosis.

Key words: biochanin A, ovariectomy, rats, osteoporosis, bones

Biochanin A (5,7-dihydroxy-4'-methoxy-isoflavone) is an isoflavone, which can be found in many clover species. In zigzag clover (*Trifolium medium*) it occurs in high concentration, and in red clover (*Trifolium pratense*), crimson clover (*Trifolium incarnatum*), haresfoot clover (*Trifolium arvense*), hungarian clover (*Trifolium pannonicum*) and red-feather clover (*Trifolium rubens*) its concentration is lower (1). It is also present in other plants such as soy, alfalfa, peanuts, and chickpea (2). This isoflavone is also reported in *Cassia fistula* and *Dalbergia odorifera* (3, 4). Biochanin A has been proven to have many pharmacological activities. Some *in vitro* studies indicated that this substance may act as a neuroprotective agent in L-glutamate-induced cytotoxicity in Parkinson's, Alzheimer's or Huntington's diseases (5), it is also an inhibitor of fatty acid amide hydrolase, the enzyme responsible for the hydrolysis of anandamide – the endogenous cannabinoid receptor ligand (6). It also inhibits allergic response in rat basophilic leukemia cells (7). There are reports that biochanin A shows antioxidative properties in cell cultures (8) as well as anti-inflammatory and anti-proliferative activities on RAW 264.7, HT-29 cell lines and mouse peritoneal macrophages (9). This isoflavone suppresses the proliferation of oral squamous carcinoma cells (10) and could be an anticancer agent which can selectively target cancer cells inhibiting multiple signaling pathways in HER-2-positive breast cancer cells (11). Moreover, it promotes apoptosis of prostate cancer cells (12, 13). Antiparasitic activity of biochanin A towards *Leishmania chagasi* and *Trypanosoma cruzi* is also confirmed (3). Furthermore, this flavonoid acts as antiviral agent in H5N1 influenza A virus-infected cells (14) and

* Corresponding author: farmafit@sum.edu.pl

shows antimicrobial properties against *Clostridium* spp. (15).

What is more, there are *in vitro* and *in vivo* studies, indicating that biochanin A may inhibit melanogenesis, thus it can prevent the abnormal skin pigmentation in mice (16). This isoflavone was also examined *in vivo* in many animal models. Some investigations on rats show that biochanin A is an antifibrotic agent which prevents liver injuries (2, 17). Moreover, it shows antihyperglicemic effect (18), and can be used as chemoprevention of mammary tumorigenesis (19). Studies on murine xenograft model indicate that this substance may also inhibit the growth of the human breast cancer cells implanted into mice (20).

Isoflavones, including biochanin A, are considered as the phytoestrogens, due to their structural and functional similarity to 17β-estradiol. Phytoestrogens are plant-derived, nonsteroidal substances, which can bind to estrogen receptors (ER) and mimics the effect of estradiol, but their activity is lower when compared to 17β-estradiol (21). During menopause in women, endogenous estrogen level decreases, what leads to disadvantageous changes, such as flushing, but one of the most dangerous effect is osteoporosis (22). Hormonal replacement therapy (HRT) is used in postmenopausal women in order to reduce these symptoms, however this therapy is known to have some adverse effects on women's health (23). Therefore, to overcome negative effects of HRT and minimize symptoms of menopause, other substances, including isoflavones, have been tested. Until now, the effects of biochanin A on skeletal system have been examined in *in vitro* and *in vivo* studies. The *in vitro* experiment was conducted on mesenchymal stem cells and the results indicate that biochanin A prevents adipogenesis and enhances the differentiation of osteoblasts (24). Oral administration of this isoflavone to ovariectomized rats at a dose of 25 mg/kg for 14 weeks showed positive results in skeletal system (25). Even though the intake of phytoestrogens is commonly considered as a safe therapy, there are also some reports that long-term administration of higher doses of these substances may result in some complications, such as abnormal uterus bleedings or the increased occurrence of endometrial hyperplasia (26, 27, 28). Thus, in our study, the effect of biochanin A on bones in ovariectomized rats was investigated while administering this isoflavone at a dose of 5 mg/kg orally (*p.o.*) for 4 weeks.

MATERIALS AND METHODS

Virgin three-month-old Wistar female rats were provided by the Centre of Experimental Medicine at the Medical University of Silesia. Animals were fed with standard laboratory chow *ad libitum* and since the day preceding the experiment, the standard chow was replaced with chow containing no soybean. The research was carried out with the approval of the Local Ethics Commission in Katowice.

Rats were divided into four groups (n = 7): I – the control group of sham-operated, vehicle treated rats (SHAM), II – the control group of ovariectomized, vehicle treated rats (OVX), III – the ovariectomized rats receiving estradiol at a dose of 0.2 mg/kg *p.o.* (OVX + ES), and IV – the ovariectomized rats receiving biochanin A at a dose of 5 mg/kg *p.o.* (OVX + BioA). Administration of analyzed substances started one week after surgery and continued for 4 weeks.

All animals were weighted at the first day or research and after 4 weeks of the administration of

Table 1. Effects of estradiol and biochanin A on the body weight gain and weight of organs in ovariectomized rats.

Parameters	SHAM	OVX	OVX + ES	OVX + BioA
Final body weight [g]	234.4 ± 4.3	245.8 ± 4.7	238.1 ± 6.4	246.4 ± 8.5
Body weight gain after 4 weeks [g]	15.9 ± 1.8	29.7 ± 2.4^{AA}	23.0 ± 1.7	37.6 ± 5.0
Uterus weight [g]	0.616 ± 0.085	0.103 ± 0.005^{AA}	0.252 ± 0.009^{BB}	0.100 ± 0.005
Thymus weight [g]	0.313 ± 0.018	0.482 ± 0.052^{AA}	0.500 ± 0.024	0.578 ± 0.048

Results are presented as the means ± SEM (n = 7). ^{AA} - $p < 0.01$ – statistically significant differences between the OVX and the SHAM groups; ^{BB} - $p < 0.01$ – statistically significant differences in comparison with the OVX group.

the substances. Moreover, the body mass gain was monitored during the whole experiment. One week before treatment with biochanin A and estradiol, sham surgery and bilateral ovariectomy were performed in general anesthesia induced by the mixture of ketamine and xylazine intraperitoreally. After 4 weeks of drugs administration, all animals were sacrificed with use of general anesthesia induced by ketamine and xylazine and cardiac exsanguination. Uterus, thymus and following bones: the right and left tibia and the right and left femur, as well as the L-4 vertebra were excised from each of the rats. The mass of analyzed bones was presented as the ratio of their mass per body mass determined after 4 weeks of the experiment.

The analysis of the mechanical properties of the bones

Investigation of the bone mechanical properties of the left femoral diaphysis, right tibial proximal metaphysis and right femoral neck was performed using the Instron apparatus, model 3342 500 N. Results were studied by using the software Bluehill 2, version 2.14.

For the femoral diaphysis, the bending test with three point loading was applied and following parameters were measured: the maximum load, the fracture load, displacement for maximum load, displacement for fracture load, and energy for maximum load. The Young's modulus and stress were also determined. Assessment of the mechanical properties of tibial proximal metaphysis was performed according to the method of Stürmer et al. (29, 30), using the three-point bending test. Likewise in femoral diaphysis, in tibial metaphysis: the maximum load, the fracture load, displacement for maximum load, displacement for fracture load, energy for maximum load, stress and Young's modulus were measured. The mechanical properties of the femoral neck were analyzed by performing a compression test and the maximum load affecting the femoral neck was determined. All methods used to examine the mechanical properties of the bones were described in previous studies (31, 32).

Assessment of the chemical content of the bones and the serum

Chemical content analysis involved the calculation of the water, organic substances and mineral substances content and the content of calcium and phosphorus in left femur (after bone mechanical properties analysis) and left tibia as well as in L-4 vertebra.

In order to determine the water content, the tibia, femur and L-4 vertebra were weighed after lyophilization in the lyophilizer Labconco Freezone 6 (USA) for seven days (temperature: -53^{o}C, pressure: 0.03 mBa). The difference between the bone mass obtained directly after the isolation and the bone mass determined after lyophilization corresponds to the water content.

Afterwards, the lyophilized bones were mineralized for 48 h in the muffle furnace type LG/11/C6 produced by Naberherm (The Netherlands) and weighed again. The bone ash obtained during the mineralization process comprised the mass of the mineral substances, and the difference between bone mass determined after lyophilization and bone mass after mineralization corresponds to the content of the organic substances in analyzed bones. The content of water, organic and mineral substances was presented as the ratio of water, organic and mineral substances mass per 100 mg of the bone mass after isolation.

In order to determine calcium and inorganic phosphorus content, mineralized bones were dissolved in 6 M HCI, diluted in distilled water, and then examined by the spectrophotometric method, using the kit Pointe Scientific, Inc. (USA) The content of calcium and phosphorus in the analyzed bones was presented as the ratio of the quantity of calcium and phosphorus per 100 mg of mineral substances.

In serum, the content of calcium and inorganic phosphorus was also determined. The assay was performed by spectrophotometric method using the kit Pointe Scientific Inc.

Statistical analysis

The results obtained during the tests were presented as the arithmetic means ± SEM.

The results were evaluated by one-way ANOVA, followed by Duncan's *post hoc* test. Nonparametric tests: Kruskal-Wallis and Mann-Whitney U test were performed, when necessary (lack of normality or of homogeneity of variance).

The differences were considered to be statistically significant if $p < 0.05$.

RESULTS AND DISCUSSION

The menopause is the mark of the end of natural female reproductive life and it is defined as the permanent cessation of menstruation resulting from the loss of ovarian follicular activity (33). The loss of the activity of ovarian follicular results in the decrease of endogenous estrogens. When such deficiency occurs, the rate of bone loss is accelerated – the osteoclasts' lifespan is increased and

osteoblasts' decreased. All these actions lead to postmenopausal osteoporosis (34). One of the most dangerous effects of postmenopausal osteoporosis are bone fractures. Due to the fact that the studies on bone mechanical resistance are unable to conduct on human, the animal model was developed. The best model for bone studies in postmenopausal period are ovariectomized rats. After ovariectomy, the osteoporotic changes similar to those in postmenopausal women are observed in these animals (35).

In our study, the ovariectomy proved to cause typical changes in rats. The body weight gain after 4 weeks in ovariectomized rats (OVX) was significantly higher than in sham-operated rats (SHAM) by 87.0%. What is more, the uterus weight was lowered by 83.2% and thymus weight increased by 53.9% (Table 1). Furthermore, weight of all analyzed bones was lower in OVX than in SHAM rats, but these changes were not statistically significant (Table 2). Estrogen deficiency also resulted in bone chemistry. The water content in analyzed bones increased, but the most significant changes were observed in femur and L-4 vertebra, where the water content was 9.9% and 11.8%, respectively, higher in OVX than in SHAM (Table 3). Furthermore, there was decrease of mineral content observed in tibia and L-4 vertebra by 4.4% and 8.9%, respectively, what is more, in L-4 vertebra, calcium content also decreased by 6.5% when compared to SHAM rats. Other studied parameters of bone chemical composition (organic fraction content as well as phosphorus content) were not affected by ovariectomy in statistically significant manner (Table 3). Ovariectomy also affected the mechanical properties of bones. The greatest changes were observed in tibia, where the following parameters were affected: maximal load, maximum stress, fracture load, energy for fracture load and stress for fracture load decreased by 43.9, 42.8, 44.5, 30.2 and 43.2%, respectively, in tibial metaphysis. Other parameters were not changed in statistically significant manner in all analyzed bones, however, the maximum stress and fracture stress were also lower in OVX group than in SHAM rats, both in femoral diaphysis as well as tibial metaphysis. There were no changes detected in femoral neck when comparing OVX to SHAM group (Table 5). Similar changes in ovariectomized rats were observed in our previous study (32), as well as in other studies (30, 36, 37).

In our experiment, the positive control was used. For this purpose, estradiol at a dose of 0.2 mg/kg *p.o.* was administered to ovariectomized rats. This substance is one of estrogens which can be used in HRT. HRT is used to overcome unfavorable effects of postmenopausal symptoms including osteoporosis (38, 39). Moreover, estradiol was also used in other studies as a positive control (30, 32, 36, 37). Ovariectomized rats receiving estradiol (OVX + ES) showed statistically insignificant decrease of body weight gain in comparison to OVX rats. What is more, in OVX + ES, the uterus weight increased by 147.7%, when compared to OVX group (Table 1). Macrometric parameters of all examined bones were not altered by administration of estradiol (Table 2). Chemical composition of analyzed bones was slightly affected by administration of estradiol at a dose of 0.2 mg/kg *p.o.* Except for mineral content in femur and L-4 vertebra, which increased by 4.3 and 6.0%, respectively, in OVX + ES when compared to OVX, other parameters in all

Table 2. Effects of estradiol and biochanin A on bone macrometric parameters in ovariectomized rats.

Parameters	SHAM	OVX	OVX + ES	OVX + BioA
FEMUR				
Weight [g]	0.663 ± 0.012	0.628 ± 0.006	0.631 ± 0.023	0.633 ± 0.023
Length [mm]	33.45 ± 0.27	33.50 ± 0.22	32.91 ± 0.25	33.37 ± 0.23
Diameter [mm]	3.25 ± 0.02	3.22 ± 0.04	3.20 ± 0.05	3.20 ± 0.05
TIBIA				
Weight [g]	0.517 ± 0.008	0.477 ± 0.014	0.489 ± 0.016	0.484 ± 0.015
Length [mm]	37.54 ± 0.34	37.59 ± 0.04	36.78 ± 0.19	37.01 ± 0.42
Diameter [mm]	2.63 ± 0.05	2.58 ± 0.05	2.62 ± 0.05	2.59 ± 0.03
L-4 VERTEBRA				
Weight [g]	0.176 ± 0.006	0.168 ± 0.011	0.163 ± 0.006	0.154 ± 0.005

Results are presented as the means ± SEM (n = 7).

Table 3. Effects of estradiol and biochanin A on bone mineral, H_2O, organic compounds, calcium and phosphorus content in ovariectomized rats.

Parameters	SHAM	OVX	OVX + ES	OVX + BioA
FEMUR				
H_2O content [mg/100 mg bone weight]	27.23 ± 0.74	29.92 ± 1.12^{A}	28.81 ± 0.52	30.47 ± 0.46
Organic compounds [mg/100 mg bone weight]	25.10 ± 0.69	24.87 ± 0.43	24.05 ± 0.17	24.13 ± 0.23
Mineral content [mg/100 mg bone weight]	47.67 ± 1.06	45.22 ± 0.81	47.14 ± 0.50	45.40 ± 0.55
Calcium content [mg/100 mg mineral substances]	39.43 ± 0.34	38.07 ± 0.94	39.55 ± 0.37	38.31 ± 0.45
Phosphorus content [mg/100 mg mineral substances]	15.79 ± 0.19	15.15 ± 0.37	15.55 ± 0.27	15.47 ± 0.17
TIBIA				
H_2O content [mg/100 mg bone weight]	25.60 ± 0.74	26.82 ± 1.16	26.32 ± 0.75	28.52 ± 0.75
Organic compounds [mg/100 mg bone weight]	25.89 ± 0.42	26.81 ± 0.34	26.24 ± 0.35	25.98 ± 0.47
Mineral content [mg/100 mg bone weight]	48.51 ± 0.43	46.37 ± 0.93^{A}	47.44 ± 0.52	45.50 ± 0.33
Calcium content [mg/100 mg mineral substances]	41.54 ± 1.53	38.03 ± 1.81	40.96 ± 0.52	41.79 ± 0.42
Phosphorus content [mg/100 mg mineral substances]	15.05 ± 1.04	14.66 ± 0.82	15.52 ± 0.26	16.00 ± 0.29
L-4 VERTEBRA				
H_2O content [mg/100 mg bone weight]	26.44 ± 0.24	29.56 ± 0.60^{AA}	27.66 ± 0.79	30.79 ± 0.58
Organic compounds [mg/100 mg bone weight]	26.29 ± 0.31	27.37 ± 0.73	26.58 ± 0.42	26.12 ± 0.33
Mineral content [mg/100 mg bone weight]	47.26 ± 0.37	43.08 ± 0.89^{AAA}	45.76 ± 0.66^{BB}	43.08 ± 0.37
Calcium content [mg/100 mg mineral substances]	43.78 ± 0.78	40.92 ± 0.65^{A}	41.06 ± 0.81	39.21 ± 0.61
Phosphorus content [mg/100 mg mineral substances]	16.71 ± 0.47	16.30 ± 0.45	15.96 ± 0.21	15.72 ± 0.21

Results are presented as the means ± SEM (n = 7). ^{A} - $p < 0.05$, ^{AA} - $p < 0.01$, ^{AAA} – $p < 0.001$ – statistically significant differences between the OVX and the SHAM groups; ^{BB} - $p < 0.01$ – statistically significant differences in comparison with the OVX group.

Table 4. Effects of estradiol and biochanin A on serum concentrations of calcium and inorganic phos-phorus in ovariectomized rats.

Parameters	SHAM	OVX	OVX + ES	OVX + BioA
Calcium mg/100 mL]	8.99 ± 0.37	9.13 ± 0.20	9.43 ± 0.59	9.49 ± 0.45
Phosphorus [mg/100 mL]	5.45 ± 0.42	6.07 ± 0.49	7.55 ± 0.64	6.29 ± 0.36

Results are presented as the means ± SEM (n = 7).

Table 5. Effects of estradiol and biochanin A administered for 4 weeks on bone mechanical properties in ovariectomized rats.

Parameters	SHAM	OVX	OVX + ES	OVX + BioA
FEMORAL DIAPHYSIS				
Maximal load [N]	100.4 ± 4.8	99.9 ± 3.7	99.1 ± 5.1	101.1 ± 4.7
Displacement for maximal load [mm]	0.494 ± 0.020	0.546 ± 0.043	0.478 ± 0.030	0.591 ± 0.040
Energy for maximum load [J]	0.029 ± 0.002	0.032± 0.004	0.027 ± 0.003	0.036 ± 0.004
Maximum stress [MPa]	172.1 ± 26.3	116.6 ± 5.0	118.9 ± 2.9	121.0 ± 4.2
Fracture load [N]	100.1 ± 4.8	99.9 ± 3.7	99.1 ± 5.1	100.9 ± 4.7
Displacement for fracture load [mm]	0.497 ± 0.022	0.546 ± 0.043	0.478 ± 0.030	0.595 ± 0.041
Energy for fracture load [J]	0.030 ± 0.003	0.032 ± 0.004	0.027 ± 0.003	0.036 ± 0.004
Stress for fracture load [MPa]	171.5 ± 26.2	116.6 ± 5.0	118.9 ± 2.9	120.8 ± 4.2
Young's modulus [MPa]	6362 ± 274	5120 ± 325	5925 ± 268	5828 ± 358
TIBIAL METAPHYSIS				
Maximal load [N]	122.3 ± 4.2	68.6 ± 5.1^{AA}	85.7 ± 12.0	69.4 ± 3.1
Displacement for maximal load [mm]	0.947 ± 0.084	0.946 ± 0.039	0.840 ± 0.061	0.941 ± 0.066
Energy for maximum load [J]	0.065 ± 0.007	0.039 ± 0.004	0.044 ± 0.008	0.045 ± 0.004
Maximum stress [MPa]	85.4 ± 7.7	48.8 ± 7.6^{A}	70.2 ± 13.3	45.5 ± 3.6
Fracture load [N]	118.9 ± 4.6	66.0 ± 5.4^{AA}	84.9 ± 11.9	68.1 ± 2.9
Displacement for fracture load [mm]	1.042 ± 0.087	1.156 ± 0.080	0.886 ± 0.066	1.038 ± 0.057
Energy for fracture load [J]	0.076 ± 0.005	0.053 ± 0.005^{A}	0.049 ± 0.009	0.051 ± 0.003
Stress for fracture load [MPa]	82.8 ± 6.9	47.0 ± 7.4^{A}	69.6 ± 13.2	44.6 ± 3.3
Young's modulus [MPa]	3692 ± 818	2762 ± 1335	4009 ± 1135	2490 ± 807
FEMORAL NECK				
Maximal load (N)	84.0 ± 9.4	80.3 ± 6.8	84.3 ± 7.2	79.4 ± 4.9

Results are presented as the means ± SEM (n = 7). ^{A} - $p < 0.05$, ^{AA} - $p < 0.01$ – statistically significant differences between the OVX and the SHAM groups.

studied bones were not changed (Table 3). Administration of estradiol to ovariectomized rats did not affect mechanical properties of bones – all analyzed parameters remained unchanged (Table 5). There were no significant changes in serum calcium and inorganic phosphorus content in OVX + ES rats, when compared to OVX rats (Table 4).

Biochanin A was previously examined on bones *in vivo* in ovariectomized rats. The authors of the study administered biochanin A at a dose of 25 mg/kg *p.o.* to the animals for 14 weeks. Their results indicated that this isoflavone shows positive effect on rodents' skeletal system, improving the bone mineral density (BMD), bone mineral content (BMC) and the percentage of the trabecular bone volume in relation to the total tissue volume (25).

There are, however, some reports indicating that the usage of isoflavones at high doses and in long-time period could be unsafe for animals and human. Some studies show that the intake of this kind of phytoestrogens may lead to uterotropic changes and even to abnormal endometrial bleedings (26-28). What is more, Leclerc et al. indicate that phytoestrogens may interact with other receptors than ER (40).

For this reason, in our study, we administered lower dose of this isoflavone for 4 weeks. This dose was evaluated on the basis of commercially available pharmacological products named Promensil® and Promensil® Forte. In Promensil®, there is 26 mg of biochanin A per 40 mg of all isoflavones. Promensil® Forte has got double dose of isoflavones, when compared to Promensil® (41). Therefore, patients may receive biochanin A at doses of 26 or 52 mg. Considering the commonly used ten-fold conversion rate resulting from faster metabolism in rats, we administered biochanin A at a dose of 5 mg/kg to animals. Similar conversion was previously used in other study (42).

The treatment with biochanin A at a dose of 5 mg/kg *p.o.* showed no results on body weight gain, uterus weight and thymus weight in OVX + BioA rats when compared to OVX rats, however, there was statistically insignificant tendency of greater body weight gain after 4 weeks of treatment in OVX + BioA rats (by 37.9%), than in OVX rats (Table 1). Our results describing uterus weight overlap with those obtained by Su et al. (25). Also, the administration of this isoflavone did not affect any of the analyzed macrometric parameters of femur, tibia and L-4 vertebra in OVX + BioA rats in comparison to OVX rats (Table 2). The analysis of chemical composition revealed that administration of biochanin A did not cause any statistically significant changes in all examined bones in OVX + BioA rats when compared to OVX rats. Neither water content, organic content, mineral content nor the calcium and phosphorus content were altered (Table 3). What is more, the examination of mechanical properties also showed that biochanin A had no influence on studied bones in treated rats when compared to untreated animals (Table 5). Our results revealed that biochanin A administered at a dose of 5 mg/kg *p.o.* to ovariectomized rats shows neither beneficial nor harmful effect on skeletal system in laboratory animals. However, in previous studies, Su et al. reported that treatment with higher dose (25 mg/kg) in long-time period (14 weeks) shows advantageous effect on the rats' bones – it prevented the bone loss in ovariectomized animals. The authors examined bone mineral density, bone mineral content and bone volume. The serum calcium and inorganic phosphorus were also studied, but their levels were unchanged (25). In our study, the concentration of inorganic phosphorus and calcium in serum was also determined, and these results overlap with those presented by Su et al. (25) – there were no significant differences between all examined groups (Table 4).

There are many reports indicating that biochanin A is demethylated to genistein in digestive system. Such mechanism was observed in sheep, administered with ^{14}C-labeled biochanin A, where labeled genistein was observed in rumen fluids (43). *In vitro* studies conducted on human liver microsomes also showed, that biochanin A is metabolized to genistein and some conjugates by cytochrome P450 (44). Pharmacokinetics and metabolism of biochanin A was also studied in rats. Moon et al. (45) demonstrated that biochanin A in male rats after oral and intraperitoneal administration was demethylated to genistein and its conjugates. Similar observations were described by Singh et al. in female rats. The authors also determined the oral bioavailability of this isoflavone and considered this parameter as low. In this study, the authors also indicated that biochanin A is rapidly converted into free genistein, as well as into genistein and biochanin A conjugates (46).

Examining the effect of biochanin A on skeletal system, this isoflavone should not be considered as single agent influencing the bone tissue. Biochanin A in digestive system metabolizes *via* demethylation to other substances including genistein, which is also a potent agent affecting the bone metabolism. The majority of scientific reports suggests that genistein shows beneficial properties on bones, preventing from osteoporotic changes both in laboratory animals (including ovariectomized rats) and women suffering from postmenopausal osteoporosis (47, 48).

There are, however, some reports indicating that genistein shows disadvantageous effect on skeletal system. *In vivo* experiments conducted by Śliwiński et al. (49) proved that genistein has harmful effects on mechanical properties of bones and their chemical composition. There is also another study revealing that genistein displays neither beneficial nor injurious effect on skeletal system in rats (50).

Śliwiński et al. examined the effect of genistein on ovariectomized rats at a dose of 5 mg/kg; he also conducted *in vitro* experiment on mice osteoclasts applying doses of 1×10^{-9}, 1×10^{-8} and 1×10^{-7} M. In *in vitro* studies, the authors observed the advantageous effects of genistein revealed by the decreased osteoclasts number and reduced RANKL/OPG ratio. The best results in *in vitro* experiment were observed at a dose of 1×10^{-8} M. In spite of beneficial effect of genistein observed in *in vitro* study, the authors demonstrated that administering this isoflavone to ovariectomized rats at a dose of 5 mg/kg for 4 weeks fails to show any beneficial influence on bones. What is more, the administration of this isoflavone caused deterioration of some mechanical parameters e.g., maximal load in bones (49).

Another article focused on the effect of genistein on bone tissue in rats administered at low doses for long time period (5 months) also demonstrated that this isoflavone does not improve the microarchitecture of bones, regardless of the age of the animals and whether they had progeny or not. The authors examined two doses administered orally – 1.6 mg/kg and 3.2 mg/kg, and used three animal models: 3-month-old virgin rats, 12-month-old retired breeder rats and 14-month-old retired breeder rats. There were no statistically significant changes observed in any of analyzed groups which might suggest that genistein shows beneficial effect on BMC, BMD or histomorphological parameters in cortical and cancellous bone (50).

The above-mentioned examples of the effect of genistein on bone tissue, as well as the reports about pharmacokinetics of biochanin A may provide the explanation of the reason why this isoflavone administered at a moderate dose (converted to average dose taken by human) – 5 mg/kg for 4 weeks did not show beneficial effect on skeletal system in ovariectomized rats.

Biochanin A is one of the major constituents in red clover herb (*Trifolii pratense herba*). Studies focused on the effect of red clover extracts on osteoporotically changed bone tissue in ovariectomized rats proved that those extracts show beneficial effect on skeletal system (37, 51). Our previous study indicated that formononetin, which is also present in red clover, also shows the advantageous effect on bones in ovariectomized rats (32). However, Cegieła et al. concluded that the effect exerted by red clover is different and more complex than the one of estradiol itself, thus it could be assumed that not only isoflavones act as a preventive agents, but other constituents may affect skeletal system in rats (37).

CONCLUSION

Due to the fact that the biological activity of biochanin A depends on many factors such as metabolism and pharmacokinetics, it is very important to examine several doses of this substance. In our research we conducted the test investigating effect of biochanin A on skeletal system of rats with ovariectomy induced osteoporosis, using the dose, which can be assumed as moderate. This dose (5 mg/kg *p.o.*) was converted into the dose that can be intake by human in dietary supplements available in pharmaceutical market.

The results obtained during studies indicate that the administration of biochanin A at a dose of 5 mg/kg *p.o.* for 4 weeks to ovariectomized rats causes neither improvement nor deterioration of mechanical parameters of bones and their chemical composition.

Acknowledgment

This study was supported by Grant No. KNW-1-043/K/3/0. The authors would like to thank the staff of Department of Pharmacology (School of Pharmacy with The Division of Laboratory Medicine, Medical University of Silesia) for making this study possible.

Conflict of interests

The authors state that they have no conflict of interests.

REFERENCES

1. Zgórka G.: J. AOAC Int. 94, 22 (2011).
2. Breikaa R.M., Algandaby M.M., El-Demerdash E., Abdel-Naim A.B.: PLoS One 8, e69276 (2013).
3. Sartorelli P., Carvalho C.S., Reimão .J.Q., Ferreira M.J., Tempone A.G.: Parasitol. Res. 104, 311 (2009).
4. Zhang D.Y., Zu Y.G., Fu Y.J., Luo M., Gu C.B. et al.: Sep. Purif. Technol. 83, 91 (2011).

5. Tan J.W., Tham C.L., Israf D.A., Lee S.H., Kim M.K.: Neurochem. Res. 38, 512 (2013).
6. Thors L., Burston J.J., Alter B.J., McKinney M.K., Cravatt B.F. et al.: Br. J. Pharmacol. 160, 549 (2010).
7. Chung M.J., Sohng J.K., Choi D.J., Park Y.I.: Life Sci. 93, 401 (2013).
8. Saviranta N.M.M., Veeroos L., Granlund L.J., Hassinen V.H., Kaarniranta K., Karjalainen R.O. et al.: Food Res. Int. 44, 109 (2011).
9. Kole L., Giri B., Manna S.K., Pal B., Ghosh S.: Eur. J. Pharmacol. 653, 8 (2011).
10. Johnson T.L., Lai M.B., Lai J.C., Bhushan A.: Evid. Based Complement. Alternat. Med. 7, 351 (2010).
11. Sehdev V., Lai J.C., Bhushan A.: J. Oncol. 2009, 121458 (2009).
12. Seo Y.J., Kim B.S., Chun S.Y., Park Y.K., Kang K.S., Kwon T.G.: J. Korean Med. Sci. 26, 1489 (2011).
13. Szliszka E., Czuba Z.P., Mertas A., Paradysz A., Krol W.: Urol. Oncol. 31, 331 (2013).
14. Sithisarn P., Michaelis M., Schubert-Zsilavecz M., Cinatl J. Jr.: Antiviral Res. 97, 41 (2013).
15. Sklenickova O., Flesar J., Kokoska L., Vlkova E., Halamova K., Malik J.: Molecules 15, 1270 (2010).
16. Lin V.C., Ding H.Y., Tsai P.C., Wu J.Y., Lu Y.H., Chang T.S.: Biosci. Biotechnol. Biochem. 75, 914 (2011).
17. Breikaa R.M., Algandaby M.M., El-Demerdash E., Abdel-Naim A.B.: Biosci. Biotechnol. Biochem. 77, 909 (2013).
18. Harini R., Ezhumalai M., Pugalendi K.V.: Eur. J. Pharmacol. 676, 89 (2012).
19. Mishra P., Kale R.K., Kar A.: Mol. Cell. Biochem. 312, 1 (2008).
20. Moon Y.J., Shin B.S., An G., Morris M.E.: Pharm. Res. 25, 2158 (2008).
21. Kuhnle G.G.C., Vogiatzoglou A., Ward H.A., Khaw K.T.: Proc. Nutr. Soc. 70 (OCE4), E254 (2011).
22. Bijak M., Połać I., Borowiecka M., Nowak P., Stetkiewicz T., Pertyński T. : Przegl. Menopauz. 6, 402 (2010) (Polish).
23. Atkinson C., Compston J.E., Day N.E., Dowsett M., Bingham S.A.: Am. J. Clin. Nutr. 79, 326 (2004).
24. Su S.J., Yeh Y.T., Su S.H., Chang K.L., Shyu H.W. et al.: Evid. Based Complement. Alternat. Med. 2013, 846039 (2013).
25. Su S.J, Yeh Y.T., Shyu H.W.: Evid. Based Complement. Alternat. Med. 2013, 594857 (2013).
26. van Hunsel F.P., Kampschöer P.: Ned. Tijdschr. Geneeskd. 156, A5095 (2012).
27. Unfer V., Casini M.L., Costabile L., Mignosa M., Gerli S., Di Renzo G.C.: Fertil. Steril. 82, 145 (2004).
28. Chandrareddy A., Muneyyirci-Delale O., McFarlane S.I., Murad O.M.: Complement. Ther. Clin. Pract. 14, 132 (2008).
29. Stürmer E.K., Seidlová-Wuttke D., Sehmisch S., Rack T., Wille J. et al.: J. Bone Miner. Res. 21, 89 (2006).
30. Folwarczna J., Pytlik M., Zych M., Cegieła U., Kaczmarczyk-Sedlak I. et al.: Mol. Nutr. Food Res. 57, 1772 (2013).
31. Kaczmarczyk-Sedlak I., Janiec W., Pytlik M., Śliwiński L., Folwarczna J. et al.: Pharmacol. Rep. 57, 203 (2005).
32. Kaczmarczyk-Sedlak I., Wojnar W., Zych M., Ozimina-Kamińska E., Taranowicz J., Siwek A.: Evid. Based Complement. Alternat. Med. 2013, 457052 (2013).
33. Burger H.G., Hale G.E., Robertson D.M., Dennerstein L.: Hum. Reprod. Update 13, 559 (2007).
34. Seeman E.: Curr. Osteoporos. Rep. 2, 90 (2004).
35. Kalu D.N.: Bone Miner. 15, 175 (1991).
36. Folwarczna J., Zych M., Burczyk J., Trzeciak H., Trzeciak H.I.: Planta Med. 75, 1567 (2009).
37. Cegieła U., Folwarczna J., Pytlik M., Zgórka G.: Evid. Based Complement. Alternat. Med. 2012, 921684 (2012).
38. Rossouw J.E., Anderson G.L., Prentice R.L., LaCroix A.Z., Kooperberg C. et al.: J. Am. Med. Assoc. 288, 321 (2002).
39. Bowring C.E., Francis R.M.: Menopause Int. 17, 63 (2011).
40. Leclercq G., de Cremoux P., This P., Jacquot Y.: Maturitas 68, 56 (2011).
41. Dębski R., Paszkowski T., Pertyński T., Zgliczyński W.: Przegl. Menopauz. 3, 189 (2013).
42. Pytlik M., Cegieła U., Folwarczna J., Nowińska B.: Pharmacol. Rep. 64, 625 (2012).
43. Batterham T.J., Shutt D.A., Hart N.K., Braden A.W.H., Tweedale H.J.: Aust. J. Agric. Res. 22, 131 (1971).
44. Tolleson W.H., Doerge D.R., Churchwell M.I., Marques M.M., Roberts D.W.: J. Agric. Food Chem. 50, 4783 (2002).
45. Moon Y.J., Sagawa K., Frederick K., Zhang S., Morris M.E.: AAPS J. 8, E433 (2006).
46. Singh S.P., Wahajuddin Ali M.M., Jain G.K.: J. Sep. Sci. 33, 3326 (2010).

47. Wang Z.L., Sun J.Y., Wang D.N., Xie Y.H., Wang S.W., Zhao W.M.: Phytomedicine 13, 718 (2006).
48. Bitto A., Burnett B.P., Polito F., Marini H., Levy R.M. et al.: Br. J. Pharmacol. 155, 896 (2008).
49. Śliwiński L., Folwarczna J., Nowińska B., Cegieła U., Pytlik M. et al.: Acta Biochim. Pol. 56, 261 (2009).
50. Turner R.T., Iwaniec U.T., Andrade J.E., Branscum A.J., Neese S.L. et al.: Menopause 20, 677 (2013).
51. Occhiuto F., Pasquale R.D., Guglielmo G., Palumbo D.R., Zangla G. et al.: Phytother. Res. 21, 130 (2007).

Received: 12. 05. 2014