Glycyrrhiza glabra L. (licorice) is a medicinal plant belonging to Fabaceae family, whereof the root is used for the pharmaceutical purposes. Its extracts are applied as expectorant, antiinflammatory, laxative, hepatoprotective or antioxidative agents (1-8) and the antiulcer activity of Glycyrrhiza glabra root is also proven (9, 10). Moreover, in hypercholesterolemic patients, administration of licorice induces a decrease of triglycerides, LDL and cholesterol as well as an increase of HDL levels in serum (11).

In both in vivo and in vitro studies, the extracts or isolated substances from the licorice root show antidiabetic (12, 13), antithrombotic (14), anticancer (8, 15, 16), antibacterial (17), antiviral (18) and antifungal (19) activities. In folk medicine, the diuretic, antipyretic, sedative and antiarrhythmic effects are also mentioned (20).

There are several substances responsible for pharmacological activity of licorice, such as flavonoids (including glabridin) and triterpenoid saponins (e.g., glycyrrhizic acid and glycyrrhizin) (21). In recent years, there are some reports indicating, that glabridin and its derivatives act as phytoestrogens (22, 23). The other Fabaceae members such as soy (Glycine max), red clover (Trifolium pratense), broad bean (Vicia faba), pea (Pisum sativum) and common bean (Phaseolus vulgaris) are also source of the phytoestrogens. These substances are used for soothing the effects of menopause as well as in the cardiovascular diseases prevention. Recently, phytoestrogens are also in the spotlight, due to their profitable effect on the bone tissue, including antiosteoporotic action. Since phytoestrogens have similar structure as female sex hormone - 17β-estradiol, their basic mechanism of action is to bind to the estrogen receptors α and β (ERα and ERβ) (24).

Recently conducted studies show, that glabridin can act as agonist or antagonist towards ERs (25-30). Other phytoestrogens found in licorice root – glabrene, glabraone, glabrol, 4’-O-methylglabridin, 3’-hydroxy-4’-O-methylglabridin, hispaglabridin A and B – in comparison with glabridin,
show similar but weaker affinity to ERs (25, 26, 28, 29).

Estrogen-like activity of glabridin was for the first time mentioned in 2000, when Tamir et al. assumed, that this flavonoid, due to its chemical structure similar to estradiol’s, can bind to ER. Both compounds contain 3 rings of phenanthrene structure and have hydroxyl groups connected with aromatic rings (glabridin at positions 2’ and 4’, while estradiol at positions 3 and 17β). What is more, both glabridin and estradiol are lipophilic substances. The authors showed that glabridin binds to the ERs in human breast cancer cells (26).

One year after this report, Tamir et al. proved, that glabridin has agonistic activity towards ERs, in both in vitro and in vivo tests (27). In the latest in vitro studies conducted by Simons et al., it has been shown, that glabridin at a concentration of $6 \times 10^{-6}$ M inhibits cellular response to estradiol administration by 80%. Thus it can be assumed, that this substance reveals antagonistic activity towards ERs (29). The authors concluded, that glabridin may act as a selective estrogen receptor modulator (SERM), regarding to its dual mode of action (30).

Glabridin shows the majority of actions typical of phytoestrogens: estrogen-like, protective to cardiovascular system, antioxidative and antiinflammatory. There are also studies indicating that this substance shows neuroprotective, antiatherogenic effects, it may regulate energy metabolism, and reveals antitumorigenic, antinephritic, antibacterial and skin-whitening activities (23, 31). However, there are no reports about the effect of glabridin (or any other phytoestrogen occurring in licorice root) on development of osteoporosis induced by estrogen deficiency in rats.

Glycyrrhizic acid and its glycoside (glycyrrhizin) are triterpenoid saponins that are not classified as the phytoestrogens. Available literature data clearly show, that glycyrrhizic acid reveals expectorant, antiulcer, antiinflammatory, neuroprotective, antitumor and hepatoprotective activities (32-34). Up till now there are no studies on effect of glycyrrhizic acid on bone tissue in ovariectomized rats.

In animals experimental ovariectomy (removal of the ovaries) causes estrogen deficiency and induces osteoporotic changes that can be compared to changes observed in postmenopausal women or female patients who underwent ovariectomy due to health reasons (35, 36). Thus, the aim of this study was to investigate the effect of glabridin and glycyrrhizic acid on bone remodeling processes in ovariectomized rats.

MATERIALS AND METHODS

The experiment was carried out on sexually mature female Wistar rats. The animals were provided by the Centre of Experimental Medicine at the Medical University of Silesia. The study was conducted with the approval of the Local Ethics Commission in Katowice (approval no. 70/2010; 22.11.2010). During the whole experiment the rats were fed with standard laboratory chow containing no soybean and had unlimited water supply. Animals were divided into following groups (n = 5):

- Group C – control group of non-ovariectomized rats, vehicle treated (water, 2 mL/kg per os)
- Group OVX – control group of ovariectomized rats, vehicle treated (water, 2 mL/kg po)
- Group OVX + E – ovariectomized rats receiving estradiol (0.1 mg/kg po)
- Group OVX + G – ovariectomized rats receiving genistein (5 mg/kg po)
- Group OVX + GL – ovariectomized rats receiving glabridin (5 mg/kg po)
- Group OVX + GA – ovariectomized rats receiving glycyrrhizic acid (15 mg/kg po)
- Both estradiol and genistein served as the positive controls in this experiment.

All analyzed substances and water were administered orally once a day for 4 weeks. During the experiment body mass gain of animals was controlled. After 4 weeks of drugs administration, rats were sacrificed with the use of general anesthesia induced by mixture of ketamine and xylazine. Each of the rats had the right tibia and the right femur as well as the L-4 vertebra excised. The uterus and the thymus of every rat were also collected. After cessation, mass of the organs was recorded. Obtained bones were used in order to conduct the analysis of macroometric and histomorphometric parameters. Measurements of macrometric parameters included: mass of the tibia, the femur and the L-4 vertebra as well as length and diameter of both the tibia and the femur.

The histological specimens were prepared and measured as previously described (37, 38). Briefly, from the tibia the transverse cross-sections and from the femur longitudinal sections of the distal epiphysis were made, then the sections were ground on the tarnished glass. In these histological specimens following parameters were analyzed: the width of the trabeculae in the distal femoral epiphysis and metaphysis, the width of the femoral epiphyseal cartilage, the area of the transverse cross-sectional of the tibial and femoral diaphysis, the area of the transverse cross-sectional of the cortical part of the
Effect of glabridin and glycyrrhizin on histomorphometric parameters of the tibia and the femur, as well as the width of the marrow cavity of the thia and the femur as well as the width of the periosteal and endosteal osseous of the thia and the femur, the area of the transverse cross-sectional area of the periosteal and endosteal osseous of the thia and the femur, as well as the width of the periosteal and endosteal osseous of the thia and the femur. The measurements of histomorphometric parameters were carried out with the use of an optical microscope connected with an RGB camera and computer (software: Lucia G 4.51, Laboratory Imaging), with final magnification of 200-400 times. The area of the transverse cross-sectional area of the periosteal and endosteal osseous of the thia and the femur were conducted with the use of an optical microscope with magnification of 200 times.

RESULTS

Effect of estradiol, genistein, glabridin and glycyrrhizin on body mass gain and the uterus mass in ovariectomized rats

After 4 weeks of the experiment the body mass gain in the ovariectomized rats (OVX) was greater in the statistically significant manner by 102% (p < 0.001) than in the non-ovariectomized control rats (C). Administration of estradiol and genistein to the ovariectomized rats (OVX + E) and (OVX + G) resulted in a statistically significant decrease of body mass gain by 29.8% (statistically significant, p < 0.05) and 14.2%, respectively, when compared to the OVX rats. Administration of glabridin to the ovariectomized rats (OVX + GL) showed no statistically significant effect on body mass gain in comparison with the OVX rats, and glycyrrhizin (OVX + GA) showed no statistically significant effect on body mass gain in comparison with the OVX rats.

Table 1. Effect of estradiol, genistein, glabridin and glycyrrhizin acid on body mass gain and mass of organs in ovariectomized rats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>C</th>
<th>OVX</th>
<th>OVX + E</th>
<th>OVX + G</th>
<th>OVX + GL</th>
<th>OVX + GA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body mass gain after 4 weeks [g]</td>
<td>24.06 ± 3.23</td>
<td>48.64 ± 3.49***</td>
<td>34.12 ± 4.54*</td>
<td>41.71 ± 4.75</td>
<td>43.26 ± 4.71</td>
<td>50.12 ± 3.83</td>
</tr>
<tr>
<td>Uterus mass [g]</td>
<td>0.39 ± 0.03</td>
<td>0.09 ± 0.01***</td>
<td>0.18 ± 0.01***</td>
<td>0.11 ± 0.01*</td>
<td>0.09 ± 0.01</td>
<td>0.09 ± 0.01</td>
</tr>
<tr>
<td>Uterus mass/body mass [g/kg]</td>
<td>1.53 ± 0.08</td>
<td>0.33 ± 0.02***</td>
<td>0.73 ± 0.06***</td>
<td>0.41 ± 0.05</td>
<td>0.34 ± 0.02</td>
<td>0.35 ± 0.02</td>
</tr>
<tr>
<td>Thymus mass [g]</td>
<td>0.39 ± 0.04</td>
<td>0.68 ± 0.02***</td>
<td>0.62 ± 0.05</td>
<td>0.61 ± 0.01</td>
<td>0.66 ± 0.02</td>
<td>0.67 ± 0.06</td>
</tr>
<tr>
<td>Thymus mass/body mass [g/kg]</td>
<td>1.55 ± 0.17</td>
<td>2.52 ± 0.08***</td>
<td>2.44 ± 0.16</td>
<td>2.32 ± 0.09</td>
<td>2.48 ± 0.06</td>
<td>2.47 ± 0.17</td>
</tr>
</tbody>
</table>

Results are presented as the means ± SEM (n = 5). *** p < 0.001, **** p < 0.001 – statistically significant differences between the OVX and the C groups; * p < 0.05, *** p < 0.001 – statistically significant differences in comparison with the OVX group.
Table 2. Effect of estradiol, genistein, glabridin and glycyrrhizic acid on the macrometric parameters of the bones in ovariectomized rats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>C</th>
<th>OVX</th>
<th>OVX + E</th>
<th>OVX + G</th>
<th>OVX + GL</th>
<th>OVX + GA</th>
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<td><strong>FEMUR</strong></td>
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<tr>
<td>Mass [mg]</td>
<td>728.7 ± 20.2</td>
<td>678.1 ± 3.49$^*$</td>
<td>670.4 ± 22.2</td>
<td>648.4 ± 11.3</td>
<td>688.1 ± 36.8</td>
<td>677.6 ± 31.7</td>
</tr>
<tr>
<td>Mass/body mass [g/kg]</td>
<td>2.88 ± 0.07</td>
<td>2.49 ± 0.03$^{**}$</td>
<td>2.65 ± 0.03$^{**}$</td>
<td>2.47 ± 0.07</td>
<td>2.58 ± 0.03</td>
<td>2.48 ± 0.04</td>
</tr>
<tr>
<td>Length of diaphysis [mm]</td>
<td>35.55 ± 0.30</td>
<td>35.53 ± 0.21</td>
<td>35.00 ± 0.32</td>
<td>34.53 ± 0.41</td>
<td>35.38 ± 0.29</td>
<td>34.96 ± 0.39</td>
</tr>
<tr>
<td>Diameter of diaphysis [mm]</td>
<td>2.98 ± 0.01</td>
<td>2.86 ± 0.02$^*$</td>
<td>2.90 ± 0.06</td>
<td>2.96 ± 0.07</td>
<td>3.02 ± 0.07</td>
<td>2.88 ± 0.08</td>
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<tr>
<td><strong>TIBIA</strong></td>
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<tr>
<td>Mass [mg]</td>
<td>527.6 ± 13.9</td>
<td>495.1 ± 5.5</td>
<td>478.7 ± 17.7</td>
<td>487.6 ± 8.7</td>
<td>508.4 ± 7.1</td>
<td>494.2 ± 21.7</td>
</tr>
<tr>
<td>Mass/body mass [g/kg]</td>
<td>2.08 ± 0.05</td>
<td>1.82 ± 0.02$^{**}$</td>
<td>1.89 ± 0.03</td>
<td>1.86 ± 0.05</td>
<td>1.91 ± 0.05</td>
<td>1.81 ± 0.03</td>
</tr>
<tr>
<td>Length of diaphysis [mm]</td>
<td>37.55 ± 0.38</td>
<td>37.75 ± 0.20</td>
<td>37.57 ± 0.30</td>
<td>37.83 ± 0.05</td>
<td>38.65 ± 0.51</td>
<td>38.46 ± 0.77</td>
</tr>
<tr>
<td>Diameter of diaphysis [mm]</td>
<td>2.27 ± 0.04</td>
<td>2.10 ± 0.02$^{**}$</td>
<td>2.23 ± 0.07</td>
<td>2.18 ± 0.05</td>
<td>2.23 ± 0.03$^{**}$</td>
<td>2.12 ± 0.04</td>
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<tr>
<td><strong>L-4 VERTEBRA</strong></td>
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</tr>
<tr>
<td>Mass [mg]</td>
<td>185.3 ± 10.5</td>
<td>161.8 ± 8.6</td>
<td>172.7 ± 7.3</td>
<td>170.6 ± 8.6</td>
<td>158 ± 6.6</td>
<td>175.4 ± 14.6</td>
</tr>
<tr>
<td>Mass/body mass [g/kg]</td>
<td>0.73 ± 0.03</td>
<td>0.59 ± 0.03$^*$</td>
<td>0.68 ± 0.02</td>
<td>0.65 ± 0.04</td>
<td>0.59 ± 0.00</td>
<td>0.64 ± 0.03</td>
</tr>
</tbody>
</table>

Results are presented as the means ± SEM (n = 5). $^*$ p < 0.05, $^{**}$ p < 0.01, $^{***}$ p < 0.001 – statistically significant differences between the OVX and the C groups; $^{**}$ p < 0.01 – statistically significant differences in comparison with the OVX group.
and 62% (p < 0.01), respectively as compared to the C rats. In the OVX + E and the OVX + G rats, the loss of the thymus mass by 9.7% and 11.3%, respectively, as well as the loss in thymus mass/body mass ratio by 2.9% and 7.4%, respectively, was recorded, when compared to the OVX rats. Glabridin and glycyrrhizic acid administration to the ovariectomized rats revealed no effect either on the thymus mass or the thymus mass/body mass ratio in comparison with the OVX rats (Table 1).

Effect of glabridin and glycyrrhizic acid on histomorphometric parameters of bones in ovariectomized rats

Ovariectomy induced statistically significant reduction of the femur mass by 6.9% (p < 0.05) and the femur mass/body mass ratio by 13.5% (p < 0.001) in the OVX rats, when compared to the C rats. In the OVX + E group, the mass of the femur was similar to this in the OVX group. However, the femur mass/body mass ratio in this group was statistically significantly higher by 6.5% (p < 0.01) than in the OVX group. There were no significant changes in the femur mass or the femur mass/body mass ratio recorded between the OVX + GE and the OVX rats. In both the OVX + GL and the OVX + GA groups the femur mass and the femur mass/body mass ratio remained almost unchanged as compared to the OVX group (Table 2).

In the control OVX rats reduction of the tibia mass (by 6.1%) and the tibia mass/body mass ratio (statistically significant by 12.9%; p < 0.01) occurred when compared to the C rats. In the OVX + E group the tibia mass/body mass ratio increased by 4.1%, while the tibia mass itself was unaffected, as compared to the OVX. In the OVX + GE rats there were no changes in the tibia mass or the tibia mass/body mass ratio in comparison to the OVX rats. In the OVX + GL group there were no changes in the tibia mass, but the tibia mass/body mass ratio increased by 5.3% when compared to the OVX group. Administration of glycyrrhizic acid showed no effect either on the tibia mass or the tibia mass/body mass ratio, when compared to the non-treated ovariectomized rats (Table 2).

The mass of L-4 vertebra as well as the L-4 vertebra mass/body mass ratio in the OVX rats were statistically significantly lower by 12.6% and 18.7%, respectively, (p < 0.05) in comparison to the C rats. In the OVX + E and the OVX + GE groups the mass of the L-4 vertebra was higher by 6.6% and 5.4%, respectively, and the L-4 vertebra mass/body mass ratio by 14.8% and 9.7%, respectively, when confronted with OVX group. Treatment with glabridin did not affect the L-4 vertebra mass or the L-4 vertebra mass/body mass ratio in ovariectomized rats as compared to the untreated OVX animals. In the OVX + GA group the L-4 vertebra mass increase by 8.4% and the L-4 vertebra mass/body mass ratio increase by 7.9% were recorded in comparison with the OVX (Table 2).

In the OVX rats the length of the femur and the tibia was comparable to the values recorded in the C rats. None of the examined substances administered to the ovariectomized rats (estradiol, genistein, glabridin or glycyrrhizic acid) affected the length of the studied bones when confronted with the control OVX group.

In comparison to the C group, the diameter of the femur and the tibia in the OVX rats was statistically significantly lower by 3.8% (p < 0.05) and 7.5% (p < 0.01), respectively. The diameter of the femur did not change after administration of estradiol, genistein or glycyrrhizic acid to the ovariectomized rats. Only treatment with glabridin resulted in the increase of the femoral diameter by 5.3%, when compared to the OVX rats.

In the OVX + E and the OVX + GE rats the diameter of the tibia was higher by 6.1% and 3.9%, respectively, than in the OVX rats. In the OVX + GL group the diameter of the tibia was statistically significantly higher by 6.3% (p < 0.01) than in the OVX group, whereas in the OVX + GA group no changes were observed (Table 2).

Effect of glabridin and glycyrrhizic acid on histomorphometric parameters of bones in ovariectomized rats

The width of the trabeculae in the femoral epiphysis and metaphysis was lower in the OVX group by 10.2% and 8.4%, respectively, than in C group. Administration of estradiol and genistein to the ovariectomized rats effected in increased width of the trabeculae in the femoral epiphysis by 11.6% and 13.1%, respectively, and in the femoral metaphysis by 16.0% and 34.1% (statistically significant, p < 0.01), when compared to the OVX rats. Treatment with glabridin resulted in increased width of the trabeculae of the femoral epiphysis by 19.5% and the femoral metaphysis by 27.4% (statistically significant, p < 0.05) when confronted with the non-treated ovariectomized rats. Glycyrrhizic acid revealed no effect on the width of the trabeculae in both the femoral epiphysis and metaphysis (Table 3).

In comparison to the C rats, in the OVX group there was a statistically significant increase of the width of the epiphyseal cartilage of the femoral bone by 26.1% (p < 0.01). Administration of genistein to
Table 3. Effect of estradiol, genistein, glabridin and glycyrrhizic acid on the histomorphometric parameters of the bones in ovariectomized rats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>C</th>
<th>OVX</th>
<th>OVX + E</th>
<th>OVX + G</th>
<th>OVX + GL</th>
<th>OVX + GA</th>
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<tr>
<td>FEMUR</td>
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</tr>
<tr>
<td>Width of trabeculae in epiphysis [µm]</td>
<td>68.93 ± 3.37</td>
<td>61.91 ± 2.20</td>
<td>69.11 ± 2.63</td>
<td>70.06 ± 3.92</td>
<td>73.86 ± 5.93</td>
<td>63.89 ± 4.33</td>
</tr>
<tr>
<td>Width of trabeculae in metaphysis [µm]</td>
<td>35.26 ± 2.29</td>
<td>32.31 ± 2.23</td>
<td>37.48 ± 3.14</td>
<td>43.31 ± 1.72**</td>
<td>41.16 ± 2.32*</td>
<td>32.16 ± 1.18</td>
</tr>
<tr>
<td>Width of epiphyseal cartilage [µm]</td>
<td>69.07 ± 1.70</td>
<td>87.17 ± 3.77**</td>
<td>89.94 ± 0.95</td>
<td>77.95 ± 2.69</td>
<td>79.15 ± 5.17</td>
<td>78.89 ± 1.96</td>
</tr>
<tr>
<td>The area of the transverse cross-sectional -</td>
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<td></td>
</tr>
<tr>
<td>of the cortical part [mm²]</td>
<td>5.09 ± 0.08</td>
<td>4.86 ± 0.17</td>
<td>5.15 ± 0.35</td>
<td>4.81 ± 0.04</td>
<td>4.95 ± 0.17</td>
<td>4.76 ± 0.13</td>
</tr>
<tr>
<td>of the marrow cavity [mm²]</td>
<td>3.58 ± 0.09</td>
<td>3.85 ± 0.08</td>
<td>3.26 ± 0.21*</td>
<td>3.39 ± 0.05**</td>
<td>3.58 ± 0.14</td>
<td>3.75 ± 0.09</td>
</tr>
<tr>
<td>- marrow cavity/diaphysis ratio [mm²]</td>
<td>0.41 ± 0.01</td>
<td>0.44 ± 0.01*</td>
<td>0.39 ± 0.02</td>
<td>0.41 ± 0.01*</td>
<td>0.42 ± 0.01</td>
<td>0.44 ± 0.01</td>
</tr>
<tr>
<td>TIBIA</td>
<td></td>
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<tr>
<td>The area of the transverse cross-sectional -</td>
<td></td>
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</tr>
<tr>
<td>of the cortical part [mm²]</td>
<td>3.55 ± 0.04</td>
<td>3.24 ± 0.03***</td>
<td>3.29 ± 0.15</td>
<td>3.27 ± 0.22</td>
<td>3.22 ± 0.15</td>
<td>3.21 ± 0.11</td>
</tr>
<tr>
<td>of the marrow cavity [mm²]</td>
<td>0.88 ± 0.05</td>
<td>0.96 ± 0.03</td>
<td>0.84 ± 0.05</td>
<td>0.87 ± 0.07</td>
<td>0.98 ± 0.04</td>
<td>0.94 ± 0.09</td>
</tr>
<tr>
<td>- marrow cavity/diaphysis ratio [mm²]</td>
<td>0.19 ± 0.01</td>
<td>0.23 ± 0.01*</td>
<td>0.21 ± 0.02</td>
<td>0.21 ± 0.01</td>
<td>0.23 ± 0.01</td>
<td>0.22 ± 0.01</td>
</tr>
<tr>
<td>Width of periosteal osteoid [µm]</td>
<td>14.63 ± 0.24</td>
<td>17.29 ± 0.19***</td>
<td>17.84 ± 0.36</td>
<td>17.51 ± 0.33</td>
<td>15.35 ± 0.52**</td>
<td>17.79 ± 0.21</td>
</tr>
<tr>
<td>Width of endosteal osteoid [µm]</td>
<td>12.17 ± 0.37</td>
<td>13.65 ± 0.65</td>
<td>13.93 ± 0.29</td>
<td>12.5 ± 0.44</td>
<td>11.62 ± 0.45*</td>
<td>11.60 ± 0.31*</td>
</tr>
</tbody>
</table>

Results are presented as the means ± SEM (n = 5). ∞ p < 0.05, ∞∞ p < 0.01, ∞∞∞ p < 0.001 – statistically significant differences between the OVX and the C groups; * p < 0.05, ** p < 0.01 – statistically significant differences in comparison with the OVX group.
the ovariectomized rats effected in a decrease of the width of the epiphyseal cartilage of the femoral bone by 10.5%, whereas administration of estradiol did not influence significantly this parameter, when compared to the OVX rats. In rats treated with glabridin and glycyrrhizic acid the decrease of the width of the femoral epiphyseal cartilage (by 9.1% and 9.5%, respectively) was observed, when confronted with the OVX rats (Table 3).

The area of the transverse cross-sectional of the cortical part of the femur and the area of the transverse cross-sectional of the marrow cavity of the femur decreased by 4.5% and 7.5%, respectively, in the OVX rats when confronted with the C rats. Also, in this group, the area of the transverse cross-sectional of the marrow cavity/area of the transverse cross-sectional of the cortical part of the femur ratio was statistically significantly higher by 7.2% (p < 0.05). In the ovariectomized rats treated with estradiol, the area of the transverse cross-sectional of the cortical part of the femur increased by 5.8% and the area of the transverse cross-sectional of the marrow cavity of the femur decreased by 15.3% (statistically significant, p < 0.05), when compared to the control OVX rats. The area of the transverse cross-sectional of the marrow cavity/area of the transverse cross-sectional of the cortical part of the femur ratio in the OVX + E group was lower by 11.9% than in the OVX group. Administration of genistein to the ovariectomized rats resulted in decrease of area of the transverse cross-sectional of the marrow cavity of the femur by 11.9% (statistically significant, p < 0.01) and a decrease of the area of the transverse cross-sectional of the marrow cavity/area of the transverse cross-sectional of the cortical part of the femur ratio by 6.5% (statistically significant, p < 0.05) as compared to the OVX group. Glabridin administration to the ovariectomized rats did not alter the area of the transverse cross-sectional of the cortical part of the femur, however the area of the transverse cross-sectional of the marrow cavity and the area of the transverse cross-sectional of the marrow cavity/area of the transverse cross-sectional of the cortical part of the femur ratio decreased by 6.8% and 4.9%, respectively, was noted when confronted with the control OVX group. Treatment with glycyrrhizic acid did not influence the area of the transverse cross-sectional of the cortical part of the femur, the area of the transverse cross-sectional of the marrow cavity or of the area of the transverse cross-sectional of the marrow cavity/area of the transverse cross-sectional of the cortical part of the femur ratio (Table 3).

In the tibial bone of the OVX rats, the area of the transverse cross-sectional of the cortical part was lower by 8.7% (statistically significant, p < 0.001), the area of the transverse cross-sectional of the marrow cavity was higher by 8.9%, the area of the transverse cross-sectional of the diaphysis was lower by 5.2% (statistically significant, p < 0.05), and the area of the transverse cross-sectional of the marrow cavity/area of the transverse cross-sectional of the diaphysis was higher by 15.3% (statistically significant, p < 0.05). There were no significant changes in the area of the transverse cross-sectional of the cortical part of the tibia after administration of estradiol or genistein to ovariectomized rats, however other parameters were altered. The area of the transverse cross-sectional of the marrow cavity of the tibia decreased by 11.7% and 8.6%, respectively, and the area of the transverse cross-sectional of the marrow cavity/area of the transverse cross-sectional of the tibial diaphysis was lower by 9.9% and 7.9%, respectively, when confronted with the control OVX group. Administration of glabridin or glycyrrhizic acid did not affect the area of the transverse cross-sectional of the cortical part, the area of the transverse cross-sectional of the marrow cavity or the area of the transverse cross-sectional of the marrow cavity/area of the transverse cross-sectional of the diaphysis of the tibia, when compared to the OVX rats (Table 3).

In the OVX rats the width of the tibial periosteal osteoid was significantly higher by 18.2% (p < 0.001), and the width of the tibial endosteal osteoid was higher by 12.1%, when compared to the C rats. Treatment with estradiol and genistein did not effect in changes in the width of periosteal osteoid of tibia, however the width of the endosteal osteoid of the tibia decreased after administration of genistein by 8.4%. Estradiol did not alter the width of the tibial endosteal osteoid in comparison with the control OVX group. Administration of glabridin to the ovariectomized rats caused a decrease of the width of the periosteal osteoid of the tibia by 11.2% (statistically significant, p < 0.01) and the width of the endosteal osteoid of the tibia by 14.8% (statistically significant, p < 0.05). There was no effect on the width of the periosteal and osteoid of the tibia after administration of glycyrrhizic acid to the ovariectomized rats, but the width of the tibial endosteal osteoid decreased by 14.9% (statistically significant, p < 0.05), when compared to the control OVX rats (Table 3).

**DISCUSSION**

In this paper, the results demonstrating the effect of substances occurring in licorice root –...
glabridin and glycyrrhizic acid – on bone remodeling in ovariectomized rats are presented for the first time.

In order to conduct the studies, the experimental model was used in which the osteoporosis was induced in sexually mature female rats by bilateral ovariectomy (bilateral cessation of the ovaries) (39). After 4 weeks from the surgery, the changes in rat’s bone tissue are similar to those observed in menopausal women (35, 36). During the menopause, the level of endogenous estrogen decreases which leads to intensified bone remodeling with the dominance of resorption process. As a result, the loss in bone tissue is observed and risk of fractures becomes higher (40).

In control ovariectomized group of rats, the increase of body mass gain, thymus mass and decrease of the uterus mass after 4 weeks from the surgery was observed. These results indicate that the ovariectomy was performed properly. The mass of all analyzed bones: the femur, the tibia and the L-4 vertebra as well as the ratio of these bones to body mass decreased in this group of animals. The decrease of mass of the femur and the tibia may be related to the reduced diameter of these bones.

As a consequence of estrogen deficiency in female rats, the increase of width of both the periosteal and endosteal osteoid in the tibia was noted. The increase of the osteoid’s width may indicate the bone formation disorders. In this group of rats, the area of the transverse cross sectional of the cortical part of the femur and the tibia decreased, while the increase of the area of the transverse cross-sectional of the marrow cavity for these bones was recorded. Furthermore, the observed increase in the area of the transverse cross-sectional of the marrow cavity/area of the transverse cross-sectional of the tibial diaphysis ratio, illustrates the adverse disproportion between the marrow cavity area and the diaphysis area. This may suggest that bone formation is inhibited or bone resorption is accelerated in ovariectomized rats. Reduction of the width of the femoral trabeculae in the metaphysis and the epiphysis also indicates that the suppression of bone formation and/or intensification of bone resorption occurs in control ovariectomized rats. Even though, the width of the femoral epiphyseal cartilage in ovariectomized rats increased, there were no changes in the length of this bone.

Basing on the observations in the changes the macrometric and histomorphometric parameters of the bones, it can be assumed that due to estrogen deficiency osteoporotic changes have been developed in ovariectomized rats. There are literature data available showing similar changes in skeletal system in rats with estrogen deficiency, thus they confirm the results obtained in our study (39, 41, 42).

Due to the fact, that osteoporotic changes occurred in the ovariectomized rats, the positive control in experiment was necessary. For this purpose estradiol was administered to rats with estrogen deficiency at a dose of 0.1 mg/kg p.o. for 4 weeks. Results obtained from this group were helpful to evaluate the effect of tested substances – glabridin and glycyrrhizic acid – on skeletal system in ovariectomized rats.

Administration of estradiol to ovariectomized rats prevented bone damage induced by ovariectomy, but its effect was not noted in all analyzed parameters. In the femoral bone an increase in the width of the trabeculae in epiphysis and metaphysis was observed. What is more, an increase of the area of the transverse cross-sectional of cortical part of this bone and a decrease of the area of the transverse cross-sectional of the marrow cavity were recorded. As a consequence, the ratio of the area of the transverse cross-sectional of the marrow cavity/area of the transverse cross-sectional of the cortical part of the femur decreased. Additionally, positive effect of estradiol on bone tissue was observed in the tibia. The area of the transverse cross-sectional of the marrow cavity as well as the area of the transverse cross-sectional of the tibial diaphysis/area of the transverse cross-sectional of cortical part of the tibia decreased. These results indicate that administration of estradiol to ovariectomized rats induces the acceleration of bone formation and/or inhibition of bone resorption processes. Also, as an effect of estradiol’s actions, the mass of the tibia, the femur and the L-4 vertebra per body mass ratios as well as diameter of the femoral diaphysis increased. However, administration of estradiol to the ovariectomized rats did not prevent the increase of periosteal and endosteal osteoid in the tibia and the increase of the femoral epiphyseal cartilage width. The protective effect of estradiol on bone tissue remodeling in ovariectomized rats was also described in other studies (41, 42). Furthermore, beneficial effects of estradiol was revealed by the reduced body and the thymus mass as well as the increase of the uterus mass in ovariectomized rats in comparison with untreated ovariectomized rats.

Genistein was another substance used in this study as a reference for evaluation of glabridin and glycyrrhizic acid effects on skeletal system of ovariectomized rats. Among all phytoestrogens, this substance was chosen due to its well documented
antiosteoporotic activity and well established position in menopause symptoms soothing. Previous studies revealed that genistein inhibits osteoporosis development in women, and in consequence reduces loss of bone material. In these women increased bone mineral density (BMD) in the femoral neck and in the lumbar spine was observed, what was proven by means of the bone markers (43, 44).

In our study, genistein administered at a dose of 5 mg/kg p.o. for 4 weeks to ovariectomized rats caused reduction in the increase of the body mass gain, the increase of the uterus mass and the uterus mass/body mass ratio as well as a reduction of the increase of the thymus mass and the thymus mass/body mass ratio when compared to control ovariectomized rats. These observations indicate that genistein reveals estrogen-like activity.

Analysis of the histomorphometric parameters of bones demonstrated that treatment with genistein resulted in partial stabilization of bone formation and resorption processes in ovariectomized rats. As evidence, an increase of the width of the trabeculae of the femoral metaphysis and epiphysis was observed, while a decrease of the width of endosteal osteoid in the tibial bone and width of epiphyseal cartilage was noted. Moreover, a decrease of the area of the transverse cross-sectional of the femoral and tibial marrow cavity was recorded. Additionally the decrease of their ratios per area of the transverse cross-sectional of the cortical parts of these bones occurred.

In spite of profitable effect of genistein on the tibial and femoral structure, there were no changes in mass of these bones. Only the diameter of the tibia increased. As for the L-4 vertebra, its mass and the ratio per body mass increased slightly after administration of genistein to ovariectomized rats. Similar observations were described by Sliwiński et al. – the results obtained in their research indicated that genistein shows beneficial activity towards bone remodeling processes, but this effect is weaker than estradiol’s (45).

Bitto et al. administered genistein subcutaneously to ovariectomized rats at doses of 1 and 10 mg/kg. The authors noted a significant increase of BMD and mineral substances content in the femur of tested animals. The biochemical markers of bone turnover such as bone-alkaline phosphatase, collagen C-telopeptide, osteoprotegerin and soluble receptor activator of nuclear factor-kB ligand assayed by these authors revealed that genistein displays profitable effect on bone tissue in ovariectomized rats. Moreover, in this study, in case of some analyzed parameters, genistein showed stronger effect than estradiol (46). There are also other papers presenting osteoprotective activity of genistein in ovariectomized rats (47, 48).

The next step of the presented study was to evaluate the effect of glabridin and glycyrrhizin acid on skeletal system of estrogen-deficient female rats. Glabridin was administered to ovariectomized rats at the same dose as genistein – 5 mg/kg p.o. After treatment with glabridin, no significant changes in the mass of the thymus and the uterus were noted. The intensified body mass gain observed in untreated ovariectomized rats also did not undergo reduction after administration of glabridin. No differences in the mass of the femur or the L-4 vertebra and only a slight increase of the tibial mass occurred in ovariectomized rats receiving glabridin. Despite the fact, that the diameter of analyzed bones increased, there were no changes in the area of the transverse cross-sectional of their cortical part. As regards the femoral bone, there was a slight decrease of the area of the transverse cross-sectional of the marrow cavity/area of the transverse cross-sectional of the diaphysis what may be result of the reduced area of the transverse cross-sectional of the marrow cavity. It is worth to note that after administration of glabridin to ovariectomized rats the width of the trabeculae in the femoral metaphysis and epiphysis significantly increased and the epiphyseal cartilage significantly decreased. These histomorphological parameters indicate that after treatment with glabridin, inhibition of the bone resorption process in ovariectomized rats may occur.

In 2004, Somjen et al. proved that glabridin has ability to bind to the estrogen receptors located in bones. The authors applied glabridin at a dose of 3000 nM to the cell cultures of osteoblasts obtained from trabeculae of long bones of pre- and post-menopausal women. They noted that after treatment with glabridin the activity of the creatine kinase (the marker of estrogen activity) increased in osteoblasts, but the dose of this substance had to be 100 times higher than estradiol’s. The authors also administered glabridin to immature female rats by intraperitoneal injection at a dose of 3 µg. In the epiphyseal cartilage and diaphyseal bone of examined animals the stimulation of activity of creatine kinase was observed. Similar observations were noted, when the authors administered glabridin to ovariectomized rats at a dose of 100 µg (49).

In the research where murine osteoblast cells MC3T3-E1 were used, the enhancement of osteoblasts function was noted after treatment with glabridin. This profitable effect of glabridin on osteoblasts may be ER mediated, associated with
cellular protection against oxidative damage, through an up-regulation of antioxidant enzymes (superoxide dismutase-1 and glutathione peroxidase 4) and preventing the disturbance of mitochondrial functions. Glabridin intensifies the expression of osteoblastic differentiation genes and increases the expression of the phosphatidylinositol-3’kinase and protein kinase B 2 genes, which are factors that determine the growth and survival of osteoblasts (50-52). The results of the experiment conducted by Kim et al. indicate that glabridin inhibits RANKL induced osteoclastogenesis in murine macrophages (53).

In the presented study, glycyrrhizic acid administered at a dose of 15 mg/kg p.o. to ovariectomized rats did not affect the body mass gain, the uterus mass or the thymus mass. Analysis of the macrometric and histomorphometric parameter of bones led to the conclusion that after administration of glycyrrhizic acid to ovariectomized rats there were no significant beneficial effects of this substance on bone remodeling processes. Only a slight increase of the mass of the L-4 vertebra, a decrease of the width of the epiphyseal cartilage and a decrease of the tibial endosteal osteoid can be recorded after treatment with glycyrrhizic acid. The other of the examined parameters remained unchanged after administration of this substance to ovariectomized rats. Due to this fact it cannot be unambiguously defined if glycyrrhizic acid reveals profitable or harmful effect on bone formation and resorption processes in estrogen-deficient rats.

Up till now there is only one research describing the effect of glycyrrhizic acid on bone tissue. Ramli et al. tested the effect of glycyrrhizic acid in rats with glucocorticoid-induced osteoporosis. The authors noted, that this substance administered to the animals induced an increase of bone volume, trabecular number, trabecular thickness and a decrease of the trabecular separation in femur. The mechanical properties of bones also improved – the increase of flexure modulus, flexure stress, and energy at break was observed. What is more, glycyrrhizic acid reduced serum concentration of the pyridinoline (bone resorption marker) and induced 11β-hydroxysteroid dehydrogenase type 1 activity in the bone (enzyme that catalyzes the interconversion of active cortisol to inactive cortisone in calcified tissue). This profitable effect of glycyrrhizic acid on bone tissue in rats with glucocorticoid-induced osteoporosis can be explained by activation of 11β-hydroxysteroid dehydrogenase type 1 (54).

Administration of quercetin and Glycyrrhiza glabra rhizome extract (consisting of 0.05% glabridin and about 1% glycyrrhizic acid) at a dose of 13 mg/kg resulted in enhancement of the bone formation rate, an increase of the volumetric bone mineral density, trabecular number and a decrease of the trabecular separation in the proximal tibiae in parathyroid hormone-treated rats (55).

CONCLUSIONS

The results obtained in our study indicate, that glabridin shows beneficial effect on skeletal system in ovariectomized rats when the macrometric and histomorphometric parameters are analyzed. However, this effect is lower when compared to the activity of estradiol or genistein.

Basing on the analysis of macrometric and histomorphometric parameters, it could be assumed that glycyrrhizic acid shows meager influence on bone tissue in ovariectomized rats and its activity has no preventive significance.

In order to accurately investigate the effect of glabridin and glycyrrhizic acid on bone tissue in ovariectomized rats, further studies, including assessment of bone mechanical properties, are needed.

Conflict of interest

The authors declare no conflict of interest.

REFERENCES


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