INTRODUCTION

Many plant species contain one of the largest groups of secondary plant metabolites, called cyanogenic glycosides. These compounds occur in approximately 2650 plant species (1), e.g., bitter almonds (2), apricot seeds, apples and peaches (3). Cyanogenic glycosides are capable of generating highly toxic hydrocyanic acid (cyanide) when degraded by enzymes (4). The released hydrogen cyanide has a faint, bitter, almond-like odor which some people are unable to detect. Therefore, the consumption of seeds containing these substances can cause acute or chronic toxicity in animals and humans (5). Apricot seeds, depending on the variety, contain poisonous most commonly occurring cyanogenic glycoside - amygdalin (6). Amygdalin (D-mandelonitrile-β-D-gentiobioside) is composed of two molecules of glucose, one benzaldehyde, which is an analgesic agent, and one hydrocyanic acid, that is considered an antineoplastic compound (7). This natural substance itself is non-toxic, but when decomposed by some enzymes, it produces hydrogen cyanide (8).

Amygdalin has been promoted as an alternative treatment in patients with asthma, diabetes, bronchitis and as alternative cancer cure (9, 10). The use of amygdalin in human treatment, especially in a cancer therapy, has been widely supported by in vitro studies (11-14). Almost all cell culture investigations demonstrated amygdalin’s anti-tumor properties. In contrast to in vitro observations, amygdalin effects in animal studies are inconsistent; however, later studies (published later than 2000) propagate amygdalin as a cancer cure (15). Despite the fact that Food Drug Administration (FDA) had banned the sale of amygdalin as a medicinal product, the compound continues to be manufactured and administered as an anticancer drug worldwide. A serious drawback connected with peroral use of amygdalin is the risk of cyanide poisoning. Particular attention should be paid to any effects of amygdalin on general physiology and morphology, since number of available data is limited. It is not still clear whether amygdalin acts directly in cancer cells than in normal cells (15).

Our investigation is a part of a larger study aimed at determining the effects of amygdalin on different aspects of health, physiology and reproduction. Using rabbit as an animal model, the effects of amygdalin application on renal structure (16), plasma levels of endocrine regulators (17), spermatozoa characteristics (18), health status (19) has been determined.

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SUBACUTE EXPOSURE TO AMYGDALIN INFLUENCES COMPACT BONE REMODELING OF RABBITS

Amygdalin is most commonly occurring cyanogenic glycoside. It is found in seeds of many plant species. Our study was aimed to reveal whether pure intramuscularly injected amygdalin or apricot seeds peroral exposure cause changes in bone microstructure of rabbits. Twenty clinically healthy 5 months-old male rabbits were segregated into five groups. Animals from groups A1 and A2 were intramuscularly injected with amygdalin at doses of 0.6 and 3 mg/kg b.w. daily for 28 days. The groups S1 and S2 received commercial feed for rabbits mixed with crushed bitter apricot seeds at doses of 60 and 300 mg/kg b.w. during 28 days. The control (C) group did not receive any amygdalin. Intramuscular and peroral amygdalin administration did not affect total body weight, femoral length and femoral weight of rabbits. Similarly, microcomputed tomography (3D analysis) has shown that amygdalin had insignificant effect on relative bone volume, bone mineral density, cortical bone thickness, bone surface, trabecular thickness, trabecular number, trabecular separation. However, histological (2D analysis) revealed evident changes in compact bone microstructure of amygdalin-exposed rabbits consistent with a different vascularity and changed biomechanical properties. We can conclude that subacute exposure to amygdalin (both intramuscular and peroral) at the doses used in our study influenced compact bone remodeling.

Key words: amygdalin, apricot seeds, bone microstructure, compact bone remodeling, bone mineral density, osteons
However, the impact of amygdalin on bone microstructure has not been investigated yet. Therefore, the present study was aimed at revealing whether pure intramuscularly injected amygdalin or apricot seeds peroral administration cause changes in bone microstructure of rabbits using 2D and 3D imaging methods. In addition, in approximately 35% of musculoskeletal research studies, rabbits are the first choice of animal mainly due to their reasonable anatomical size and lower costs in terms of purchase and maintenance as compared to larger animals (20). Also, they reach skeletal maturity shortly after sexual maturity, around 5 – 6 months of age (21).

MATERIALS AND METHODS

Ethical approval

The State Veterinary and Food Institute of Slovak Republic and Ethic Committee approved all of the experimental procedures. The protocol of this study was reviewed and approved specifically, with the approval number 3398/11-221/3. Institutional and national guidelines for the care and use of animals were followed appropriately.

Animals

In the experiment, twenty clinically healthy 5 months-old male rabbits of meat line P91 were used. The animals were obtained from the Experimental farm of the Animal Production Research Centre (NPPC) in Nitra (Slovakia). The males were used because they are less susceptible to skeletal damage than females (22). The rabbits were randomly segregated into five groups, of 4 animals each. They were housed in individual flat-deck wire cages under temperature 20 – 24°C, constant photoperiod of 12 h of daylight, and humidity 55 ± 10%. Animals had free access to water and feed. No toxic or side effects or death were observed throughout the study. Rabbits from groups A1 and A2 were intramuscularly injected by amygdalin at doses 0.6 and 3 mg/kg b.w. daily for 28 days. The experimental groups S1 and S2 received commercial feed for experimental groups S1 and S2 received commercial feed for amygdalin at doses 0.6 and 3 mg/kg b.w. daily for 28 days. The control (C) group did not receive any amygdalin or apricot seeds. The peroral and intramuscular doses of amygdalin were calculated to not exceed the acute medium lethal peroral doses (LD₅₀) values for cyanide. These doses ranged from 2.13 to 6 mg/kg b.w., considering that 1 g of amygdalin releases 59 mg HCN (23, 24). The intramuscular doses of amygdalin (0.6 and 3.0 mg/kg b.w.) release 0.035 and 0.177 mg/kg HCN, respectively. Based on the content of amygdalin in apricot seeds, the experimental groups S1 and S2 received 3.12 and 15.6 mg/kg b.w. of amygdalin which is consistent with 0.18 and 0.92 mg/kg HCN, respectively.

Chemicals

Amygdalin from apricot seeds (≥ 99% purity) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Amygdalin was freshly dissolved in sterile saline and 0.5 ml was applied intramuscularly to *musculus biceps femoris* on a daily basis. Bitter apricot seeds were provided by Trasco (Ziar n. Hronon, Slovakia). Chemical composition of apricot seeds is mentioned in the study of Kovacikova *et al.* (19).

Procedures

At the end of treatment period, rabbits were euthanized by electrocution and bone specimens (both femurs) were sampled during necropsy. Before 2D and 3D imaging, all femurs (n = 40) were weighed on analytical scales and their length was measured with a digital sliding instrument. For quantitative 3D analyses of compact and trabecular bone tissues, microcomputed tomography (microCT; µCT 50, Scanco Medical) was used. All volume parameters of compact bone were assessed in a region of interest starting 40.0 mm from the end of the growth plate (distal epiphysis) and extending 5.0 mm at femoral midshaft. Relative bone volume with marrow cavity (BV/TV, %), relative bone volume without marrow cavity (BV/TV*, %), bone mineral density (BMD, mg HA/ccm), cortical bone thickness (Ct.Th., mm), and bone surface (Bs., mm²) were measured. Trabecular bone parameters and microarchitecture were analysed in a region of interest starting 3.0 mm from the distal end of the growth plate and extending 5.0 mm. Following parameters were measured: relative bone volume (BV/TV, %), trabecular number (Tb.N., 1/mm), their thickness (Tb.Th., mm) and separation (Tb.Sp., mm), bone surface (BS, mm²).

For macroscopical 2D analyses, right femurs were sectioned at the diaphyseal midshaft and segments were fixed in HistoChoice fixative (Amresco, USA). These segments were then dehydrated, degreased and embedded in Biodur epoxy resin (Gunter von Hagens, Heidelberg, Germany) following the methodology of Martiniakova *et al.* (25). Transverse thin sections (70 – 80 µm thick) were cut with a sawing microtome (Leitz 1600, Leica, Germany) and fixed onto microscopic glass slides by Eukitt (Merck, Darmstadt, Germany) (26). The qualitative 2D characteristics of compact bone tissue were determined in accordance with the internationally accepted classification systems of Enlow and Brown (27) and Riquèles *et al.* (28). The quantitative 2D parameters of the compact bone were assessed by Motic Images Plus 2.0 ML software (Motic China Group Co., Ltd.). Following parameters were measured: area (µm²), perimeter (µm), maximum and minimum diameters (µm) of the primary osteons’ vascular canals, Haversian canals and secondary osteons in all views (anterior, posterior, medialis, lateralis) of thin sections. The rabbits were kept mainly for other investigations (e.g., health status, biochemical and reproduction analyses) at the Animal Production Research Centre in Nitra (Slovakia). The present research was performed as an additional study focused on bone microstructure.

Statistical analysis

General statistics and comparisons were performed using SPSS 17.0 software. The measured values were expressed as mean ± standard deviation (SD). The differences in quantitative 2D and 3D parameters of both compact and trabecular bone tissues among all groups were determined using Games-Howell and/or Tukey’s tests. The P-value for statistical significance was set to 0.05.

RESULTS

Macroskopical analysis of bones

Our results showed non-significant impact on both intramuscular and peroral administrations of amygdalin on total body weight, femoral length, and femoral weight in rabbits. Statistically significant differences were observed only for femoral weight between A1, S1 and S2 groups (Table 1).

Quantitative 3D analysis of compact bone tissue

Microcomputed tomography revealed that amygdalin had insignificant effect on relative bone volume (with and without...
marrow cavity), BMD, cortical bone thickness and bone surface (Fig. 1, Figs. 2f-3j).

Quantitative 3D analysis of trabecular bone tissue

Similarly for the trabecular bone, no significant differences related to relative bone volume, bone surface, trabecular number, their thickness and separation were determined among all groups (Fig. 2k-2o, Fig. 3).

Qualitative 2D analysis of compact bone tissue

The periosteal surface of femurs in rabbits from the C group consisted of primary vascular longitudinal bone tissue with vascular canals running in a direction parallel to long axis of the bone. Dense Haversian bone tissue with a large density of secondary osteons was observed in the middle part of the compact bone. Endostal surfaces of femurs consisted either of non-vascular bone tissue (with osteocytes and cellular lamellae) or irregular Haversian bone tissue (with scattered and isolated secondary osteons) (Fig. 2a).

Rabbits intramuscularly and perorally exposed to amygdalin have similar and/or almost identical bone microstructure. The middle part of the compact bone in rabbits from A1, S1 groups was formed not only by Haversian bone tissue but also by primary vascular longitudinal bone tissue (it extended there from periosteum). Therefore, a decreased density of secondary osteons in the central area of the compact bone was identified in these animals (Fig. 2b and 2d).

In rabbits from A2, S2 groups evident changes related to bone vascularization associated with the presence of plexiform bone tissue were observed. Plexiform bone tissue with osteon banding was identified near endostal border and in the middle part of substantia compacta in A2 group, whereas it was exhibited throughout entire sections of the femurs in S2 group (Fig. 2c and 2e). Osteon banding consisted of linearly oriented primary and/or secondary osteons, where osteons line up in rows of five osteons or more.

Quantitative 2D analysis of compact bone tissue

Altogether, 855 primary osteons’ vascular canals, 380 Haversian canals and 380 secondary osteons were measured. The results are summarized in Table 2. We observed that the primary osteons’ vascular canals and secondary osteons were significantly lower (P < 0.05) in rabbits from A1, A2, S1, S2 groups compared to those from the C group.
groups as compared to the C group. On the other hand, sizes of Haversian canals did not differ significantly among rabbits from all groups.

**DISCUSSION**

According to our results, subacute exposure to amygdalin (both intramuscular and peroral) had no significant effect on total body weight, femoral weight and length. Similarly, no differences in the body weight and weight of various organs (e.g. brain, liver, spleen, kidney, lungs and heart) were reported in Wistar rats (120 – 150 g) following subacute peroral administration of potassium cyanide for 14 days (29). Also, Basu (30) observed no changes in body weight and liver weight of guinea pigs (weighing 200 – 250 g at the start of experiment) perorally administered by laetrile for 24 days. Ibebunjo et al. (31) analysed the effects of sodium cyanide on bone growth (humerus, radius, ulna, femur, tibia, fibula) and development of dogs. Their results have shown that this cyanide had no adverse impact on weights and lengths of all bones studied after 14 weeks administration.

Quantitative 3D analyses of compact and trabecular bone tissues revealed no significant effect of amygdalin on all measured parameters. These findings provide the first information in this area of research, therefore comparison with other studies is impossible.

Our results of qualitative 2D analysis of the compact bone in rabbits from the C group are consistent with those of other researches (28, 32, 33). However, subacute exposure to amygdalin leads to several changes such as the presence of plexiform bone tissue with osteon banding, reduction or absence of irregular and/or dense Haversian bone tissues and a decreased density of secondary osteons in the middle part of the compact bone. These differences can be attributed to intensive remodeling and formation of bone tissue as an adaptive response to bone against
Amygdalin toxicity. Bone is a living and metabolically active organ that undergoes continuous remodeling throughout life (34). Osteoblasts and osteoclasts are responsible for bone formation and resorption, including growth, modeling, repair and remodeling of the skeleton (35). Bone remodeling plays an important role in adjusting bone architecture to meet changing mechanical and metabolic needs. It helps to repair microdamages in bone matrix and is involved in bone regeneration (34, 36). Numerous

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**Table 2.** Data of quantitative 2D analysis of compact bone tissue in rabbits from all experimental groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>N</th>
<th>Area (μm²)</th>
<th>Perimeter (μm)</th>
<th>Max. diameter (μm)</th>
<th>Min. diameter (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary osteons' vascular canals</strong></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>C (1)</td>
<td>165</td>
<td>240.46 ± 41.18</td>
<td>55.57 ± 4.75</td>
<td>9.62 ± 1.08</td>
<td>7.94 ± 0.86</td>
</tr>
<tr>
<td>A1 (2)</td>
<td>181</td>
<td>185.86 ± 21.23</td>
<td>48.79 ± 3.01</td>
<td>8.38 ± 0.82</td>
<td>7.07 ± 0.53</td>
</tr>
<tr>
<td>A2 (3)</td>
<td>173</td>
<td>197.09 ± 22.31</td>
<td>50.26 ± 3.11</td>
<td>8.63 ± 0.84</td>
<td>7.28 ± 0.60</td>
</tr>
<tr>
<td>S1 (4)</td>
<td>170</td>
<td>193.08 ± 21.50</td>
<td>49.65 ± 2.91</td>
<td>8.49 ± 0.72</td>
<td>7.25 ± 0.58</td>
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<tr>
<td>S2 (5)</td>
<td>166</td>
<td>197.98 ± 22.70</td>
<td>50.26 ± 3.12</td>
<td>8.57 ± 0.80</td>
<td>7.36 ± 0.58</td>
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<tr>
<td>Tukey/Games-Howell test</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>C (1)</td>
<td>83</td>
<td>316.47 ± 63.87</td>
<td>63.46 ± 6.34</td>
<td>10.94 ± 1.27</td>
<td>9.15 ± 1.06</td>
</tr>
<tr>
<td>A1 (2)</td>
<td>77</td>
<td>302.14 ± 45.36</td>
<td>62.33 ± 4.88</td>
<td>10.87 ± 1.16</td>
<td>8.83 ± 0.76</td>
</tr>
<tr>
<td>A2 (3)</td>
<td>76</td>
<td>307.13 ± 67.98</td>
<td>62.74 ± 6.67</td>
<td>10.92 ± 1.39</td>
<td>8.88 ± 1.13</td>
</tr>
<tr>
<td>S1 (4)</td>
<td>74</td>
<td>300.63 ± 51.85</td>
<td>62.20 ± 5.57</td>
<td>10.90 ± 1.20</td>
<td>8.75 ± 0.93</td>
</tr>
<tr>
<td>S2 (5)</td>
<td>70</td>
<td>306.38 ± 57.11</td>
<td>62.62 ± 5.48</td>
<td>10.86 ± 0.96</td>
<td>8.95 ± 1.12</td>
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<tr>
<td>Tukey/Games-Howell test</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C (1)</td>
<td>83</td>
<td>7448.02 ± 1895.99</td>
<td>312.56 ± 39.48</td>
<td>56.24 ± 8.10</td>
<td>41.86 ± 7.00</td>
</tr>
<tr>
<td>A1 (2)</td>
<td>77</td>
<td>6405.06 ± 1911.65</td>
<td>292.53 ± 43.16</td>
<td>54.17 ± 8.53</td>
<td>37.07 ± 6.98</td>
</tr>
<tr>
<td>A2 (3)</td>
<td>76</td>
<td>6851.79 ± 1582.01</td>
<td>303.21 ± 37.02</td>
<td>55.88 ± 8.25</td>
<td>38.80 ± 5.70</td>
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<tr>
<td>S1 (4)</td>
<td>74</td>
<td>6269.52 ± 1530.82</td>
<td>289.01 ± 33.57</td>
<td>52.83 ± 7.09</td>
<td>37.55 ± 6.10</td>
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<tr>
<td>S2 (5)</td>
<td>70</td>
<td>6665.96 ± 1222.01</td>
<td>300.07 ± 28.68</td>
<td>55.32 ± 6.90</td>
<td>38.36 ± 5.06</td>
</tr>
<tr>
<td>Tukey/Games-Howell test</td>
<td></td>
<td></td>
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</tbody>
</table>

**Abbreviations:** C (1), control rabbits; A1 (2), 0.6 mg amygdalin/kg b.w. intramuscular administration; A2 (3), 3.0 mg amygdalin/kg b.w. intramuscular administration; S1 (4), 60 mg apricot seeds/kg b.w. peroral administration; S2 (5), 300 mg apricot seeds/kg b.w. peroral administration. The values are shown as mean and SD (standard deviation). N, number of measured structures; P < 0.05 (+); NS, non-significant differences.
signalling pathways are consistent with bone remodeling or bone morphogenesis. The central role of the receptor activator of nuclear factor κB (RANK) - RANK ligand (RANKL) - osteoprotegerin (OPG) signalling system was highlighted in bone remodeling. An imbalance between RANKL and OPG synthesis is considered one the key mechanisms of glucocorticoid-induced bone loss (37). In our previous in vitro study on cultivated human osteoblasts, the highest concentration of amygdalin (10 000 µg/ml) increased the expression of TNFSF11 gene which codes RANKL (38). Therefore, an accelerated bone resorption was associated with amygdalin exposure in our study. According to Hamel (39) cyanide causes intracellular hypoxia by reversibly binding to mitochondrial cytochrome oxidase a3. Hypoxia can negatively influence osteoblast activity and thus plays a key role in bone remodeling and mineralization. On the other hand, hypoxia can cause progressive increase in the resorption and formation of bigger osteoclasts. Low oxygen concentration can alter bone homeostasis, leading to osteolysis (40). In general, plexiform bone tissue is found in many rapidly growing animals (41). This type of bone tissue contains rectilinear residual vascular spaces, which results in a “brick wall” appearance (33, 42). Therefore, it derives its name from this interconnecting vascular plexus (43). This special arrangement allows increasing bone strength rapidly (44). Haversian bone tissue has lower stiffness, fatigue strength and fracture toughness as compared to plexiform bone tissue. In addition, Haversian remodeling occurs at locations of high compressive stresses. Plexiform bone tissue is observed under low tensile stresses (45).

Our results also revealed a vasoconstriction of primary osteons’ vascular canals in amygdalin-exposed rabbits. It is known that blood vessels (provide nutrition for the bone) are present in primary osteons’ vascular canals (46). The results from Munoz et al. (47) have shown that the exposure to cyanide increased cortisol concentrations in the plasma. Cortisol, the major glucocorticoid, is the primary hormone responsible for the stress response (48). According to Ullian (49) and Baum and Moe (50), glucocorticoids act directly on blood vessels, thereby induce vasoconstriction which is connected with an increased blood pressure and hypertension.

On the other hand, the sizes of Haversian canals (which also contain blood vessels) were not influenced by amygdalin application. This finding could be associated with different structure of primary and secondary osteons (51). According to Burr and Allen (43), primary osteons do not have a well-defined boundary separating them from the rest of the existing matrix. However, secondary osteons (and also Haversian canals) are separated from the matrix by a highly mineralized cement line (52). Therefore, we speculate that different results in histomorphometry of both canals can be associated with the cement line.

We have also found that intramuscular and peroral exposure to amygdalin significantly decreased the sizes of secondary osteons (except for A2 group). Secondary osteons are formed by concentric lamellae (43) in which collagen fibers run parallel to each other (51). According to Pidaparti and Burr (53), collagen fibers contribute to the mechanical and structural integrity of the compact bone. Suchard et al. (54) have shown that acute cyanide toxicity (ingestion of apricot seeds) caused metabolic acidosis in woman which can be consistent with an inhibited alkaline phosphatase activity and osteoblastic collagen synthesis (55). Generally, compact bone microstructure of adult humans consists mainly of dense Haversian bone tissue with a large density of secondary osteons. Primary osteons are only occasionally identified in their bones. Therefore, the information related to primary osteons’ vascular canals vasoconstriction after amygdalin application is limited in humans. The other changes in the compact bone demonstrated in our study might be observed in humans consuming amygdalin.

**Conclusions**

Subacute exposure to amygdalin (both intramuscular and peroral) at the doses used in our study did not affect total body weight, femoral weight and femoral length of male rabbits. Also, microcomputed tomography (3D analysis) did not reveal a significant effect of amygdalin on all measured parameters of compact and trabecular bone tissues. However, evident changes in compact bone microstructure after histological (2D analysis) were detected. These changes are consistent with the presence of plexiform bone tissue, decreased density of secondary osteons in the middle part of the compact bone, vasoconstriction of primary osteons’ vascular canals and reduced sizes of secondary osteons. Our results indicate that short - term intramuscular and peroral application of amygdalin affected compact bone remodeling including vascularization and biomechanical properties.

**Study limitations**

There are some limitations in the present study. First, this is a study with restricted numbers of rabbits per groups. This was related to our ethical rules regarding keeping to the principle of reducing animal experiments; however, studies with larger populations would be required to find minimal histopathological differences among groups. Second, only subacute effects of amygdalin on bone microstructure were investigated. Therefore, to generalize the results, the treatment period should take a longer time. Third, we used only males because they are less susceptible to skeletal damage than females. Fourth, all rabbits were healthy. It would be interesting to investigate the impact of amygdalin on bone microstructure of unhealthy animals (e.g. rabbits with osteosarcoma). To avoid the complexity of the subject it would be necessary to study these limitations in separate studies.

**Abbreviations**: BMD, bone mineral density; Bs., bone surface without marrow cavity; BT/TV*, relative bone volume without marrow cavity; b.w., body weight; cm, centimetre; Ct.Th., cortical bone thickness; FDA, Food Drug Administration; g, gram; kg, kilogram; mg, milligram; µCT, microcomputed tomography; µm, micrometre; mm, millimetre; NS, non-significant differences; SD, standard deviation; Tb.N, trabecular number; Tb.Sp., trabecular separation; Tb.Th., trabecular thickness.

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