BONE ADAPTATION TO SIMULTANEOUS CADMIUM AND DIAZINON TOXICITY IN ADULT MALE RATS

Hana Chovancová, Radoslav Omelka, Ivana Boboňová, Grzegorz Formicki, Róbert Toman, Monika Martiniaková

ABSTRACT
Food contamination from natural or anthropogenic sources poses severe risks to health of human and animals. Bone is a metabolically active organ, which can be affected by various toxic substances, such as cadmium (Cd) and diazinon (DZN), leading to disruption in bone metabolic processes. The present study was designed to investigate the effect of simultaneous peroral administration to Cd and DZN on femoral compact bone structure in adult male rats. A total of twenty 1-month-old male Wistar rats were randomized into two experimental groups. In the first group (EG), young males were dosed with a combination of 30 mg CdCl₂/L and 40 mg DZN/L in drinking water, for 90 days. Ten 1-month-old males without Cd-DZN intoxication served as a control group (CG). After 90 days of daily peroral exposure, evaluations of femoral bone macro- and micro-structure were performed in each group. We found no significant differences in body weight, femoral weight, femoral length and cortical bone thickness between both groups (EG and CG). Our results suggest an adaptive response of compact bone tissue to Cd-DZN-induced toxicity in adult male rats in order to prevent osteonecrosis.

Keywords: bone; osteotoxicology; cadmium; diazinon; rats

INTRODUCTION
Most foods contain natural or synthetic chemicals that could represent a toxic hazard for the consumers (Nassreddine and Parent, 2002). Cadmium (Cd) is a heavy metal that is widely present in the environment as pollutant (Mouls and Thévenod, 2010). It still attracts the attention of researchers and the public because its level in food products often exceeds the maximum allowable limits (Toman et al., 2011). According to Järup and Akesson (2009), the diet is the major source (~ 99%) of Cd exposure in the general non-smoking population. Upon absorbed, Cd irreversibly accumulates in the human body, in particularly in kidneys and other vital organs such as lungs and liver (Bernard, 2008). In addition to its cumulative properties, it is also highly toxic metal which can disrupt a number of biological systems, usually at doses that are much lower than most toxic metals (Järup et al., 1998). One of the target organs for Cd toxicity is also bone (WHO, 1992). Exposure to Cd has been linked to bone loss, low bone mass and osteoporosis, and even to an increased incidence of bone fractures (Wilson et al., 1996; Wang et al., 2003). The results obtained by Brzó ska and Moniuszko-Jakoniuk (2005a) have shown that chronic, even low-level exposure to Cd disturbs bone metabolism during skeletal development and maturity by affecting bone turnover most probably through a direct influence on bone formation and resorption, and indirectly via disorders in Ca metabolism. Besides interfering with Ca, Cd also alters the metabolism of other metals essential for bone health, mainly zinc (Zn), iron (Fe) and copper (Cu); Noël et al., 2004).

Diazinon (DZN) is a contact organophosphate (OP) pesticide, which is extensively used in agriculture (Salehi et al., 2009). Like other organophosphates (OPs), the main toxic action of DZN is inhibition of acetylcholinesterase (AChE) activity, which results in accumulation of acetylcholine (ACh) and associated neurotoxicity (Oruc and Usta, 2007). Diazinon toxicity varies widely within and among species, and is modified by organism age, sex, body size, climatic conditions, pesticide formulation, chemistry of the environment, and other factors (Montz, 1983). According to Garg et al. (2004), a potential target of pesticide toxicity is the skeletal system. Marked impairment in the development of the backbone in ducklings due to OPs toxicity has been observed in the study by Ludle et al. (1979). Higher amounts of DZN caused additional defects in quail and chicken including folding of the spinal cord, shortening of the neck (Wyttenbach and Hwang, 1984), fusing and twisting of vertebrae, abnormal development of ribs and breastbone (Meneely and Wyttenbach, 1989), curled claws, reduced
growth of leg and wing bones (Cho and Lee, 1990), and reduced bone calcification (Cho and Lee, 1991). In addition, OPs cause a significant reduction in bone mass and density in individuals following chronic low-level intoxication (Compston et al., 1999). Results by Lari et al. (2011) indicate that DZN exposure is associated with decrease in trabecular and cortical bone density and might be one of the causes for worldwide increasing prevalence of osteoporosis.

The aim of the current study was to investigate the effect of co-administration to Cd and DZN as food contaminants on femoral bone structure in adult male rats.

**MATERIAL AND METHODOLOGY**

Our experiment was conducted on twenty 1-month-old male Wistar rats obtained from the accredited experimental laboratory (number SK PC 50004) of the Slovak University of Agriculture in Nitra. The animals were housed individually in plastic cages under constant temperature (20-22 °C), humidity (55 ±10%), and 12/12 h cycle of light and darkness with the provision of food (feed mixture M3, Machal, Bonargo, Czech Republic) and water ad libitum. Clinically healthy rats (free of typical rodent pathogens) were randomly divided into two groups, of 10 animals each. In the first group (EG), young males were dosed with a daily intake of 30 mg CdCl₂/L in combination with 40 mg DZN/L in drinking water for 90 days. The second group without Cd and DZN supplementation served as a control (group CG). The water consumption was daily monitored during the whole experiment. The xenobiotics used in our experiment were chosen on the basis of their possible occurrence in the human and animal food (Toman et al., 2011). Indeed, correlation coefficients found between Cd and DZN in men (0.70) and women (0.69) indicate high probability of exposure to both compounds (Toman et al., 2012). The doses of Cd and DZN (chosen based on studied literature and our previous experiments with tested dose-response effects) were high enough to reach a toxicity level but also safe enough to prevent animal mortality (non-lethal doses). All procedures were approved by the Animal Experimental Committee of the Slovak Republic. At the end of the experiment, all animals were killed, weighed, and both their femurs were used for macroscopic and microscopic analyses. After cleaning all soft tissues, right femurs were weighed on analytical scales with an accuracy of 0.01g and the length was measured with a sliding instrument. The unpaired Student’s T-test was used for establishing statistical significance between both experimental groups. The significance level was accepted at P <0.05. For histological investigation, each right femur was sectioned at the midshaft of its diaphysis. The obtained segments were placed in Histocutix fixative (Amresco, USA). Specimens were then dehydrated in ascending grades of ethanol and embedded in epoxy resin Biodur (Günter von Hagens, Germany) according to Martiniakova et al. (2007). Transverse thin sections (70-80 μm) were prepared with a sawing microtome (Leitz 1600, Leica, Wetzlar, Germany) and affixed to glass slides by Eukitt (Merck, Darmstadt, Germany) as previously described (Martiniaková et al., 2008). The qualitative histological characteristics of the compact bone were determined according to the internationally accepted classification systems of Enlow and Brown (1956) and Ricoles et al. (1991), who classified bone into three main categories: primary vascular tissue, non-vascular tissue and Haversian bone tissue. Various patterns of vascularization can occur in primary vascular bone: longitudinal, radial, reticular, plexiform, laminar, lepidosteoid, acellular, fibriform and protohaversian. There are three subcategories identified in Haversian bone tissue: irregular, endosteal and dense.

**RESULTS**

Body weight, femoral weight, femoral length and cortical bone thickness did not differ significantly between both experimental groups (Tab. 1). Femoral diaphyses of rats from the control group had the following microstructure in common. The internal layer surrounding the medullary cavity (i.e. endosteal border) was formed by non-vascular bone tissue in all views of the thin sections. The bone tissue contained cellular lamellae and osteocytes. Primary and/or secondary osteons were not present. Additionally, there were also identified some areas of primary vascular radial bone tissue in anterior, posterior and lateral views. This type of bone tissue was created by branching or non-branching vascular canals radiating from the bone marrow cavity. Some primary and secondary osteons were also found especially in the anterior and posterior views near the endosteal surfaces.

**Table 1** Body weight, femoral weight, femoral length and cortical bone thickness in adult male rats co-administered to 30 mg CdCl₂/L and 40 mg DZN/L in drinking water (group EG) and control rats (group CG)

<table>
<thead>
<tr>
<th>Rat’s group</th>
<th>N</th>
<th>Body weight (g)</th>
<th>Femoral weight (g)</th>
<th>Femoral length (cm)</th>
<th>Cortical bone thickness (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EG</td>
<td>10</td>
<td>427.78±19.22</td>
<td>1.03±0.07</td>
<td>3.98±0.09</td>
<td>0.573±0.066</td>
</tr>
<tr>
<td>CG</td>
<td>10</td>
<td>405±52.65</td>
<td>1.05±0.17</td>
<td>3.94±0.09</td>
<td>0.575±0.048</td>
</tr>
<tr>
<td>T-test</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

N: number of rats; NS: non-significant changes
In the middle part of the compact bone, a few primary and secondary osteons were identified. However, dense Haversian bone tissue characterized by dense concentration of secondary osteons was not observed. Finally, the periosteal border of analysed bones was again composed of non-vascular bone tissue, mainly in the anterior and posterior views (Fig. 1).

The rats simultaneously exposed to Cd and DZN displayed a similar microstructure to rats from the control group, except for the middle part of the compact bone where primary vascular radial bone tissue was observed. Vascular canals expanded into the central area of the bones from endosteal surfaces. The canal expansion was in some cases so enormous that the canals also occurred near periosteal surfaces. As a result of this process, a smaller number of primary and secondary osteons was identified in the Cd-DZN-intoxicated rats (Fig. 2). But no clinical manifestations of osteoporosis (i.e. resorption lacunae or osteoporotic fractures) were revealed in these rats.

**DISCUSSION**

Our results showed non-significant effect of simultaneous peroral application of Cd and DZN on body weight, femoral weight and femoral length in adult male rats. Correspondingly, no demonstrable changes in body weight (Brzóska and Moniuszko-Jakoniuk, 2005b; 2008; Martiniaková et al., 2013) and femoral length (Brzóska et al., 2007; 2008; 2010; Martiniaková et al., 2013) have also been reported in adult male rats after their peroral exposure to 5, 30 or 50 mg Cd/L in drinking water. Also, subchronic intoxication with a sole dose of DZN (the same level as it was used in our study) did not induce significant alterations in body weight of rats (Cabaj, 2012). Weight of femoral bone in rats perorally receiving mixture of Cd and DZN were similar to those from the control group; however, in our previous study (Martiniaková et al., 2013) we have found that Cd administered in single dose had a positive impact on femoral weight in adult male rats. On the basis of these findings we can conclude that beneficial effect of Cd on femoral weight in adult male rats is in interaction with DZN suppressed.

The thickness of cortical bone is generally accepted as an important parameter in the assessment of cortical bone quality and strength. According to Garn et al. (1991), cortical thickness of femoral shaft is a good measuring site for evaluation of bone mass. Our research demonstrates no significant differences in cortical bone thickness between rats co-administered to Cd and DZN, and those of the control group. In the study by Comelekoglu et al. (2007), cortical thickness in the femoral diaphysis was also unchanged in adult female rats after common low intraperitoneal administration of Cd for 18 weeks. On the other hand, cortical bone thickness in rats from our control group was higher in comparison with the value published by Comelekoglu et al. (2007) for 4 month-old female rats (0.45 ±0.0057 mm). This discrepancy may be influenced by the different gender and strain of the animals in the two experiments. It is well known that earlier completion of longitudinal growth and earlier inhibition of periosteal apposition produces a smaller bone in females (Seeman, 2008).

The results of the qualitative histological analysis of femurs from the control rats correspond with previous works (Enlow and Brown, 1958; Martiniaková et al., 2005; Reim et al., 2008; Martiniaková et al., 2009). The basic structural pattern of compact bone tissue was non-vascular. In addition, there were some areas of primary vascular radial and/or irregular Haversian bone tissues. However, there was no evidence of true Haversian intracortical bone remodeling. It is generally known that aged rats and mice lack true Haversian cortical bone remodeling but not cancellous bone remodeling (Erben et

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**Fig. 1 Microscopic structure of compact bone in rats from the control group:**
1 non-vascular bone tissue; 2 vascular canals radiating from marrow cavity; 3 primary and secondary osteons in middle part of compact bone; 4 non-vascular bone tissue.

**Fig. 2 Microscopic structure of compact bone in Cd-DZN-exposed rats:**
1 enormous vascular canals radiating from marrow cavity; 2 smaller number of primary and secondary osteons in middle part of compact bone.
al., 1996; Reim et al., 2008). Therefore, some secondary osteons can be observed in the long bones (near the endostea). In our study, the newly formed remodeling units within compact bone originated from the endocortical surface and extended deep into the underlying compact bone. The same findings have also been documented in the study of Reim et al. (2008) in 13 month-old male rats.

Prolonged intake of Cd and DZN mixture resulted in induction of demonstrable changes in the middle part of compact bone where vascular canals expanded from endostea and led to the formation of primary vascular radial bone tissue. In some cases, vascular canals were also present near periosteal surfaces. The final result of this process was a smaller number of primary and secondary osteons indicating the reduced bone mechanical properties. In general, bone is dynamic tissue that is continuously remodeled to remove microfractures, to adapt to changing mechanical strains and metabolic demands (Hofstetter, 2007; Chen et al., 2009). Disappearance of the Haversian canal system, which was replaced by a large quantity of degenerated, necrotic, and restorative tissues have been demonstrated in the study by Li et al. (1997) for ovariectomized rats after a long-term Cd administration. Also, Cd-induced apoptosis of bone cells was documented in many studies (Coone et al., 2007; Lévesque et al., 2008; Chen et al., 2009; Smith et al., 2009; Arbon et al., 2012; Bram et al., 2012). Furthermore, decreased number of active osteons in broiler chicks was found after exposure to OP pesticides (Garg et al., 2004). In general, DZN exerts its toxicity through inhibition of AChE. According to Genever et al. (1999) and Inkson et al. (2004), this enzyme is also expressed by osteoblasts suggesting a role for AChE (i.e. bone matrix protein) in bone tissue. Thus, the expression of high levels of AChE in bone-forming osteoblasts and their progenitors supports a toxic effect of AChE inhibitors (including DZN) on these cells (Genever et al., 1999; Grisaru et al., 1999; Inkson et al., 2004; Hoogduijn et al., 2006). Based on all mentioned aspects we propose that the formation of primary vascular bone tissue, mainly in the central area of the femur, could be explained as an adaptive response of bone to Cd-DZN toxicity, in order to protect the tissue against death of cells and subsequent necrosis. Interestingly, changes in qualitative histological characteristics of compact bone tissue were in these rats less pronounced than in those exposed to only Cd in sole dose (Martiniaková et al., 2013). Indeed, Cd-intoxicated rats displayed a presence of resorption lacunae near endosteal surfaces indicating an early stage of osteoporosis while in rats co-intoxicated with Cd and DZN these structures were absent. This finding suggests that the significant effect of Cd on rat bone microstructure is in combination with DZN partially eliminated. We speculate that the fact could be associated with a molecule of AChE expressing also in bone tissue. According to Compston et al. (1999), this molecule might have a role in the regulation of cell-matrix interactions and in the coupling of bone resorption to bone formation. In addition, the demonstration of Chfa - 1 and other osteogenic factor-binding motifs on the AChE gene promoter offers further support for a physiological role of AChE in bone formation (Grisaru et al., 1999). Hence, the presence of AChE in bone matrix provides a possible mechanism for OP-induced effects in bone (Genever et al., 1999). The fact was confirmed in the study by Compston et al. (1999), who have found that chronic OP exposure significantly decreased bone formation in agricultural workers. Reduced bone mineral density (BMD) was also observed in rats after application of 15 and 30 mg DZN/kg for 4 weeks (Lari et al., 2011). In respect to all mentioned findings and available scientific papers we propose that absence of resorption lacunae in Cd-DZN-intoxicated rats can be attributed to an opposite impact of Cd and DZN on AChE activity. Indeed, there is evidence that while DZN inhibits AChE, Cd is able to stimulate it. The experiment by Carageorgiou et al. (2004) has shown that effect of Cd on brain AChE activity in rats is dose- and exposure duration-dependent. Results revealed that short-term treatment of rats with Cd induces a dose-dependent decrease of brain AChE activity, while long-term Cd administration stimulates it. In accordance with this finding, Toman et al. (2012) observed decreasing in AChE activity in rats 36 hours exposed to Cd. On the other hand, Cd administered in drinking water for 3 weeks led to considerable increased the enzyme function in rats (Srinivasan and Ramprasath, 2011). Therefore, we consider that opposite (competitive) effect of Cd and DZN on AChE activity resulted in elimination of DZN adverse impact on function of AChE in bone metabolism that was in microstructural level reflected by absence of resorption lacunae in Cd-DZN treated rats.

CONCLUSION

Our study demonstrates that simultaneous peroral exposure to Cd and DZN did not influence macroscopic structure of femoral bone in adult male rats. However, it has significant impact on bone microstructure in these animals. Co-administration to Cd and DZN affected mainly the middle part of rats’ bones where primary vascular radial bone tissue was identified as a result of adaptive response to xenobiotic-induced osteonecrosis. On the other hand, the vascular canal expansion into central area of substantia compacta led to a smaller number of primary and secondary osteons signaling weakened mechanical properties of the bones. Moreover, our study showed that Cd in combination with DZN had less expressive effect on bone microstructure in male rats than Cd in a sole dose.

Food chain contamination is one of the important pathways for the entry of toxic pollutants such as Cd and DZN into the human or animal body. Therefore, monitoring of xenobiotic presence in foods is the first step to prevent their toxic effects on human health including bones.

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Contact address:
Hana Chovancová, Constantine the Philosopher University, Faculty of Natural Sciences, Department of Zoology and Anthropology, 949 74 Nitra Slovakia, E-mail: hchovancova@ukf.sk.
Radoslav Omelka, Constantine the Philosopher University, Faculty of Natural Sciences, Department of Botany and Genetics, 949 74 Nitra Slovakia, E-mail: romelka@ukf.sk.
Ivana Boboňová, Constantine the Philosopher University, Faculty of Natural Sciences, Department of Zoology and Anthropology, 949 74 Nitra Slovakia, E-mail: ivana.bobonova@ukf.sk.
Grzegorz Formicki, Krakow Pedagogical University, Institute of Biology, Department of Zoology, Krakow 31 054 Poland, E-mail: formicki@ap.krakow.pl
Róbert Toman, Slovak University of Agriculture, Faculty of Agrobiology and Food Resources, Department of Veterinary Sciences, 949 76 Nitra Slovakia, E-mail: robert.toman@uniag.sk.
Monika Martiniaková, Constantine the Philosopher University, Faculty of Natural Sciences, Department of Zoology and Anthropology, 949 74 Nitra Slovakia, E-mail: mmartiniakova@ukf.sk.