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Protective effect of zinc supplementation against cadmium-induced oxidative stress and the RANK/RANKL/OPG system imbalance in the bone tissue of rats

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ABSTRACT

It was investigated whether protective influence of zinc (Zn) against cadmium (Cd)-induced disorders in bone metabolism may be related to its antioxidative properties and impact on the receptor activator of nuclear factor (NF)-κB (RANK)/RANK ligand (RANKL)/osteoprotegerin (OPG) system. Numerous indices of oxidative/antioxidative status, and Cd and Zn were determined in the distal femur of the rats administered Zn (30 and 60 mg/l) or/and Cd (5 and 50 mg/l) for 6 months. Soluble RANKL (sRANKL) and OPG were measured in the bone and serum. Zn supplementation importantly protected from Cd-induced oxidative stress preventing protein, DNA, and lipid oxidation in the bone. Moreover, Zn protected from the Cd-induced increase in sRANKL concentration and the sRANKL/OPG ratio, and decrease in OPG concentration in the bone and serum. Numerous correlations were noted between indices of the oxidative/antioxidative bone status, concentrations of sRANKL and OPG in the bone and serum, as well as the bone concentrations of Zn and Cd, and previously reported by us in these animals (Brzóska et al., 2007) indices of bone turnover and bone mineral density. The results allow us to conclude that the ability of Zn to prevent from oxidative stress and the RANK/RANKL/OPG system imbalance may be implicated in the mechanisms of its protective impact against Cd-induced bone damage. This paper is the first report from an *in vivo* study providing evidence that beneficial Zn impact on the skeleton under exposure to Cd is related to the improvement of the bone tissue oxidative/antioxidative status and mediating the RANK/RANKL/OPG system.

Introduction

Recently, due to the growing human exposure to chemical substances (Nawrot et al., 2010) and increasing prevalence of osteoporosis in industrialized countries (Garriguet, 2011; Orwig et al., 2011), much attention has been paid to xenobiotics as risk factors for bone diseases (Cho et al., 2012; Pollack et al., 2013). One of them is cadmium (Cd) belonging to the main and most toxic environmental and occupational pollutants (Kah et al., 2012; Nawrot et al., 2010). Epidemiological studies provide strong evidence that even a relatively low long-term exposure to Cd disturbs bone metabolism contributing to low bone mineral density (BMD), and thus this metal has been hypothesized as an environmental risk factor for osteoporosis (Engström et al., 2012; Sughis et al., 2011; Suwazono et al., 2010; Thomas et al., 2011). The epidemiological and experimental (Brzóska, 2012; Brzóska and Moniuszko-Jakoniuk, 2004, 2005) data on the unfavorable impact of low and relatively low Cd exposure on bone metabolism and prognoses that general population exposure to this metal will show an increasing trend (Nawrot et al., 2010) persuade researches for searching for effective ways of protection from its damaging impact on the skeleton.

Recent results of our own studies (Brzóska et al., 2007, 2008, 2011b) and sparse data provided by other authors so far (Bulat et al., 2008; Malekpouri et al., 2011; Suzuki et al., 1990) show that zinc (Zn), being a bone essential element, may play an important role in the protection from the Cd-induced bone damage. We have reported, on a rat model of moderate and relatively high chronic human exposure to Cd (long-term administration of 5 and 50 mg Cd/l in drinking water, respectively), that an enhancement of the daily intake of Zn has beneficial impact on bone metabolism and strength properties (Brzóska et al., 2007, 2008, 2011b).

Zn plays an important role in the proper bone growth, development, and maintenance of healthy bones in human and animals (Hadley et al., 2010; Sun et al., 2011; Yamaguchi, 2010). It promotes bone formation

Abbreviations: BMD, bone mineral density; CAT, catalase; Cd, cadmium; CTX, carboxy-terminal cross-linking telopeptides of type I collagen; Cu, copper; Fe, iron; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; H_2O_2, hydrogen peroxide; LPO, lipid peroxides; Mn, manganese; OC, osteocalcin; OPG, osteoprotegerin; OSI, oxidative stress index; PC, protein carbonyl groups; P-SH, protein thiol groups; ROS, reactive oxygen species; SOD, superoxide dismutase; sRANKL, soluble receptor activator of nuclear factor-κB; sRANKL/OPG, the ratio of sRANKL and OPG; TAS, total antioxidative status; TOS, total oxidative status; T-SH, total thiol groups; Zn, zinc; 8-OHdG, 8-hydroxy-2′-deoxyguanosine.

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via stimulating osteoblasts proliferation and differentiation, and improves osteoblastic bone mineralization *via* activating alkaline phosphatase and stimulating collagen synthesis (Hadley et al., 2010; Liang et al., 2012a; Sun et al., 2011; Yamaguchi and Weitzmann, 2011). Moreover, it suppresses osteoclasts differentiation, inhibiting bone resorption (Hadley et al., 2010; Yamaguchi and Weitzmann, 2011). Zn supplementation has been noted to have positive impact on bone mineral content and to prevent osteoporosis (Sun et al., 2011; Yamaguchi, 2010), whereas this bioelement deficiency inhibits synthesis of the extracellular bone matrix and its calcification (Alcantara et al., 2011; Sun et al., 2011). It has been suggested that the main mechanism by which Zn promotes osteoblastogenesis and suppresses osteoclastogenesis may consist in antagonism of the nuclear factor (NF)-κB activation and in the stimulation of osteoprotegerin (OPG) activity (Hie et al., 2011; Liang et al., 2012a; Yamaguchi and Weitzmann, 2011); however, the molecular mechanism explaining how this bioelement regulates bone turnover is still poorly understood. Cd affects bone tissue directly *via* influencing differentiation and activity of bone cells as well as by disturbing the body mineral status in an indirect way, and this metal-induced disorders in the metabolism and biological functions of Zn are implicated in the mechanisms of its damaging action in the bone tissue (Bhattacharyya, 2009; Brzóska, 2012; Brzóska and Moniuszko-Jakoniuk, 2004, 2005; Brzóska et al., 2007, 2011a; Bulat et al., 2008; Chen et al., 2009, 2011, 2012; Smith et al., 2009; Suzuki et al., 1990). Cd decreases Zn concentration in bones and inhibits an activity of this bioelement-dependent enzyme—alkaline phosphatase playing an important role in bone mineralization (Brzóska, 2012; Brzóska and Moniuszko-Jakoniuk, 2004, 2005; Brzóska et al., 2007, 2011a). Moreover, recently we have noted for the first time an inhibition by Cd of Zn-dependent antioxidative enzyme—superoxide dismutase (SOD) in the bone tissue and revealed oxidative stress involvement in the mechanism of this heavy metal action in bone *in vivo* (Brzóska et al., 2011a). The latest *in vitro* data (Chen et al., 2009, 2011, 2012) suggests that Cd may disturb the bone turnover *via* influencing the receptor activator of NF-κB (RANK)/RANK ligand (RANKL)/OPG system. RANKL, its cellular receptor—RANK, and the decoy receptor—OPG play an important role in the regulation of bone turnover (Lloyd et al., 2008; Wright et al., 2009). However, the involvement of the RANK/RANKL/OPG system in the pathways of Cd impact on the bone tissue has not been investigated *in vivo* until now.

Based on our own findings (Brzóska et al., 2007, 2008, 2011b) and data by other authors (Bulat et al., 2008; Malekpouri et al., 2011; Suzuki et al., 1990) we have concluded that the protective influence of Zn on bone during chronic exposure to Cd may arise from its direct action in the bone tissue as well as an indirect impact related to the lower body burden of Cd, including its lower accumulation in bones. However, the way how Zn protects against Cd impact on the skeleton is still poorly understood. Taking into account a small number of data from *in vivo* and *in vitro* studies conducted so far on the oxidative stress involvement in the mechanisms of Cd action in the bone tissue (Brzóska et al., 2011a; Smith et al., 2009) and the suggested RANK/RANKL/OPG system implication in these mechanisms (Chen et al., 2009, 2011) as well as antioxidative Zn properties (Galażyn-Sidorczuk et al., 2012; Messaoudi et al., 2010) and its ability to influencing the RANK/RANKL/OPG system (Hie et al., 2011; Liang et al., 2012a; Yamaguchi and Weitzmann, 2011), we have hypothesized that the beneficial impact of this bioelement on the skeleton under exposure to Cd might result, at least partly, from its action as an antioxidant and from the mediation of the RANK/RANKL/OPG system. The aim of this study was to investigate this hypothesis. For this purpose, the bone tissue oxidative/antioxidative status, including oxidative protein, lipid and DNA damage, the serum and bone tissue concentrations of soluble RANKL (sRANKL) and OPG as well as the bone concentrations of Cd and Zn were evaluated in the same rats in which we have previously reported the protective Zn impact (Brzóska et al., 2007, 2008, 2011b). Moreover, the dependences between the indices of the bone status determined in the present paper and indices of bone turnover and BMD previously reported in these animals (Brzóska et al., 2007, 2008) were estimated. According to our knowledge a similar study has not been conducted until now.

Materials and methods

Animals and experimental protocol. The study was approved by the Local Ethics Committee for Animal Experiments in Bialystok (Poland) and performed according to the ethical principles and institutional guidelines and international Guide for the Use of Animals in Biomedical Research.

Seventy-two adult (10-week old) male Wistar rats, randomly assigned into nine groups of 8 animals each, were used. Two groups received Zn alone, two were treated with Cd alone and four groups were supplemented with Zn during the whole course of Cd exposure. Zn and Cd were administered in drinking water at the concentrations of 30 or 60 mg Zn/l (as $ZnCl_2$; POCh, Gliwice, Poland) and 5 or 50 mg Cd/l (as $CdCl_2 \cdot 2½H_2O$; POCh) alone and in combination for 6 months. The remaining experimental group drank water without Cd and Zn addition and it served as a control. Redistilled water (containing <0.05 μg Cd/l and not completely deprived of bone necessary bioelements) was used as drinking water to eliminate Cd intake in the groups of rats not intoxicated with this metal according to the experimental protocol.

Throughout the experiment, the animals were housed under controlled conventional conditions (12-h light–dark cycle, temperature 22 ± 2 °C, relative humidity 50 ± 10%) and fed a standard rodent LSM dry chow (Agropol, Motycz, Poland) and drinking water *ad libitum*. The LSM diet contained 1.11% Ca, 0.72% phosphorus, 48 μg Zn/g and 0.098 μg Cd/g. The total daily intake (with food and water) of elements necessary for proper bone metabolism and having antioxidative properties covered the animals' requirement. The experimental protocol has been described in details in our previous reports from studies in these rats (Brzóska et al., 2007, 2008, 2011b; Galażyn-Sidorczuk et al., 2012; Rogalska et al., 2011).

The daily doses of Zn and Cd were within the same ranges of values irrespective of whether these elements were administered alone or together. The mean (mean ± SE; SE—standard error) daily intakes of Zn in the rats that received 30 and 60 mg Zn/l (alone or with Cd) for the 6-month experimental period were 2.240 ± 0.236 mg/kg b.wt and 4.420 ± 0.649 mg/kg b.wt, respectively. The mean daily doses of Cd in the animals exposed to 5 and 50 mg Cd/l (alone or with Zn) were 0.430 ± 0.089 mg/kg b.wt and 2.752 ± 0.239 mg/kg b.wt, respectively. Detailed data on Zn and Cd intakes in particular groups has already been presented (Galażyn-Sidorczuk et al., 2012; Rogalska et al., 2011). Since diet consumption in all groups receiving Zn or/and Cd was similar as in the control group (24.7 ± 0.39 g/rat/24 h), the mean daily intakes of these metals with the LSM diet in the rats that received Cd or/and Zn in drinking water were within the range of their intakes in the control ones (1.186 ± 0.019 mg Zn/rat and 2.421 ± 0.038 μg Cd/rat). Administration of 30 and 60 mg Zn/l (alone or with Cd) enhanced the daily intake of this bioelement by an average of 79% and 151% of its intake with the standard LSM diet, respectively.

At termination, the whole blood was collected by cardiac puncture with and without anticoagulant (heparin), and different organs and tissues, including femur, were dissected under barbiturate anesthesia (Vetbutal, 30 mg/kg b.wt., *ip*). A portion of the whole blood collected without anticoagulant was centrifuged after coagulation and serum was separated. The bones, after cleaning of the surrounding muscles and tissues, were weighted. The biological material not used immediately was stored frozen at −70 °C until all measurements were performed.

The serum samples and slices of the bone tissue isolated from the distal epiphysis of the right femur (trabecular bone region) were used in the present study to estimate the impact of Zn supplementation during exposure to Cd on the oxidative/antioxidative status of the bone tissue and the RANK/RANKL/OPG system. With the aim of evaluating the antioxidative bone status, numerous indices of the enzymatic and non-enzymatic antioxidative barrier as well as the total antioxidative