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Original Research

Morphological Changes in the Liver and the Response of Antioxidant Enzymes after Turkeys' Chronic Exposure to Cadmium

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Abstract

We observed the effect of cadmium (Cd group) and cadmium+zinc (Cd/Zn group) in the liver of turkeys after a 71-day exposure. The experimental birds were divided to three groups; control group (Control), group exposed to cadmium (group Cd) as $CdCl_2$ (aqueous solution, 0.5 mg/kg feed); and group Cd/Zn exposed to the same dose of cadmium as Cd group plus zinc as $ZnSO_4$ (aqueous solution, 90 mg/kg feed). Light and electron microscopy revealed pronounced changes in the liver of turkeys from the Cd group, such as hyperaemia, dilatation of sinusoids and accumulation of inflammatory cells, including macrophages, heterophils, and lymphocytes in sinusoids. Necrotizing hepatocytes were observed sporadically. The ultrastructural changes included swollen mitochondria with injured cristae, dilated cisternae of rough endoplasmic reticulum, and damaged intercellular contacts between hepatocytes. In the Cd/Zn group, Zn was not able to completely protect the liver, but the changes were less pronounced.

The specific activity of superoxide dismutase was significantly increased in the Cd group. Glutathione peroxidase was significantly increased in all experimental groups. In the Cd/Zn group, zinc co-administration had a protective effect on the activity of antioxidant enzymes. Exposure of turkeys to cadmium did not affect the content of TBARS in the liver.

Keywords: morphology, antioxidant enzymes, liver, cadmium, zinc, turkey

Introduction

Cadmium is a toxic metal and an important environmental pollutant. It is present in the soil, water, air, and in food. The wide environmental distribution of cadmium led has to an increased interest in its toxicity and biological effects. This metal is resistant to biodegradation and has extremely long biological half-life in the body, which results in accumulation in the tissues [1]. Cadmium affects adversely a number of organs in humans and other mammals, including liver, kidneys, lungs, pancreas, testis, placenta, and bone, and causes poisoning of various tissues of humans and animals [1-6].

In the liver, a variety of mechanisms have been described involving Cd-induced cytotoxicity. Cadmium ions have high affinity to thiol groups. Primary damage is caused by binding of Cd^{2+} to thiol groups on critical molecules in mitochondria. Inactivation of thiol groups causes inhibition of energy metabolism, membrane damage, and dysfunction of mitochondria, oxidative stress, and altered gene expression [7, 8]. Chemical similarity of cadmium to some essential elements

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enables Cd to replace those elements in biological structures. This metal is interacting in many ways with the metabolism of essential metals such as calcium (Ca), zinc (Zn), cooper (Cu), and iron (Fe) [9, 10]. Martelli et al. [10] reported that disturbances of calcium, zinc or iron homeostasis, individually or most probably collectively, play a key role in the toxicological action of cadmium. Cd intoxication induces expression of a number of stress genes [11] and might affect the activity of some antioxidant enzymes. Cadmium induces oxidative stress. This process has been associated with the production of excessive reactive oxygen species (ROS) that interact with the cellular macromolecules causing lipid peroxidation, DNA damage, and membrane protein degradation [7, 8, 12-15].

Cadmium is not essential for organisms but can be present in all tissues, predominantly in the liver and kidneys. In the liver, cadmium is stored as a metallothionein complex. Metallothionein (MT) is easily induced by Cd and various metal ions, particularly by Zn [16-18]. The excess Cd, which could not be detoxified with induced MT, is toxic to the liver and results in severe structural changes, even at very low concentrations [11, 19, 20].

The changes in the structure and ultrastructure of mammalian liver after cadmium administration were the subject of extensive studies [7, 19-25]. Relatively few reports dealt with ultrastructural changes in the poultry liver after Cd exposure [26].

Skalická et al. [27] presented that cadmium content in poultry meat from the polluted area of Eastern Slovakia was slightly higher, in comparison to maximum permissible hygiene limits for cadmium in poultry meat (0.1 mg/kg) and liver (0.5 mg/kg) according to Codex Alimentorum of the Slovak Republic No. 98/1996. Contamination by cadmium often has direct toxic effects. Therefore, in the present study, the damage to the liver of turkeys resulting from Cd administration was evaluated by histological studies and by measuring the activities of intracellular antioxidant enzymes, superoxide dismutase (SOD), glutathione peroxidase (GPx-cum, GPx-H_2O_2), and the content of thiobarbituric acid reactive substances (TBARS) as an indicator of lipid peroxidation. The protective effect of Zn was evaluated with respect to histological changes in the liver and the activities of the respective antioxidant enzymes.

Experimental Procedures

Animals and Diets

The experiment was carried out on 18 (female) turkeys of BIG-6 breed at the age of 35 days. The birds were divided to 3 groups of 6 animals after 30 days of acclimatization. The turkeys were initially fed mixed feed HYD 14. HYD 15 was given from week 9 of age. Two weeks before finishing the experiment the animals received mixed feed HYD 16. The composition of the diets was in compliance with the Regulation of the Government of the Slovak Republic No. 440/2006. Food and water were offered to turkeys *ad libitum*. The first group was the control (Control) without any treatment. The second group (group Cd) received cadmium as $CdCl_2$ (aqueous solution) at a dose ten times exceeding the daily acceptable limit (0.5 mg/kg feed). The third group (group Cd/Zn) received the same dose of cadmium as the Cd group plus zinc as $ZnSO_4$ (aqueous solution) at a dose exceeding twice the recommended dose (90 mg/kg feed). Cadmium or zinc was administered individually to the beaks of the turkeys as aqueous solutions of cadmium chloride and zinc sulphate, respectively, for 71 days. The birds were killed on day 136 of age [28]. The experiment was carried out according to the ethical requirements on animal handling.

Methods

Histological samples of the liver for light microscopy (LM) were processed by a common histological technique. They were fixed in 4% neutral formaldehyde and embedded in paraffin. Then 5-7 μm thick slides were stained with haematoxylin and eosin and photographed under a light microscope Jenamed. The samples intended for transmission electron microscopy (TEM) were fixed in 3% glutaraldehyde, postfixed in 1% OsO_4 (both in a phosphate buffer pH 7.2-7.4), dehydrated in acetone and embedded in Durcupan ACM (Fluka). The ultrathin sections were cut on an ultramicrotome Tesla BS 490, stained with uranyl acetate and lead citrate and evaluated under a transmission electron microscope Tesla BS 500.

Preparation of tissue extracts for analysis of antioxidant enzyme activities: the liver was homogenized in 5 mmol·dm^{-3} Tris-HCl buffer, pH 7.8, containing 0.15 mmol·dm^{-3} KCl, 1 mmol·dm^{-3} Na_2EDTA and 2 mmol·dm^{-3} GSH, using a Ultra-Turrax homogenizer. Homogenates (25% w/v) were centrifuged at 105,000 g for 60 min and the supernatants were stored at -50°C until being used for enzyme assays.

Total superoxide dismutase activity (SOD, EC 1.15.1.1) was determined by measuring the inhibition of cytochrome c reduction using the xanthine/xanthine oxidase $O_2^{\cdot-}$ generating system at 550 nm (25ºC) [29]. One unit of SOD activity was defined as the amount of enzyme that causes 50% inhibition of cytochrome c reduction under the assay conditions. Glutathione peroxidase activity (GPx) was measured by monitoring the oxidation of NADPH+H$^+$ at 340 nm (37ºC), as described Flohé and Günzler [30] in a coupled assay with glutathione reductase. Cumene hydroperoxide (GPx-cum, EC 1.11.1.12) or H_2O_2 (GPx-H_2O_2, EC 1.11.1.9) were used as substrates. One unit of enzyme activity GPx is activity that catalyses the formation of 1 μmol of product per minute under respective assay conditions. Specific enzyme activities were expressed in U/mg of protein.

The protein concentration was measured by the method of Bradford [31] using bovine serum albumin as a standard.

Lipid peroxides formation was measured as malondialdehyde and other aldehydes, by reaction with thiobarbituric acid, yielding coloured products named thiobarbituric reactive substances (TBARS) that absorb at 535 nm [32]. The content of TBARS was expressed in A_{535}/mg of protein.

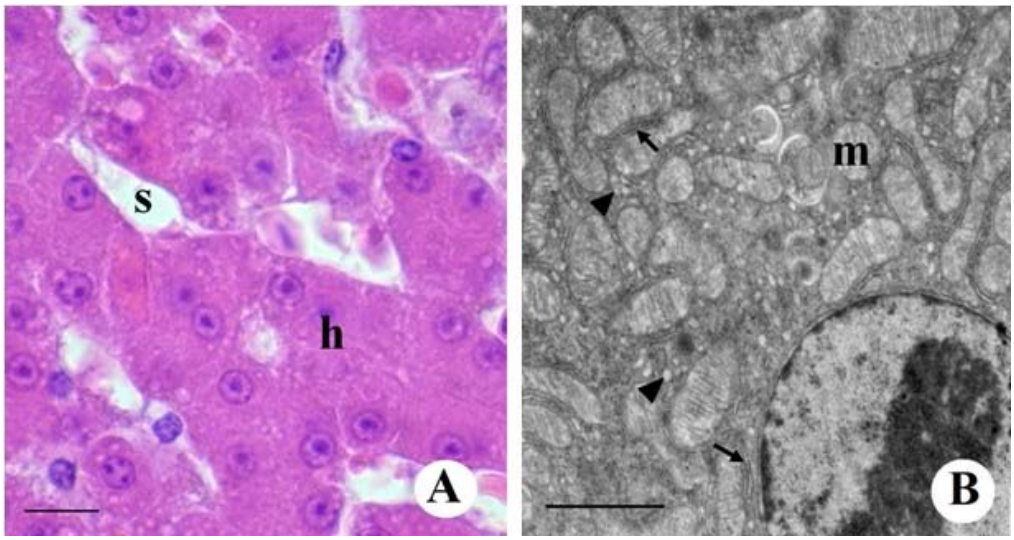

Fig. 1. Light and electron microscopy of liver from control groups. A: Hepatocytes were arranged in anastomozing two-cell thick cords – h, separated by sinusoids –s. Scale bar: 15 μm. B: The most numerous organelles were mitochondria – m. RER was closely associated with mitochondria – arrows. SER - arrowheads. Scale bar: 4.9 μm.

The content of metals (Cd, Zn) was determined by means of atomic absorption spectroscopy (AAS-SOLAR 939 UNICAM) using the method of Kocourek [33].

All reagents (the highest purity) were from Sigma, Merck, and Boehringer.

Statistics

The specific activities of antioxidant enzymes are given as means±SD of at least three independent determinations in six different batches. Statistical analysis was done by one-way analysis of variance (ANOVA) with the *post hoc* Tukey multiple comparison tests.

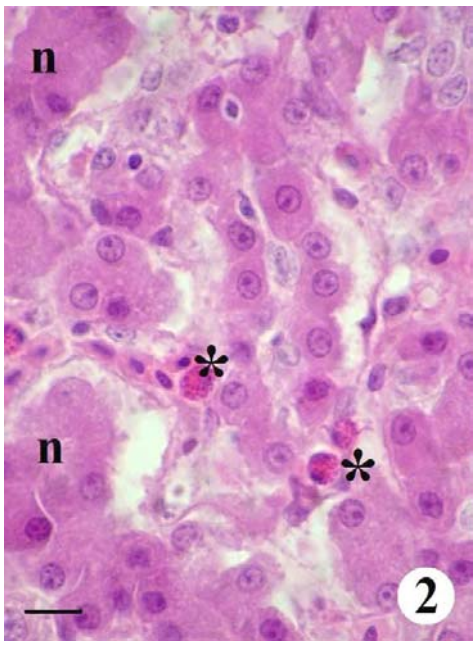

Fig. 2. Light microscopy of turkey liver, Cd group. Necrotizing hepatocytes – n, dilated sinusoids filled with heterophils – asterisk, Scale bar: 10 μm.

Results

The accumulation of cadmium was confirmed by AAS in the liver of turkeys during cadmium intoxication. The content of cadmium was 11 times higher in Cd group (0.78±0.08 $mg \cdot kg^{-1}$) compared with the control (0.07±0.03 $mg \cdot kg^{-1}$). In Cd/Zn group, the accumulation of cadmium was lower; the content of cadmium was approximately 6 times higher (0.41±0.05 $mg \cdot kg^{-1}$) compared with the control [28]. No changes were observed in the content of zinc in all experimental groups (data not shown).

The mean body weight of turkeys at the beginning of the experiment was 1.88 kg and at the end 10.53 kg. The highest mean body weight was recorded in control turkeys (11.30 kg) and the lowest in the group administered cadmium (10.06 kg). The mean body weight of birds in group Cd/Zn reached 10.56 kg [34].

Light Microscopy (LM)

In the control group, the hepatocytes were arranged in anastomozing two-cell thick cords or tubules, which were separated by sinusoids. The bile canaliculus was formed by 4-6 hepatocytes arranged around the canaliculus. The hepatocytes were large and wedge-shaped. The larger vascular pole was oriented toward the liver sinusoids and the small biliary pole was oriented toward the bile canaliculus. The hepatocytes generally had only one nucleus. They were round with a prominent nucleolus. The nucleus was usually located toward the vascular pole. The cytoplasm was acidophilic (Fig. 1A).

In the Cd group, cadmium caused hyperaemia of the liver with inflammatory infiltrate that consisted mostly of heterophils and lymphocytes. Blood sinusoids were dilated and contained an increasing number of Kupffer cells. In the places of their massive accumulation we observed changes in hepatocytes. Many of them were enlarged, dilated, with

light cytoplasm. The borders between neighboring cells were frequently absent. In these sites the liver parenchyma frequently lacked its typical appearance. Necrotizing hepatocytes were sporadically observed in the liver. These cells had shrunken nuclei with condensed chromatin and acidophilic cytoplasm. However, no inflammatory changes were observed in the portobiliary spaces (Fig. 2).

The Cd/Zn experimental group showed less pronounced histological changes compared to the previous (Cd) group. We observed no hyperaemia of the liver and the blood sinusoids were slightly dilated with sporadic occurrence of heterophils. The hepatocytes retained their pyramidal shape and were arranged into trabeculae or tubules around the centrally located biliary capillary. There were neither necrotic foci nor individual necrotizing hepatocytes in the parenchyma. The portobiliary spaces were free of inflammatory infiltrate and contained only little connective tissue (Fig. 3).

Transmission Electron Microscopy (TEM)

In the control group, the hepatocytes at the biliary poles possessed numerous microvilli. The cell membranes in these regions were joined together by tight junctions, the *zonulae occludentes*. The most numerous organelles in the hepatocytes were mitochondria. They were ovoid or rod-shaped and had a double membrane. Lysosomes were randomly distributed throughout the cytoplasm. Rough endoplasmic reticulum (RER) and free ribosomes were closely associated with the mitochondria. The smooth endoplasmic reticulum (SER) appeared as delicate membranes sparsely scattered in the cytoplasm. Thin, flattened endothelial cells and Kupffer cells formed the wall of sinusoids. Intercalated

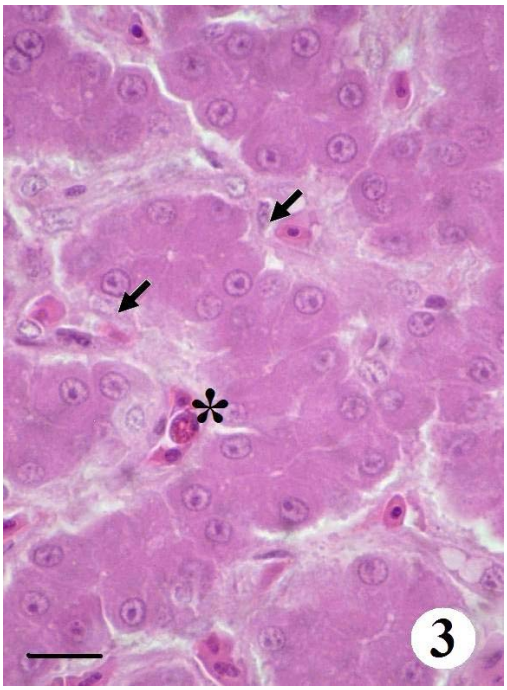

Fig. 3. Light microscopy of turkey liver, Cd/Zn group. Kupffer cells – arrows, dilated sinusoids filled with heterophils - asterisk. Scale bar: 10 μm.

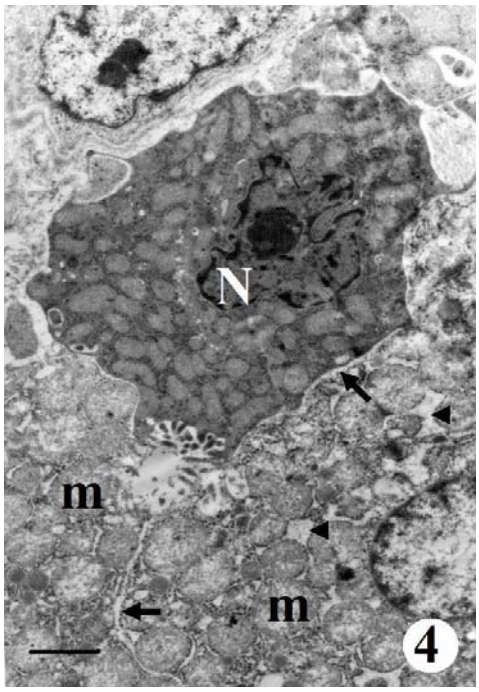

Fig. 4. Electron micrograph of Cd group. Irregular nuclei – N, dilated mitochondria – m, dilated intercellular spaces – arrows, RER – arrowheads. Scale bar: 3.3 μm.

cells were widely distributed throughout the hepatic parenchyma (Fig. 1B).

In the Cd group we observed changes in ultrastructure of hepatocytes. Damaged hepatocytes had markedly electrondense cytoplasm. Their nuclei were irregular with deep invaginations. The chromatin around nuclear membrane was condensed. These hepatocytes had only slightly dilated RER, which still contained bound ribosomes. The most pronounced changes were observed in mitochondria. They were swollen, round, dilated, with damaged cristae. The matrix contained several electrondense granules. Lipid globules were observed sporadically. Microvilli at the vascular poles of hepatocytes were short and irregular. Intercellular contacts between neighbouring hepatocytes were inconspicuous and in some places almost invisible. Due to damage to these intercellular contacts we observed extension of the intercellular space. The hepatocytes with unchanged nuclei also showed changes in RER. In some hepatocytes we observed dilatation only of peripheral cisternae RER, while in others the whole cisternae RER were dilated. These cells showed changes in mitochondria resembling those observed in hepatocytes with dark electrondense cytoplasm (Figs. 4 and 5).

The experimental group Cd/Zn showed less pronounced changes in the parenchyma. No necrotizing hepatocytes were observed in cords. All cells had cytoplasm of equal density. Intercellular contacts showed no visible changes. The hepatocytes were located close to each other without extended intercellular spaces. However, the ultrastructure of hepatocyte organelles was changed. The damage was evident particularly in mitochondria. They were dilated with damaged cristae and some contained electrondense granules. Dilatation was seen also in RER.

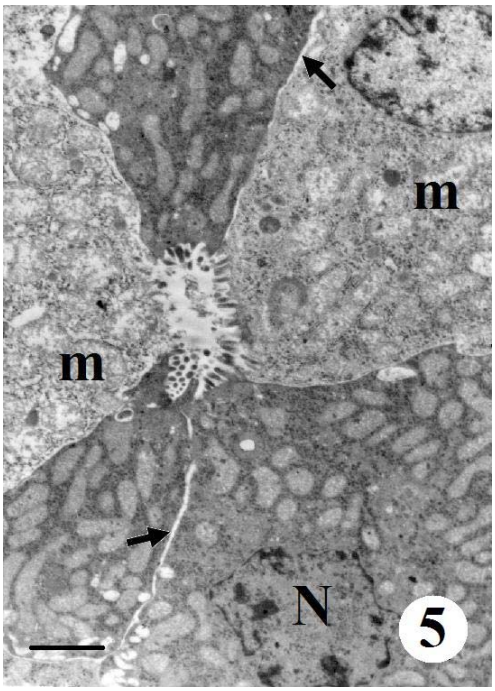

Fig. 5. Electron micrograph of Cd group. Irregular nuclei – N, dilated mitochondria – m, dilated intercellular spaces – arrows. Scale bar: 3.9 μm.

The changes were less pronounced than those caused by cadmium alone. The cells contained neither lipid globules nor autophagozones (Figs. 6 and 7).

The Activities of Antioxidant Enzymes

The high specific activity of SOD in the liver is referred to its important role in the organ. The specific activity of SOD was significantly increased in Cd group. Zinc co-administration showed a protective effect. No significant changes were seen in the Cd/Zn group as compared with the control (Fig. 7).

GPxs play a major role in protecting cells from oxidative damage, especially lipid peroxidation of biological membranes [35]. The activity of GPx-cum was significantly increased in all experimental groups as compared with the controls. The activity of GPx-H_2O_2 was decreased significantly in the Cd/Zn group (Fig. 8).

One of the markers of oxidative damage of membrane lipids is the TBARS content [36]. TBARS content showed no significant changes in experimental groups (Fig. 8).

Discussion

Cadmium is a dangerous environmental and industrial pollutant. It has extremely long biological half-life in the body and accumulates in the tissues. Liver is a major target organ of cadmium toxicity following acute exposure, but also is a target of chronic cadmium toxicity [37].

Our results indicated a significant increase in cadmium levels in the liver (0.78±0.08 mg/kg) and kidneys (1.091±0.057 mg/kg) compared with control group [28]. Despite low content of cadmium in the liver of turkeys in the Cd group compared with other animals [19, 22, 23], some histological changes were observed. Mitsumori et al. [19] investigated the relationship between hepatotoxicity and accumulation of cadmium in the liver of rats and observed apparent toxicity only after the Cd level reached Cd 164.4 μg/g. They failed to observe any histological

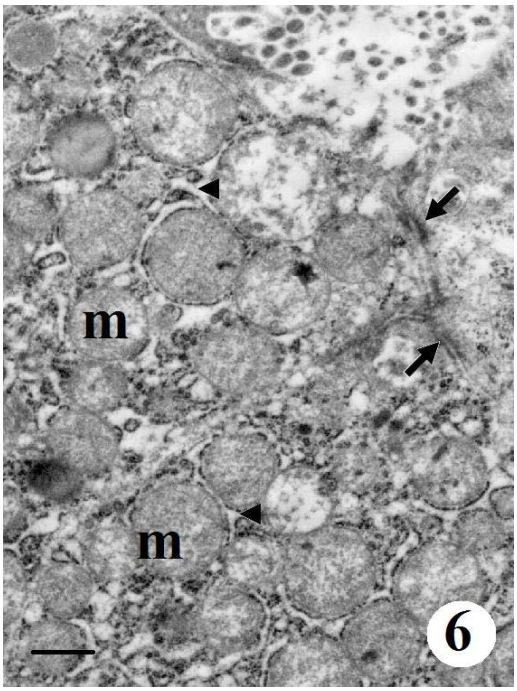

Fig. 6. Electron micrograph of Cd/Zn group. Dilated mitochondria – m, intercellular contacts – arrows, RER – arrowheads. Scale bar: 5.6 μm.

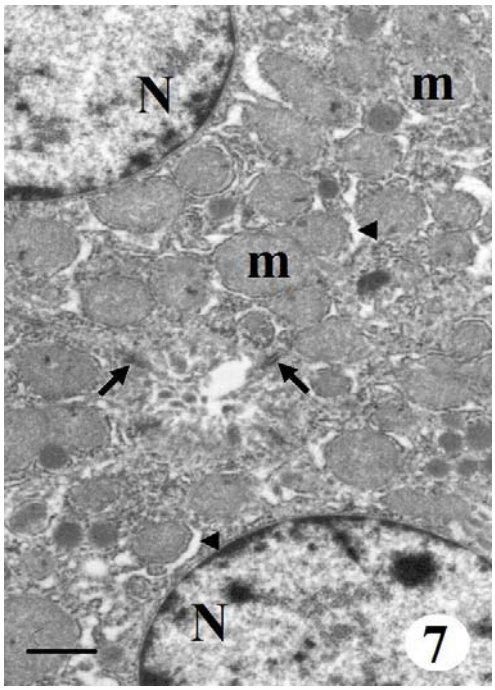

Fig. 7. Electron micrograph of Cd/Zn group. Dilated mitochondria – m, nuclei of hepatocytes – N, intercellular contacts – arrows, RER – arrowheads. Scale bar: 4.3 μm.

changes in the liver at Cd level of 125.7 μg/g. On the other hand, Brzóska et al. [22] and Jihen et al. [23] observed histological changes in rat liver at Cd levels reaching 16.96 μg/g and 28 μg/g. The liver response to injury induced by cadmium can vary extremely. Cadmium can cause, even at very low concentrations, distinct pathological changes in the liver [11].

In our study, cadmium damaged the junctional complexes. Several studies indicate that Cd^{2+} can displace Ca^{2+} in extracellular binding site on E-cadherin. This displacement changes the conformation of the molecule and the adhesive regions are no longer properly oriented to maintain the intercellular linkage. The loss of the linkage results in separation of cells from each other [38, 39]. We observed an extension of the intracellular spaces as a result of damage to these inter-cellular contacts and, consequently, separation of hepatocytes from each other, indicating E-cadherin disruption [40].

Many studies reported dilatation of the cisternae of rough endoplasmic reticulum with a loss of ribosomes, nuclear condensation, and an increase in the number of perichromatin granules [7, 21]. We observed dilatation of RER, without the loss of ribosomes in both treated groups. This change was probably due to failure of calcium homeostasis and accumulation of unfolded proteins in the endoplasmic reticulum (ER) [41]. The key intracellular targets for Cd are mitochondria [7, 11, 20, 42, 43]. Our ultrastructural analysis showed big changes in mitochondria in the Cd group. They were round and swollen with damaged cristae.

Primary damage to the liver is a result of direct effect of cadmium. Secondary damage is caused by inflammatory processes that are part of the activation of Kupffer cells [7]. It is known that activated Kupffer cells release a number of proinflammatory mediators that initiate a cascade of cellular and humoral responses leading to inflammation and secondary damage to the liver. The increased number of Kupffer cells and heterophils was observed in Cd group. Damaged hepatocytes were observed in some areas with massive accumulation of these inflammatory cells. Activated neutrophils, macrophages, and Kupffer cells are a major source of ROS during acute and chronic inflammatory diseases. However, large amounts of ROS are produced as a consequence of electron transfer reactions in mitochondria, peroxisomes, and cytosol. Generated ROS can be scavenged by the cellular defence system, e.g. superoxide dismutase, catalase, glutathione peroxidase, etc. [12].

The high specific activity of SOD in the liver of control group indicates an important role of SOD in this organ. The significantly increased activity of SOD observed in the Cd group might be a response to the accumulation of ROS.

Biological membranes are the primary sites of cadmium action. Cadmium causes alterations in the functionality of membranes by inducing changes in lipid composition and by affecting the membrane-associated enzymatic activities [44]. Excessive amount of ROS could directly react with unsaturated fatty acid on the surface of the membrane, which leads to the destruction of its structure and function [43].

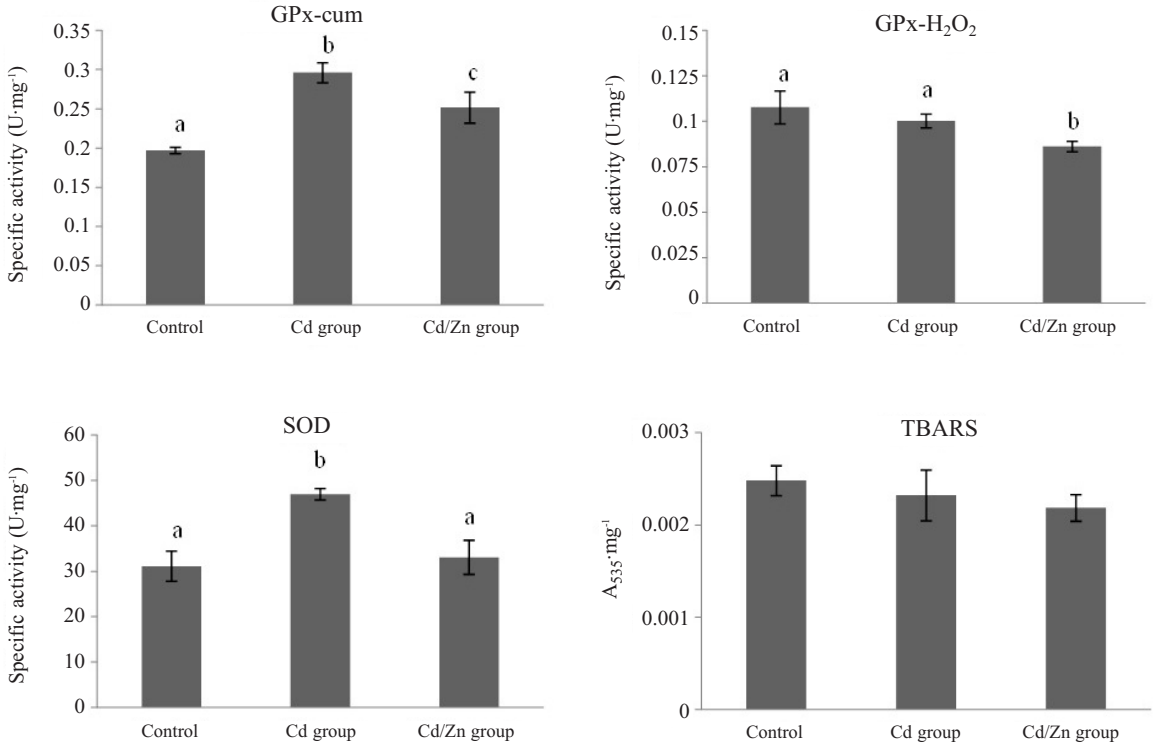

Fig. 8. Specific activities of antioxidant enzymes (SOD, GPx) and TBARS content in turkey livers after chronic exposure to cadmium. Values are means ± SD (n=6). Distinct letters above columns mean significant differences ($P<0.05$).

Cadmium through binding to the inner membrane enhances lipid peroxidation and disturbs the integrity of mitochondrial membranes [11].

GPx enzymes that catalyze GSH-dependent reduction of hydroperoxides through their peroxidase activity are the second major defence against ROS-induced lipid peroxidation. These enzymes play an important role in the detoxification of lipid hydroperoxides and thus contribute to the protection of membrane integrity. The activity of GPx-cum was also significantly increased in Cd and Cd/Zn experimental groups when compared to the control. Enhanced GPx-cum activity correlated with the structural changes in the liver.

The ultrastructural changes in hepatocytes induced by cadmium and significant changes in SOD and GPx-cum activities, in the Cd experimental group, were not accompanied with significant increases in TBARS content, the marker of the oxidative damage.

It is known that the bioaccumulation and toxicity of cadmium is modified by many dietary components. Cadmium is highly interactive with concentrations of dietary zinc, which reduces the rate of cadmium absorption from various food sources. Zinc, the element involved in cell membrane stabilization, metallothionein synthesis, and superoxide dismutase structure (Cu/Zn SOD) is an important antioxidant decreasing ROS production [15, 45, 46]. Some studies have suggested that Zn protection is due to redistribution of Cd in the organism since Zn is able to induce synthesis of metallothionein in the liver [16]. Naď et al. [28, 34] during the monitoring of the effect of higher doses of cadmium and interaction of zinc on the health of turkeys, confirmed the protective effect of zinc against cadmium. In our experiment, in the Cd/Zn group, treatment with Zn markedly reduced the level of cadmium in the liver, but it was still significantly increased when compared with the control. The activity of SOD showed no significant changes when compared with the control. The activity of GPx-cum was significantly increased but the increase of GPx-cum was lower than in turkeys receiving cadmium alone. We noticed a partial improvement in the Cd-induced damage to the liver. Some ultrastructural changes also were observed but these changes were less pronounced than in the Cd group. Light microscopy showed no hyperaemia and the number of inflammatory cells was decreased. The cadmium-induced inflammation of liver tissue is an important mechanism of Cd-induced oxidative stress. The Kupffer cells could release inflammatory cytokines in response to Cd overload, which in turn contribute to Cd-generated free radicals in the liver. Inhibition of the Kupffer cell function has been shown to decrease Cd hepatotoxicity in rats [5]. In our experiments in the group receiving zinc together with cadmium we observed a decreased number of inflammatory cells and changes in the activity of antioxidant enzymes. The protective function of zinc is reflected in lower cadmium accumulation in the liver and in the reduction of ROS production [46]. Further studies are necessary for better understanding of the protective role of zinc against cadmium hepatotoxicity.

In contrast to these findings, Jihen et al. [15, 23] observed only partial corrective effects of Zn on Cd toxicity in the liver of rats. Zn partially alleviated the damage observed in the liver, but the accumulation of Cd in the liver in the Cd/Zn group was significantly higher than in the Cd group, so was the content of TBARS.

A study by Chung et al. [47] showed that Zn provides protection against oxidative stress due to Zn-mediated transcription of antioxidants, but when administered simultaneously with hydrogen peroxide, the cytotoxic effects were even more severe than those of hydrogen peroxide administered alone. The findings indicated the complexity of the involvement of Zn, which can have indirect effects by acting as both an antioxidant and prooxidant [47]. Rogalska et al. [48] provided evidence of the hepatoprotective influence of zinc under chronic exposure to cadmium in rats and showed that excessive intake of this element may intensify liver injury.

Conclusion

This study investigated the influence of cadmium on the turkey´s liver. Liver acts as a barrier or filter between the digestive system and the rest of the body and has an essential role in metabolism of many chemicals and toxic substances entering the organism through the gastrointestinal system. Our study demonstrates that the chronic effect of cadmium causes structural and ultrastructural changes in the turkey's liver. Inflammation, massive accumulation of Kupffer cells, and necrotizing hepatocytes were the most frequent changes. Moreover, the electron microscopy revealed the most pronounced changes in mitochondria, which were swollen with injured cristae. The cadmium also caused the separation of hepatocytes from each other, which was due to the damage of intercellular contacts. The activities of some antioxidative enzymes also were changed. Zinc had a partially protective effect.

Acknowledgements

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