The effect of ozone on the expression of metallothionein in tissues of rats chronically exposed to cadmium

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ABSTRACT

Our aims were to evaluate the expression of metallothionein (MT) in an experimental rat model which experienced chronic exposure to cadmium (Cd) and to measure its expression after ozone therapy (OT) or oxygen (Ox) in the same model, as compared to the control group, which was exposed to neither cadmium nor ozone.

Forty male Wistar rats were divided into 5 groups: control, Cd, Cd and Ox, Cd and Ox, and Ox. During our research, Cd concentration (ASA) and MT concentration (ELISA) were determined in supernatants of the kidneys, liver and pancreas. SDS-PAGE analyses and immunohistochemical localization were used to evaluate the level of MT expression in the tissue. In rats intoxicated with Cd, the highest concentration of both Cd and MT was observed in the kidneys and liver, with a significantly lower concentration measured in the pancreas. Ozone therapy reduces the accumulation of cadmium in the liver and kidneys, resulting in a reduced expression of metallothionein in those tissues.

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1. Introduction

Ozone (O₃), a gas composed of three oxygen atoms, is continuously produced in the stratosphere by UV radiation or during the electric discharge of lightning from atmospheric oxygen. Ozone as a gas mixture of ozone/oxygen used in medicine is known as medical ozone therapy (OT) (Inoue et al., 2008a). The ozone/oxygen mixture has various effects on the immune system, such as the modulation of phagocytic activity of peritoneal and alveolar macrophages (Boccì, 2006, 2004; Oter and Korkmaz, 2006). Ozone causes the production of interferon, tumor necrosis factor, and interleukin-2. The production of interleukin-2 launches a cascade of subsequent immunological reactions (Elvis and Ekta, 2011). Clinical studies have so far shown that OT appears useful in treatment for peritonitis, infected wounds, and advanced ischemic diseases (Oter and Korkmaz, 2006; Re et al., 2008). It has also been demonstrated that O₃ increases the activity of antioxidant enzymes such as glutathione peroxidase, superoxide dismutase and catalase, which prepare the host to face physio-pathological conditions mediated by reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Boccì, 2006, 1996). The ameliorative effects of OT on oxidative and nitrosative stress have been reported in different experimental models of renal ischemia/reperfusion injury (Chen et al., 2008) necrotizing pancreatitis (Oztas et al., 2011; Uysal et al., 2010; Guven et al., 2009) and necrotizing enterocolitis (Guven et al., 2009; Kesik et al., 2009).

The effect of O₂ and oxygen on an organism simultaneously exposed to the influence of heavy metals, e.g. cadmium (Cd), has not been well understood so far. The distribution of Cd, a source of exogenous free radicals, in organs of animals differs depending on the chemical form of the administered Cd and the duration of exposure. However, repeated administration of cadmium salts produced histopathological changes in both the liver (Habeebu, 2000) and kidneys (Hughes et al., 2000). In one study, the severity of changes significantly decreased after the administration of an oxygen–ozone mixture (Kuryszko et al., 1995). In another, such beneficial effects of O₃ were not found (Laszczycza et al., 1996).

Cd is toxic to a number of tissues. Acute Cd poisoning causes pulmonary edema, hemorrhage, fulminate hepatitis, testicular injury, and lethality (Liu et al., 2007). Long-term exposure to high doses of Cd may cause biochemical and functional changes in some criti-
cal organs (e.g. nephrotoxicity, osteotoxicity, and immunotoxicity) (Liu et al., 2007). Cd is also classified by the International Agency for Research on Cancer as a human carcinogen causing tumors in the lungs and prostate, at the injection site, and in other tissues (Waalkes, 2003). Moreover, Cd can influence the absorption and distribution of essential elements (Cu and Zn) and can replace them in enzymes (Swiergosz-Kowalewska, 2001).

Metallothionein (MT) is a common name for a large group of low molecular weight proteins (6–7 kDa) capable of binding metal ions through the 20 cysteinyI groups that constitute part of its structure. The most widely expressed isoforms in mammals are MT-1 and MT-2, in which 60–61 amino acids form a structure with two clusters, able to bind seven metal ions. When the body is exposed to Cd, MT synthesis is induced and Cd replaces zinc (Zn) to form the structure Cd2Zn2MT (Klaassen et al., 1999; Vašák, 2005). MT binds Cd, resulting in its accumulation in various tissues and greatly reducing the biliary excretion of Cd. This ultimately affects the biological half-life of Cd in the body. However, in acute Cd poisoning, metallothionein serves as protection (Klaassen et al., 2009).

A common model used in toxicology to estimate the potential hazard of contaminants for human health is the rat, for which MT induction in several tissues, especially the liver, after exposure to Cd has been well documented (Chan and Cherian, 1992; Száková et al., 2009).

MT is speculated to be an acute phase protein participating in adaptive mechanisms induced by stress, scavenging free radicals (Hidalgo et al., 1988) and acting as cytoprotection against their toxic effects (Sato and Brenner, 1993). As pro-inflammatory cytokines, including interleukin (IL)-1, IL-6, and interferon (IFN)-γ, also induce MT expression in vivo (Inoue et al., 2008a; Waelput et al., 2001), it has been implied that MT may play a role in inflammation. Consistent with this, MT has been shown to possess both pro- and anti-inflammatory properties depending on the pathophysiological presentation (Inoue et al., 2008a,b; Milnerowicz et al., 2009; Śliwińska-Mossoń et al., 2012; Waelput et al., 2001).

Based on these data and observations, we designed this study to find out whether OT has an ameliorative effect on chronic exposure to Cd in hepatic, renal and pancreatic injuries in an experimental rat model. We also determined the expression of MT under oxidative stress generated in the organs following both Cd and O3 exposure.

2. Materials and methods

2.1. Experimental procedures

All animal procedures were approved by the institutional committee on the care and use of animals at our institution. Forty male Wistar rats (260–300g) provided by the animal laboratory were randomly assigned into 5 groups containing 8 rats each: control group (C), Cadmium group (Cd), Cadmium and oxygen group (CdOx), Cadmium and ozone group (CdOz) and Ozone group (Oz).

The animals were kept in cages at room temperature in a light cycle of 12 h light and 12 h dark. The animals were fed ad libitum feed “Muirgran” and watered with tap water. The animals in groups Cd, CdOx and CdOz were subjected to 12 weeks of intoxication by drinking water containing 50 mg of Cd2+L in the form of cadmium acetate. The CdOz and Oz groups were treated intraperitoneally with a mixture of oxygen-ozone once a day at approximately 10 a.m. for 10 days. Each subject received a 1 ml dose with a concentration of 40 μg/mL of ozone. The oxygen-ozone mixture was obtained from medical oxygen by means of the O2 generator Biozon “U” (B. Prochazka GmbH, Reutlingen, Germany). The experimentation on animals was done at the University of Silesia in Katowice.

All procedures were completed before biological measurements were taken. After 12 weeks, the animals from all five groups were sacrificed. Kidney, liver and pancreas tissues were rapidly excised and washed with cold saline. One part of each organ was preserved in paraffin for histological and immunohistochemical studies. The tissues were fixed in 10% buffered formalin solution at room temperature for 24 h, then rinsed with running tap water for 1 h, and subsequently were treated following the recommended protocol. Tissues were dehydrated through a series of ethanol solutions of increasing concentrations (first 70%, 80%, 90% and three times at 100%), then twice in xylene solvent and then were dipped three times in paraffin. The second part of the organs was frozen in liquid nitrogen and stored at −80°C for biochemical studies.

2.2. Homogenates preparation

2.2.1. Using the supernatant to determine the concentration of Cd and MT (A)

The tissues were washed several times in phosphate-buffered saline (PBS) and were homogenized at a 1:10 ratio in sterile tubes in a solution containing: 10 mM Tris/HCl pH 8.2, 250 mM sucrose (Sigma-Aldrich, ref. No 84097, Germany), 0.1 mM PMSF (phenylmethylsulphonyl fluoride, Sigma-Aldrich, ref. No 329-98-6 Germany) and 2 mM β-mercaptoethanol (Sigma-Aldrich, ref. No M6259, Germany), using a Potter-Elvehjem homogenizer at 4°C. The homogenates were centrifuged at 10.000g for 10 min at 4°C. The supernatants were then carefully separated from the pellet and transferred to new sterile tubes, heated at 100°C for 5 min, rapidly cooled in ice water and then centrifuged at 12.000g for 20 min at 4°C (Milnerowicz et al., 2000).

2.2.2. Supernatants in SDS-PAGE analyses (B)

Supernatants designed for electrophoretic separation prior to their application to gels were subjected to carboxymethylation according to method described by Kimura et al. (1991). The carboxymethylation of proteins was performed on each of the supernatants with 0.1 M Tris–glycine buffer of pH 8.8 containing: 25% glycerol (Sigma-Aldrich, ref. No 56-81-5, Germany), 60 mM DDT (dithiothreitol, Sigma-Aldrich, ref. No 3483-12-3, Germany), 40 mM EDTA (Ethylene-dinitro-triacetic acid, Sigma-Aldrich, ref. No 60-00-4, Germany), 4% SDS (Sodium lauryl sulfate, Sigma- Aldrich, ref. No 151-21-3, Germany) and 0.05% bromophenol blue (Sigma-Aldrich, ref. No B0126, Germany). The samples were incubated at 100°C for 15 min and cooled in ice water for 3 min; then, a 1 M recrystallized iodoacetic acid solution (IA) of pH 8.0 was added. Then samples were incubated at 50°C for 15 min and cooled again. The final concentrations of SDS, DDT and IA were 2%, 20 mM and 150 mM in the reaction mixture, respectively (Kimura et al., 1991). An aliquot of samples, prepared as described above, were separated on a 15% polyacrylamide gel.

2.3. Method for the determination of MT, Cd and proteins

In the supernatants (A), the concentrations of MT, Cd and proteins were determined.

The Cd concentration was estimated by an atomic absorption spectrometry (ASA) method as previously described (Milnerowicz et al., 2000), using a GFAAS (SOLAR M6, ThermoElemental Co.). The following working parameters were applied: lamp current: 6.0 mA, spectral bandpass: 0.8 nm, and wavelength: 228.8 nm. The reference materials BCR-194, −195, −196 by IRMM, UE were used. Reference curves were plotted for certified reference materials for each element separately. Validation was performed occasionally, on a random basis.

The protein concentration was determined by the Lowry method (Lowry et al., 1951).

ELISA was employed to measure the MT concentrations in the supernatants, based on a previously described method for mea-
suring this protein in rats (Gasull et al., 1994). The two standards of MT (MT-1 and MT-2) were obtained from the human liver by employing the isolation method described by Hidalgo et al. (1989). Polystyrene plates (Greiner Labortecnich, Germany) were coated overnight with 15 ng rat liver zinc/cadmium MT-2 (in 100 µl) in a 100 mM carbonate buffer of pH 9.6 at room temperature and in a water-saturated atmosphere. The plates were rinsed three times with a 0.05% Tween 20 solution (Sigma-Aldrich, ref. No 9005-64-5, Germany) in PBS, and the remaining active groups were blocked by 2 h incubation with 200 µl/well of PBS containing 1% bovine serum albumin (BSA) (Sigma-Aldrich, ref. No 232-936-2, Germany). The plates were then washed two times as above, and 100 µl of the solution containing the unknown or standard (1–100 ng MT) and 100 µl of antibody (both diluted in PBS containing 1% BSA) were added (final dilution of antibody 1:1000). The plates were then incubated for 2 h at 37 °C while being mixed in a humid chamber (Analoco Statfax 2200 incubator). The plates were then washed three times as above; 200 µl/well of anti-rabbit IgG-HRP conjugate (1:10,000) (Sigma-Aldrich, ref. No RABHRP1, Germany) were added; and the plates were incubated for another 2 h at 37 °C. After washing the plates, the substrate solution – 0.04% o-phenylenediamine dihydrochloride (Sigma-Aldrich, ref. No 615-28-1, Germany) and 0.012% hydrogen peroxide in phosphate-citrate buffer, pH 5.0 – was added. The enzymatic reaction was stopped after a 30-min incubation in a dark chamber (50 µl 2 M H2SO4 (Sigma-Aldrich, ref. No. 339741, Germany)). The dye reaction was read using a Statfax 2100 microplate reader (Analoco Co.) at a wavelength of 492 nm.

Each assay was repeated three times. MT concentrations in the successive standard dilutions and corresponding inhibition percentages developed a standard logarithmic curve, where axis Y = inhibition percentage, and axis X = MT ng/well (Milnerowicz and Slowińska, 1997).

2.4. SDS-PAGE analyses

Total protein content was quantified by the Lowry method modified by Peterson, using bovine serum albumin (BSA) (Sigma-Aldrich, ref. No 232-936-2, Germany) as a standard (Peterson, 1979). Protein separations were carried out on sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) following the Laemmli protocol (Laemmli, 1970), using a Mini Protean Tetra apparatus with a gel dimension of 8.3 × 7.3 cm (Bio-Rad USA). A gel containing 15% acrylamide for separating (1.5 M Tris–HCl pH 8.8, 10% SDS, DI water, 10% ammonium persulfate (APS), and TEMED) and one with 4% acrylamide for stacking (0.5 M Tris–HCL pH 6.8, 10% SDS, DI water, 10% APS, and TEMED) were used.

The protein solutions (of both standard proteins and supernatants) were added to the reaction mixture and applied to the gel. Gels were run, in triplicate, for approximately 2.5 h, at 45 mA/gel (Power Basic, Bio-Rad) in a Tris-glycine buffer (0.025 M Tris–base, 0.19 M glycine, and 0.0035 M SDS, pH 8.3). Gels were stained by silver staining as described previously (Kimura et al., 1991).

Gels were scanned using an optical densitometer GS-800 (Bio-Rad, USA) operating at 300 dpi resolution. The Quantity One 4.6.6 software (Bio-Rad Laboratories, USA) was employed for gel-imaging densitometric analysis.

2.5. Histopathological examinations

In histological sections routinely stained with hematoxylin and eosin (H&E), histopathological changes were noted in the following organs: kidneys, liver and pancreas.

The extent of tissue lesions was evaluated under an Olympus BX41 light microscope (Olympus, Japan). Blind analysis of the histological samples was performed by two independent pathologists.

2.5.1. Immunohistochemistry

The tissue samples were dewaxed and rehydrated in a series of alcohol solutions. The sections were then incubated with 3% hydrogen peroxide to block the intracellular activity of peroxidase. Non-specific binding was blocked with Antibody Diluent (Dako Cytometry, ref. No.: S0808, UK). The sections were then covered with a solution of mouse monoclonal anti-metallothionein antibodies dissolved in 1% bovine serum albumin in a ratio of 1:500 and incubated in a humid chamber overnight at a temperature of 4 °C. After washing, the samples were incubated at room temperature for 30 min, with rabbit immunoglobulins against the mouse antibodies marked with biotin. Another 10 min incubation followed, in a 50 mM Tris/HCl, pH 7.8 solution with streptavidin-peroxidase complex and 3,3’-diaminobenzidine (DAB). The preparations were closed in glycerin gel and allowed to dry. A negative control was performed for each tissue section, replacing the primary antibody with the anti-rabbit immunoglobulin control IgG antibody (Negative Control DAKO, Carpenteria, USA, Code: X0903).

The stained tissue sections were evaluated qualitatively by a pathologist under a high-powered Olympus BX41 light microscope (Olympus Optical Co. Ltd, Japan) that interfaced with an Olympus DP70 digital camera (Olympus Optical Co. Ltd, Japan) to digitize the image.

2.5.2. Evaluation of results of staining by immunohistochemistry

The intensity of MT immunoreactions within the cells was graded semi-quantitatively in the following manner: negative (no immunohistochemical reaction: −); weakly positive (lightly stained but clearly differentiated from negative background: ±); moderately positive (between weak and strong: +); strongly positive (dark brown with high contrast: +++) and very strongly positive (+++++).

2.5.3. Digital imaging methodology

To determine the MT content in the immunohistochromatically stained sections, a digital imaging method for DAB quantification was used on slides with hematoxylin counterstain. The image analysis allows for quantitative numerical comparisons of staining intensity assessed based on the value of the signal read from the DAB staining. There exists a linear relationship between staining intensity and the concentration of the antigen. Stained tissue sections were viewed under a light microscope that interfaced with a camera for scientific applications. Both units were controlled by a personal computer. The Fiji measurement system (runs on Windows) was used to perform quantitative analysis on the digitized images. The system quantified the average darkness of the image due to the DAB signal from staining for MT. The system then converted the signal staining into a mean gray value, and results were reported as DAB signal intensity units. The reaction intensity was evaluated in arbitrary units (A.U.) of optical density (OD). A total of ten areas were acquired from each tissue section of the organs – kidneys, liver and pancreas – taken from the rats of each study group.

2.5.4. Statistical analysis

Results of the concentrations of MT, Cd and proteins in the supernatants and the digital imaging methodology determination and densitometric analysis of gels were analyzed using Student’s t-test, as well as the Shapiro-Wilk test for normality and the Levene test for homogeneity of variance. If the assumption of homogeneity of variance was not met, the Cochran-Cox test was used. If the distribution of variables was not normal and nonparametric, the Mann-Whitney U test was used. Spearman rank correlation analysis was used to evaluate the correlations.
For all of these analyses, a p value of ≤0.05 was considered to be statistically significant. The Statistica 12.5 PL program (StatSoft, Warsaw, Poland) was used in calculations.

3. Results

3.1. Kidneys

In the kidneys of the CdOx and CdOz groups, similar Cd concentrations were found. A statistically significant decrease in the Cd concentration in these groups compared to Cd group was observed (Fig. 1A).

The greatest difference in concentration of MT in the kidneys of rats was observed in the Cd group compared to the C group. A statistically significant increase was noted in the MT concentration in the study groups which had been intoxicated by Cd compared to the C group (Table 2, Fig. 1A).

In the Cd, CdOx and CdOz groups, there were strong positive correlations between MT and Cd concentrations in the kidneys. Interestingly, no significant relationship was found between the concentrations of Cd and MT in the C and Oz groups (Table 3).

The SDS-PAGE results for the kidneys from the C group showed that both MT-1 and MT-2 isoforms were generated, with a slightly higher proportion of MT-2 isoforms (Fig. 2A,C; Table 4).

In lane 2 (Cd group), we observed an increase in the concentrations of the two isoforms, although a greater increase in MT-1 isoforms appeared compared to the control. In lane 3 (CdOx group), we found a slight increase in the concentrations of both isoforms compared to the control and a slight decrease compared to the Cd group. We observed similar staining intensity gel in lane 2 (Cd) and 4 (CdOz) for both isoforms of MT. In the Oz group, we found the highest proportion of MT-1 isoforms compared to the control (Fig. 2A,C). In summary, after conducting electrophoretic separation of the isoforms of MT in the kidneys, we found that salts of Cd and O3 individually and the administration of Cd and oxygen or Cd and O3 together in the kidney result in more of the MT-1 isom isoform being produced.

The kidneys from the C and Oz groups showed normal morphology, whereas in the group of rats exposed to Cd, changes in the clinical pathology of nephropathy were clearly seen (Fig. 3b–f). A histological examination detected glomerular vacuolization and glomerulosclerosis, dilatation and atrophy of the tubules, marked protein deposits in the tubular lumen, as well as interstitial lymphocyte infiltration.

The Cd and CdOx groups showed similar nephrotoxic effects of Cd exposure. Cd intake affected the proximal tubules of the nephron more than the glomerular parts. Hydropic degeneration of the epithelia, acidophilic changes of the renal tubules, interstitial mononuclear cell infiltration, and desquamation of the epithelium into tubular lumen (tubular cell apoptosis) were noted. Slight glomerular swelling was seen in some of the rats in the CdOz group. Although Cd intake had a clear impact on tubular function in the CdOz group compared to the C group, it was not as prominent compared to the aforementioned groups. Histopathological changes were similar, but with slightly decreased intensity.

An immunohistochemical reaction for MT in the kidneys from rats of the both groups control and O3 was not observed. In the rats intoxicated with cadmium acetate, the strongest reaction for MT was observed in the cellular nuclei, while a weaker reaction was noticed in the cytoplasm, mainly in the area under the brush border of proximal convoluted tubules (Fig. 3c). A strong reaction for MT was also noted in the cells of the vascular endothelium of the kidney glomeruli. In other parts of the kidney, the immunohistochemical reaction was weakly positive. Similar results in the kidneys of rats from CdOx were observed. The MT expression in the cells was assessed as strong or moderately positive (Fig. 3d–e; Table 7).

A significantly weaker MT reaction was observed in the kidneys of rats intoxicated with Cd and given intraperitoneal injections of oxygen-ozone mixture (Fig. 3f). A semiquantitative estimation of the location of MT in the kidney cells of the experimental rats was performed; the results are presented in Table 7. The highest intensity of staining for MT was found in the kidneys of rats in the Cd group. Their MT content was, on average, 1.5 times higher than that noted in the rats from the CdOz group. In addition, a statistically significant increase in the intensity of MT staining was found in these rats when compared with the CdOx and CdOz groups (respectively: p = 0.0118; p = 0.000034).

A statistically significant difference between the intensity of staining of MT in the CdOx group was observed compared to the CdOz group (p = 0.002) (Fig. 4). It shows the protective role of the O3 from the toxic effect of Cd on the kidneys.
Fig. 2. The SDS/PAGE, silver-stained proteinograms of tissues: kidneys (A); liver (B); (amount of proteins about 50 ng/lane) and quantitative densitometric analysis of the intensity of silver-stained isoforms metallothionein-1 and 2 in SDS/PAGE: kidney (C), liver (D).

Lanes: (1) C, (2) Cd, (3) CdOx, (4) CdOz, (5) Oz, (6) samples containing two isoforms of MT from human liver, molecular mass approx. 6.5 kDa.

3.2. Liver

In the liver, the highest concentrations of Cd were found in the Cd and CdOx groups of rats. A lower concentration of Cd was noted in the CdOz group than in the Cd and CdOx groups. (Table 1, Fig. 1B).

In the liver, the highest concentrations of MT were observed in the Cd group. Similar MT concentration values were found in the liver of the CdOz and Oz groups. A statistically significant increase in MT concentration in the study groups exposed to Cd was observed compared to the control group. In the Cd and CdOz groups, there

<table>
<thead>
<tr>
<th>Organ</th>
<th>Group</th>
<th>C</th>
<th>Cd</th>
<th>CdOx</th>
<th>CdOz</th>
<th>Oz</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidneys</td>
<td>2.9 $^{a1,2}$</td>
<td>432 $^{b(1/2)*}$</td>
<td>235.8 $^{c(1)*}$</td>
<td>268.0 $^{b(b)*}$</td>
<td>3.8 $^{a1}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(2.4–3.9)</td>
<td>(387.6–468.9)</td>
<td>(183.9–401.1)</td>
<td>(208.2–456.7)</td>
<td>(2.9–4.2)</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>0.4 $^{a1}$</td>
<td>195.6 $^{b(b)*}$</td>
<td>198.7$^{c(1)*}$</td>
<td>142.9$^{a(b)*}$</td>
<td>3.7 $^{a2}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0.3–0.5)</td>
<td>(178.3–201.6)</td>
<td>(182.1–250.9)</td>
<td>(115.9–167.8)</td>
<td>(2.9–3.9)</td>
<td></td>
</tr>
<tr>
<td>Pancreas</td>
<td>0.5 $^{a2}$</td>
<td>21.7 $^{a(b)*}$</td>
<td>35.9 $^{a(b)*}$</td>
<td>76.0 $^{a(b)*}$</td>
<td>0.6 $^{a2}$</td>
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<tr>
<td></td>
<td>(0.4–0.5)</td>
<td>(19.7–24.3)</td>
<td>(26.8–43.2)</td>
<td>(63.4–82.6)</td>
<td>(0.4–0.7)</td>
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</tbody>
</table>

Statistical significance:
- In the each group between organs: *p < 0.001; **p < 0.01; ***p < 0.02.
- In the organ between groups: *p < 0.01.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Group</th>
<th>C</th>
<th>Cd</th>
<th>CdOx</th>
<th>CdOz</th>
<th>Oz</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidneys</td>
<td>10.2 $^{a1}$</td>
<td>59.4</td>
<td>53.1</td>
<td>43.7 $^{b(b)}$</td>
<td>25.6 $^{a1}$</td>
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<tr>
<td></td>
<td>(9.5–12.6)</td>
<td>(55.8–61.4)</td>
<td>(37.9–57.6)</td>
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<td>(20.3–30.2)</td>
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<tr>
<td>Liver</td>
<td>2.6 $^{a1}$</td>
<td>5.7</td>
<td>4.1</td>
<td>3.5 $^{a1}$</td>
<td>3.0 $^{a1}$</td>
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<td></td>
<td>(1.9–2.6)</td>
<td>(4.8–6.9)</td>
<td>(3.1–5.2)</td>
<td>(1.8–4.2)</td>
<td>(1.1–3.5)</td>
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</tr>
<tr>
<td>Pancreas</td>
<td>0.3 $^{a2}$</td>
<td>1.4</td>
<td>0.7</td>
<td>0.4</td>
<td>0.2 $^{a2}$</td>
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<tr>
<td></td>
<td>(0.2–0.8)</td>
<td>(0.9–1.9)</td>
<td>(0.2–1.1)</td>
<td>(0.2–0.9)</td>
<td>(0.1–0.3)</td>
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</tr>
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</table>

Statistical significance:
- In the each group between organs: *p < 0.001; **p < 0.01; ***p < 0.02.
- In the organ between groups: *p < 0.01; **p < 0.02.
were strong positive correlations between MT and Cd concentrations in the liver (Tables 2 and 3, Fig. 1B).

In the SDS-PAGE analyses of the liver of the control group, a similar intensity level of the MT-1 and MT-2 isoforms was observed. In lane 2 (Cd group), both isoforms were 30% stronger in intensity than the control, while in the liver of the CdOx group, a slight increase in concentration of both MT isoforms was observed compared to the control (MT-1 by 20%, and MT-2 by 10%). Both isoforms of the CdOx group were still lower than the Cd group (Table 4, Fig. 2B,D).

Also, a decrease in protein concentrations in the CdOx group of about 20–30% was found compared to the control, with a greater decrease in the intensity of staining for MT-1 than MT-2. When compared to the intensity of staining for isoforms of MT in the CdOx group, the Oz group featured a higher concentration of MT-2 isoforms and a lower one of MT-1 isoforms.
Table 3
Correlated cadmium and metallothionein concentrations in supernatants of tissues in the study groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Organ</th>
<th>Kidneys</th>
<th>Liver</th>
<th>Pancreas</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cd</td>
<td></td>
<td>R = 0.82; p = 0.000</td>
<td>R = 0.71; p = 0.000</td>
<td>R = 0.89; p = 0.003</td>
</tr>
<tr>
<td>CdOx</td>
<td></td>
<td>R = 0.76; p = 0.02</td>
<td></td>
<td>R = 0.66; p = 0.04</td>
</tr>
<tr>
<td>CdOz</td>
<td></td>
<td>R = 0.85; p = 0.006</td>
<td>R = 0.85; p = 0.01</td>
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</tr>
</tbody>
</table>

Table 4
Quantitative densitometric analysis (color intensity) of the intensity of isoforms metallothionein-1 and -2 silver staining in the SDS-PAGE in samples prepared from kidneys.

<table>
<thead>
<tr>
<th>Group</th>
<th>C</th>
<th>Cd</th>
<th>CdOx</th>
<th>CdOz</th>
<th>Oz</th>
<th>Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT-1</td>
<td>120.3</td>
<td>183.8</td>
<td>161.1</td>
<td>188.3</td>
<td>192.8</td>
<td>105.9</td>
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<tr>
<td>MT-2</td>
<td>132.0</td>
<td>170.9</td>
<td>145.7</td>
<td>165.9</td>
<td>150.2</td>
<td>63.5</td>
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</tbody>
</table>

In the Oz group, a decrease in the concentration of both forms of MT was observed compared to all groups analyzed. The administration of cadmium salts to animals induces the synthesis of MT-1 in the liver, but after exposure to a mixture of oxygen-ozone, the incidence of induction of this isomer is reduced. The greatest decrease in concentration of MT-2 was found in the Oz group; the greatest increase was found in the Cd group, compared to the C group (Table 4, Fig. 2D).

Livers from the C and Oz groups showed normal morphology. Histopathological examination showed progressive and diverse lesions of liver injury induced by chronic Cd exposure. The most severe lesions observed in both the Cd and CdOx groups included focal necrosis of parenchymal cells, apoptosis, and focal inflammation (collections of polymorphonuclear leukocytes, mainly neutrophils) in the parenchymal and portal tracts, as well as around central veins (Fig. 4h and i). Lymphocytic infiltration of the portal tracts occurred in the majority of hepatocytes, whereas the thickening of blood vessels with hyperplastic lining was noted in three-quarters of the rats. Hepatocyte degenerative cloudy swelling appeared in all cells, as did hepatocyte/hyperplasia, focal necrosis and apoptosis. In addition, Kupffer cell hyperplasia was noted. Histopathological findings of the liver of rats which received Cd plus the oxygen-ozone mixture were weaker. Mild lymphocytic infiltration in the portal system occurred in only half of the cases and although there was mild degenerative cloudy swelling of hepatocytes, the following did not appear in any of the rats: mild hepatocyte hyperplasia, focal necrosis or apoptosis. Kupffer cell hyperplasia occurred in only a few of the tissues of rats, with mild severity (Fig. 3).

In the control group, the immunohistochemical reaction for MT was present in a few hepatocytes. The MT expression in the cells was assessed as weakly positive or negative (no immunohistochemical reaction). A statistically significant difference was observed between the intensity of staining of MT in the control group compared to all study groups (Table 7). An immunohistochemical reaction for MT in the liver tissue of rats from the Oz group was not observed.

For most rats intoxicated with Cd, there was a stronger immunohistochemical reaction for MT in liver tissue than in other study groups. A higher reaction intensity of MT was observed in hepatocytes than in Browicz-Kupffer cells in the liver. Strong staining of MT was observed in both the nuclei and cytoplasm of the cells. In addition, strong expression of MT around the interlobular vein was observed (Fig. 3h). The intensity of the reactions and the amount of MT-secreting cells in the tissue of the CdOx group were differentiated. A similar effect was observed, but to a smaller degree, when O3 was administered (Fig. 3j and Fig. 4). A statistically significant decrease in the intensity of staining of MT in the CdOx and CdOz groups was observed compared to the Cd group (respectively: p = 0.00007; p = 0.00006). Furthermore, a statistically significant difference between the intensity of staining of the MT in the CdOx group was noted compared to the CdOz group (p = 0.0013) (Fig. 4).

3.3. Pancreas

In the C and Oz groups, similar Cd concentrations were found in the pancreas. A significantly higher concentration was found in the Cd, CdOx and CdOz groups in comparison to the control group. The highest concentration of Cd in the pancreas was observed in the CdOx group (Table 1, Fig. 1C).

In the pancreatic supernatants of the C and Oz groups, similar MT concentrations were found. Higher MT concentrations in the Cd and CdOx groups were also found in comparison to the C group (Table 2, Fig. 1C). In the pancreas, the highest concentration of MT was noted in the Cd group, while the lowest was found in the Oz group. In the Cd and CdOx groups, correlations between MT and Cd concentrations were strongly positive (Table 3).

The electrophoretic study did not demonstrate clearly the induction of MT in the pancreas in the CdOx and CdOz groups. A similar color intensity for MT staining was observed in the groups C, OT and CdOz. In the Cd and CdOx groups, a slight decrease in the intensity for MT staining was found compared to the C group (Table 6). In addition, we did not observe a clear separation of MT isoforms.

Moreover, the intensity of the staining of the two MT isoforms was similar to that of a single MT isoform as observed in the liver and kidney.

The histopathologic examination confirmed the regularity of the pancreatic exocrine and endocrine cells from the C and Oz groups. Pancreatic islets were clearly separated. There were no changes such as calcification and fibrosis to indicate the presence of inflammation. The histopathologic diagnosis confirmed inflammatory characteristics in all tissues of the rats intoxicated by Cd. In the exocrine and endocrine part of the pancreas, fibrous connective tissues were observed. The ducts were generally dilated and focally proliferated, and some were filled with partially calcified protein plugs. Although the histopathologic lesions of the pancreas from CdOx group did not show prominent differences compared to the Cd and CdOz groups, there was a slight decrease in intensity.

The MT expression in the acinar and islet cells was assessed as moderately positive or strongly positive in the pancreas samples from the Cd and CdOz groups. MT-positive cells were found in both the islet and acinar cells in the pancreas from rats intoxicated with Cd. The MT-positive acinar cells had a much stronger immunohistochemical reaction than the islet cells.

The intensity of the immunohistochemical staining for MT was statistically lower in the pancreas than in the kidneys and liver of rats intoxicated with Cd (respectively: p = 0.0009; p = 0.0043). The following immunohistochemical reactions for MT were observed: no reaction, weak and moderate reaction. Interestingly enough, single MT-positive cells were also noted in the dilated pancreatic ducts. There was no reaction in the pancreas of rats treated only with O3 (Table 7, Fig. 4).

4. Discussion

Cd is an environmental pollutant ranked eighth on the Priority List of Hazardous Substances, and human activity has markedly increased the presence of Cd in the global environment. Food is the major source of Cd exposure for the general population, and cigarette smoking significantly adds to the body burden of Cd (Järup et al., 1998; Klaassen et al., 1999; Milnerowicz, 1997; Milnerowicz et al., 2015). Occupational exposure arises mainly from Cd fume.
inhalation, the cadmium-nickel battery industry, electroplating, and paint pigments (Bizon et al., 2013; Klaassen et al., 2009; Lei et al., 2007; Ścisialka et al., 2014).

The major target organs in animals for the accumulation of Cd and its toxicity are the liver, kidney and reproductive system. Acute Cd poisoning produces primarily hepatic and testicular injury, whereas chronic exposure results in renal damage and osteotoxicity (Lei et al., 2007). Cd in appreciable quantities is also found in the pancreas (Han et al., 2003; Lei et al., 2007).

The kidney is a major target of chronic Cd exposure (Järup et al., 1998). Cadmium salts are nephrotoxic to both humans and animals and cause degeneration and atrophy in the proximal tubules and, in some, the interstitium. This gives rise to such clinical presentations as polyuria, glycosuria, aminoaciduria, and high and low mass proteinuria. Similarly, the liver accumulates significant amounts of Cd when exposed to the metal both acutely or chronically, and is its target organ (Dudley et al., 1982, 1985). The Cd accumulated in the pancreas causes a change in the level of zinc that affects both endocrine and exocrine functions in this organ.

4.1. Kidneys

In our studies of chronically exposed animals, we demonstrated that due to intoxication, the Cd concentration increased mostly in the kidneys and liver and, to a smaller degree, in the pancreas. In animals chronically exposed to CdCl₂ for 10 months, nephrotoxicity and Cd accumulation were observed in the kidneys (Groten et al., 1994). The highest concentration of Cd in the kidneys was observed in the group of rats intoxicated with Cd only; a lower value was recorded in the group of rats exposed to cadmium and ozone. This may indicate a protective effect of O₃ against the accumulation of Cd in these organs.

Therapeutic effects of O₃ treatment are difficult to explain, with the exception of the transient, local effects of increased oxygen concentration in hypoxic states. On the other hand, the injurious, prooxidative action of O₃ inhaled or diffused into solutions and biomembranes is well documented (Laszczycza et al., 1996).

Although adverse health effects induced by O₃ exposure have been ascertained in humans and in animals as mentioned above, their mechanisms and/or endogenous molecular targets have not been fully identified. In our studies, the role of MT in oxidative stress generated in the organs following both Cd and O₃ exposure was determined.

It has been long thought that Cd-induced nephrotoxicity is mediated by the Cd-MT complex. The induction of MT by Cd and the subsequent sequestration of Cd by MT protects tissues from Cd toxicity. In our studies, a 6-fold increase in MT concentrations was observed in the kidneys of rats in the Cd group. The MT concentrations obtained in our study were similar to those found by other authors (Chan and Cherian, 1992).

The presence of the MT isoforms and their molecular masses were confirmed by polyacrylamide gel electrophoresis. The detection limit of SDS-PAGE silver staining was approximately 1.5 ng/lane for mouse liver MT-1 and MT-2. Both calibration curves for densitometric analysis were similar and of good linearity, at least up to 50 ng/lane. The results highlighted two important points: the isoforms could be separated by SDS-PAGE and silver staining.

MT is substantially better highlighted by silver staining than are other low-molecular-mass, heat-stable proteins (McCormick and Lin, 1991).

In the SDS-PAGE analyses of the kidneys in the Cd group, we observed an increase in the concentrations of the two isoforms. There was a greater increase of the MT-1 isomer in comparison to the control. Furthermore, a strong positive correlation was found between MT concentration and Cd concentration in the Cd group in kidneys. It shows that an increase in MT concentration is a response to Cd exposure.

The Cd-MT complex is acutely nephrotoxic to experimental animals after intravenous injection (Nordberg et al., 1975). The working theory was that Cd-MT was formed in the liver in response to Cd exposure and was released into the bloodstream from damaged hepatocytes. The Cd-MT complex in the blood was then filtered by the kidney and taken up into proximal tubule cells, where it degraded, releasing locally high levels of “free” Cd to produce tubular injury.

This thesis was accepted for three decades, until being recently challenged by two lines of experimental data. Firstly, MT-null mice, which are unable to produce MT and thus to form the Cd-MT complex, are hypersensitive to chronic cadmium nephropathy (Liu, 1998a). Kidneys of Cd-treated mice were enlarged and the histopathology showed various types of lesions, including proximal tubular degeneration, apoptosis, atrophy, interstitial inflammation, and glomerular swelling. These lesions were more severe in MT-null mice than in the controls. These data indicate that Cd-induced renal injury is not necessarily mediated through the Cd-MT complex and that MT is an important intracellular protein for protecting against chronic Cd nephrotoxicity. Secondly, kidney pathology from a single injection of Cd-MT differs greatly from that induced by chronic oral Cd ingestion (Liu et al., 1998b). Therefore, it has been proposed that acute Cd-MT injection is not an appropriate model for the study of chronic Cd-induced nephrotoxicity.

Our research confirms the above statements. Chronic exposure to cadmium salts caused nephrotoxicity, which was observed as an increase in Cd accumulation in this organ and the induction of a high expression of MT in tissues (Figs. 3 b–d and 4). It is interesting that the histopathological changes were most evident in the kidneys from Cd and CdOx groups, which correlated with the intensity of the staining reaction for MT.

The presence of MT in both the nucleus and cytoplasm was observed (Tsujikawa et al., 1994), which is another piece of evidence of its significant role in the action against the genotoxicity of Cd. It has also been documented that Cd-induced toxic effects are closely associated with the production of ROS, which can destroy DNA, proteins, and lipid function, as well as activate signaling pathways that cause cell death (Bagchi et al., 2000). Both oxidative stress damage and the activation of MAPK pathways are associated with Cd-induced apoptosis (Chang et al., 2013). MT is a scavenger of these radicals (Hidalgo et al., 1988; Milnerowicz et al., 2009; Sato and Bremer, 1993). In the rats intoxicated with Cd, the strongest response for MT induction was observed in the cellular nuclei, while a weaker one was noticed in the cytoplasm, mainly in the area under the brush border of proximal convoluted tubules (Fig. 4c). The results obtained for MT localization confirm the opinion that its presence in the nuclei of mature cells is a result of extrinsic influences such as carcinogens, heavy metals and stress (Tohyama et al., 1993; Tsujikawa et al., 1994). A strong reaction for MT was also noted in the cells of the vascular endothelium of the kidney glomeruli.

A significantly lower MT reaction was observed in the kidneys of the CdOx group. It shows the protective role of O₃ against the toxic effects of Cd on the kidneys. A decrease in Cd accumulation and MT concentration as a consequence of the administered O₃ injections clearly demonstrates a decrease in the ability of Cd to induce MT. However, in SDS-PAGE, a similar staining intensity of MT was observed in the Cd and CdOx groups for both isoforms of MT in kidneys.

O₃ oxidizes the available antioxidants and reacts immediately with polyunsaturated fatty acids to form ROS, such as hydrogen peroxide (Ballinger et al., 2005; Pryor, 1994). Also, O₃ exposure reportedly results in oxidative stress in the airway, possibly through the devastation of iron homeostasis (Ghio et al., 2006).
iron can increase oxidant generation after O3 interaction with aqueous media and produce hydroxyl radicals (Byvoet et al., 1995). MT is able to scavenge a wide range of ROS, including superoxide, hydrogen peroxide, hydroxyl radical, and NO (Sato and Kondoh, 2002). In particular, it has been reported that the ability of MT to capture hydroxyl radicals, which are primarily responsible for the toxicity of ROS, is 300 times greater than that of glutathione (Vliagoftis et al., 2000), the most abundant antioxidant in the cytosol (Sato and Kondoh, 2002). Therefore, the higher concentration of MT observed in rats’ kidneys treated with O3 as shown in SDS-PAGE may indicate that the MT is involved in protecting against the renal damage induced by O3 exposure. Similar results were obtained in an experiment on rats, with a dose of subacute O3 (Inoue et al., 2008a).

### 4.2. Liver

The liver is a major target organ of Cd toxicity following acute exposure, but it is also a target of chronic Cd toxicity following chronic CdCl2 exposure (0.1 mg/kg.s.c. for 10 weeks) (Habeebu, 2000). In wild-type mice, hepatic Cd concentrations increase in a dose- and time-dependent manner, reaching 400 μg Cd/g liver after 10 weeks of exposure to inorganic Cd, along with a 150-fold increase in hepatic MT concentrations. In MT-null mice, hepatic Cd concentrations were less than 10 μg Cd/g liver. Despite the lower accumulation of Cd in the livers of MT-null mice, the maximum tolerated dose of Cd was only 1/8 of that for wild-type mice, and liver injury was more pronounced (Habeebu, 2000).

The degree of liver damage increases proportionally with Cd and MT levels. The greatest lesions observed in both Cd and CdOx groups included focal necrosis of parenchymal cells, apoptosis, and foci of inflammation in the parenchyma and portal tracts, as well as around the central veins (Fig. 3h and i). The presence of MT in most of the tissues proves that it plays a significant role in intracellular protection against the toxicity of Cd. An immunohistochemical study confirmed the morphological changes elicited by the exposure of animals to Cd (Dudley et al., 1985) and increased MT synthesis (Waalkes et al., 1992). In our study, for most rats intoxicated with Cd, there was a stronger immunohistochemical reaction to MT in liver tissues than in other studies. In earlier studies, repeated administration of CdCl2 to MT-null mice was found to have produced nonspecific chronic inflammation in the parenchyma and portal tracts, and higher doses produced granulomatous inflammation and preneoplastic proliferative lesions (Habeebu, 2000). Apoptosis and mitosis occurred concomitantly in the liver following repeated Cd exposure, and more apoptosis is seen in MT-null mice at a dose of 0.1 mg Cd/kg for 10 weeks than in wild-type mice that received the same dose of Cd (Habeebu, 2000). Thus, intracellular MT is an important protein protecting against chronic Cd-induced liver injury. This was also confirmed by SDS-PAGE, in which samples prepared from the liver of rats intoxicated with Cd showed an approximately 30% increase in protein concentrations compared to the control for both isoforms of MT-1 and MT-2 (Fig. 2B,D, Table 5).

The difference between the influence of oxygen and an oxygen-ozone mixture on MT concentrations in the liver under cadmium acetate intoxication is noteworthy. Oxygen injections did not diminish the MT concentration in the liver.

Similar concentrations of MT in liver tissue in the CdOx and Oz groups were found. A significantly lower immunohistochemical reaction for MT was observed in the liver of rats from the CdOx group. Additionally, SDS-PAGE analyses showed a decrease of approximately 20–30% in protein concentrations in the CdOx group compared to the control. Moreover, in the Oz group, there was a decrease in concentration of both forms of MT in comparison with all analyzed groups.

### 4.3. Pancreas

Animal studies have shown that Cd can cause pancreatic β-cell damage, suppress insulin secretion, increase glucose intolerance, and have diabetogenic effects (Han et al., 2003; Ithakissios et al., 1975). In subchronic or chronic investigations, a marked disturbance of glucose homeostasis, the destruction of pancreatic islets, a lower level of insulin secretion, an increase in gluconeogenic enzymes, and even a significant increase in Cd toxicity in diabetic conditions have been observed after rats are exposed to Cd (0.5–2 mg/kg/day), which is accompanied by significant Cd accumulation in the blood and/or pancreas (Chapatwala et al., 1982; Edwards and Prozialeck, 2009; Han et al., 2003; Lei et al., 2007). Some in vivo studies have indicated a possible link between Cd exposure and diabetogenic effects (blood glucose imbalance and pancreatic islet dysfunction); however, the precise mechanisms of Cd-induced toxicological effects on the function of pancreatic β-cells and injuries remain unclear. In our study, a significantly higher Cd concentration in the Cd, CdOx and CdOz groups was found in comparison to the control group. There was also a strongly positive correlation between the concentrations of Cd and MT in the pancreas of rats exposed to Cd.

The presence of MT in the pancreas as a result of its induction after Cd administration was first described by Yau and Mennear (Yau and Mennear, 1977). There is some controversy about the responsiveness of MT induction within different pancreatic cells. Andrews et al. (1990) reported that MT mRNA levels were elevated in both the endocrine and exocrine cells of rat pancreas following an injection of cadmium and zinc salts, whereas Minami et al. (1995) observed that MT was located immunohistochemically in the exocrine cells of mice after a Zn injection. In our study,

### Table 5

<table>
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<tr>
<th>Group</th>
<th>C</th>
<th>Cd</th>
<th>CdOx</th>
<th>CdOz</th>
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<tr>
<td>MT-1</td>
<td>135.1</td>
<td>181.1</td>
<td>142.7</td>
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<td>MT-2</td>
<td>130.0</td>
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### Table 6

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<th>CdOz</th>
<th>Oz</th>
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<tr>
<td>MT-1 + MT-2</td>
<td>167.4</td>
<td>124.1</td>
<td>147.5</td>
<td>159.4</td>
<td>157.2</td>
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### Table 7

<table>
<thead>
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<th>Group</th>
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<th>Liver</th>
<th>Pancreas</th>
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<tr>
<td>C</td>
<td>Cd</td>
<td>CdOx</td>
<td>CdOz</td>
<td>Oz</td>
</tr>
<tr>
<td>151.7 ± 32.5⁵</td>
<td>60.0 ± 26.1⁶</td>
<td>129.6 ± 20.3⁵</td>
<td>244.3 ± 6.6¹</td>
<td>256.3 ± 10.2²</td>
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<tr>
<td>Cd</td>
<td>219.2 ± 15.4⁶</td>
<td>166.5 ± 18.1⁷</td>
<td>144.5 ± 10.5⁶</td>
<td>156.6 ± 37.8</td>
</tr>
<tr>
<td>Oz</td>
<td>124.1 ± 14.0³</td>
<td>132.5 ± 10.5</td>
<td>171.7 ± 19.2³</td>
<td></td>
</tr>
</tbody>
</table>

Statistically significant differences from organs of the tissue (kidneys, liver, pancreas) between organs:

Cd: ²p = 0.012; ³p = 0.0158.
CdOx: ⁴p = 0.0009; ⁵p = 0.0043.
CdOz: ⁶p = 0.0004; ⁷p = 0.0009.
Oz: ⁸p = 0.0086.
immunohistochemical MT staining in the pancreas was definitely statistically stronger in the Cd and CdOz groups compared to the tissues of the control group. MT expression in the acinar and islet cells was assessed as moderately or strongly positive. The intensity of the immunohistochemical staining for MT was statistically lower in the pancreas as compared with the kidneys and liver of rats intoxicated with Cd. This result confirms that the main organs accumulating Cd are the kidneys and liver, although the histopathologic diagnosis confirmed the occurrence of the inflammatory process characteristics in all the tissues of rats intoxicated by Cd. Interestingly enough, single MT-positive cells were also noted in the dilated pancreatic ducts. In the exocrine and endocrine part of the pancreas, fibrous connective tissues were observed. The ducts were generally dilated and focally proliferated, and some were filled with partially calcified protein plugs. Similar results are obtained in smoking patients with chronic pancreatitis (Milnerowicz et al., 2007); Cd is one of the main components of cigarette smoke.

Pancreatic β-cells, which function in insulin biosynthesis/secretion in mammals, are at greater risk of apoptosis due to ROS attacks than other cell types. The mitochondria of β-cells can generate excessive levels of ROS and are both the major source of ROS in these cells and also a primary target for ROS attack. This, combined with a failure of the ROS defense system, results in the relatively high vulnerability of β-cells to oxidative stress damage (Kaneto et al., 2005; Newsholme et al., 2007). The histopathologic examination confirmed the regularity of the pancreatic exocrine and endocrine cells from the CdOz and Oz groups. The MT expression in the acinar and islet cells was assessed as moderately positive in the CdOz group. The obtained results correlated with the intensity of the MT staining on the gel by SDS-PAGE. This may indicate a synergistic protective effect of MT and O3 against the toxic effect of Cd on the pancreas, though the Cd concentration in the sample prepared from pancreatic tissue from the CdOz group is disturbing, as there was a concentration of Cd 40 times that of the control group.

In earlier studies, it was found that OT reduced the severity, septic complications, and mortality of acute necrotizing pancreatitis in an experimental rat model. The experiment showed a greater benefit of OT compared to hyperbaric oxygen therapy in serum amylase and lipase levels, oxidative stress, bacterial translocation, serum neopterin levels, histopathologic damage score, and mortality rate. These findings suggest that it is possible to improve the outcome of acute necrotizing pancreatitis by using Oz as an adjuvant therapy (Uysal et al., 2010). The study by Chang et al. (Chang et al., 2013) showed that Cd is capable of inducing oxidative stress damage that causes the suppression of insulin secretion and apoptosis in pancreatic islet β-cells in vivo and in vitro. In another study, the use of O3 and vitamin C treatment in diabetic rats showed significant improvement in such cellular changes when compared to diabetic untreated rats, although they were still abnormal when compared with normal rats (Helal et al., 2013).

Notably, the results of some epidemiological studies have indicated significantly higher blood and urine levels of Cd in diabetic patients compared to healthy individuals (Afridi et al., 2015; Haswell-Elkins et al., 2008). A significantly positive correlation has also been revealed between increases in urinary Cd and the prevalence of impaired fasting glucose levels (odds ratio [OR]: 1.48–2.05, 95% confidence interval [CI]: 1.21–2.95) and DM (OR: 1.24–1.45, 95% CI: 1.06–1.97) (Schwartz et al., 2003).

Therefore, the use of Oz in subjects occupationally exposed to Cd may offer an opportunity to overcome the harmful effects of exposure to this toxic metal and reduce tissue damage to important organs for health and life.

To the best of our knowledge, this is one of the first reports evaluating the effect of medical Oz on Cd-induced renal, hepatic and pancreatic injuries in an experimental rat model. Our data showed that:

1. Ozone therapy (a mixture of oxygen-ozone administered intraperitoneally) reduces the accumulation of cadmium in the liver and kidneys, resulting in a reduced expression of metallothionein in those tissues.
2. A good marker for cadmium exposure to is metallothionein.
3. The lack of a protective effect of ozone against the accumulation of cadmium in the pancreas confirms that the pancreas is sensitive to the influence of pro-oxidative factors.
4. The effects of ozone therapy in chronic exposure to cadmium are not conclusive. Ozone therapy, depending on the organ, can increase Cd toxicity or protect from Cd toxicity.

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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