THE EFFECT OF COMBINED ZINC-CADMIUM INJECTION IN OVO ON THE ACTIVITY OF INDICATIVE HYDROLASES IN ORGANS OF NEWLY HATCHED CHICKS

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ARTICLE INFO
Received 6. 2. 2014
Revised 4. 3. 2014
Accepted 30. 3. 2014
Published 1. 4. 2014

ABSTRACT
The aim of the experiment was to examine if embryotoxic effect of cadmium, evaluated by using selected lysosomal hydrolases as biomarkers, can be reduced by simultaneous zinc in ovo administration. Developing chicken eggs (n= 42 eggs per group, 4th day of incubation) were injected by 50 µL of 0.7% NaCl solution containing cadmium ions (0 or 50 nmol per egg) and/or zinc ions (0, 100 or 500 nmol per egg). The Cd and Zn distribution in tissues (liver and kidneys) of 1-day old chicks was examined by ICP-OES method, while the activities of lysosomal hydrolases: N-acetyl-β-D-glucoaminidase (NAG) and β-D-mannosidase (β-MAN), and arylsulfatase (ARYL) were tested spectrophotometrically. The hatchability of control group and chicks treated by combination of 50 nmol Cd and 500 nmol Zn per egg was about 61% (comparable to the control group 61.9%) while the hatchability of remaining groups was from 30 to 50% lower (P≤0.05). Cd accumulation in liver and kidney of chicks exposed to this metal increased significantly in both tissue to 0.07 µg/g, whereas in the presence of higher zinc dose it was reduced to 0.04 and 0.05 µg/g, respectively. Moreover the increase in activity of lysosomal hydrolases induced by treatment of separately Cd and Zn was abolished by simultaneous administration of both ions (P>0.05). Concluding, supply of Zinc seems to protect against Cd-induced disorders during chick embryo development.

Keywords: Chick, cadmium, zinc, accumulation , hydrolases activity, antagonism

INTRODUCTION
Cadmium (Cd) is an environmental contaminant that has been recognized to be a risk factor in humans (Nordberg et al. 2000). It is present in air, water, soil, food and cigarette smoke (Czezot and Skrzyczyk, 2010). Chronic exposure to this metal leads to damage of numerous organs and systems, primarily kidneys (Rogalska et al., 2009; Czezot and Skrzyczyk, 2010). The toxicity of Cd consists, generally, in its ability to disturb numerous cellular functions and cause damage of various cellular structures (Martelli et al., 2006; Thompson and Bannigan, 2008). Many toxic effects of Cd seem to be indirect and due, at least in part, to oxidative stress induced following exposure to this cation. In fact, it has been shown that Cd generates reactive oxygen species (ROS) and can reduce the level of the main antioxidant compounds in the cells by inactivating enzymes and other antioxidant molecules (Filipe et al., 2006; Rogalska et al., 2009; Thompson et al., 2010).

It is known that many effect of the toxic action of Cd result from its interactions with essential elements (Brosika and Moniuszko-Jakoniuk, 2001; Martelli et al., 2006). On the other hand, there is growing evidence that the interactions between some essential elements and Cd can be effective in protection against cadmium toxicity (Bridge et al., 1976; Jemal et al., 2007; Culliane et al., 2009). Zinc (Zn) is an important antioxidant that decreases reactive oxygen species (ROS) production (Powell, 2000). It is involved in cell membrane stabilization, metallothionein synthesis and superoxide dismutase (Cu/Zn SOD) structure. Numerous studies have shown that Zn supply may reduce Cd absorption and accumulation, and also prevent or reduce the adverse actions of Cd, whereas Zn deficiency can intensify Cd accumulation and toxicity (Martelli et al., 2006; Culliane et al., 2009).

The chick embryo is a well-known animal model, which provides an ideal scenario for the metal-metal antagonists interactions. In ovo screening procedure involves exposing chicken embryos to toxic compounds and identifying the measurements (biomarkers) how these affect their development, metabolism and survival. Therefore, many compounds can be screened under the same conditions reducing a possible interference of their environment, genetics, diet background and season, and above all at reduced time and costs (Vargas et al., 2007). The egg must supply all the nutrients that the embryo needs to access (unless manipulated by “in ovo” addition) for growth and development (Yair and Uni, 2011).

The aim of our study was to assess the possible toxic effects of Cd and to investigate the possible protective influence of Zn supplementation on cadmium distribution in the body as well as some acid hydrolases activity in the blood and tissues of 1-day old chicks treated with Cd and/or Zn during embryogenesis.

MATERIAL AND METHODS
Animals and Treatment
Hatching eggs (n=300) originating from a parental flock of Ross 308 broilers were divided randomly into 6 groups and incubated in a Masalles 65 DIGIT incubator under standard conditions (T = 37.8°C, RH = 50%). On the 4th day of incubation (E 4) the eggs were candled and those with living embryos (n=262) were used for further analyses. Next, in ovo injection according with method described by Lis et al. (2011) and modified by Dżugan et al. (2012) was performed. The eggs were treated by 50 µL of 0.7% NaCl solution containing: 50 nmol Cd ions (as CdCl₂·2H₂O Sigma Chemical Co., St. Louis, MO, USA; Cd group), 100 or 500 nmol Zn ions (as ZnCl₂, Sigma Chemical Co., St. Louis, MO, USA; 2 Zn and 10 Zn groups), combinations these ions (Cd+Zn group) and pure saline (control group).

Blood and tissues preparation
Immediately after hatching, the chicks were weighed and sexed. The blood plasma samples (1-1.5 mL) were collected from the jugular vein of decapitated chick (10 chicks per group) into heparin tubes and were centrifuged (7 min, 2000×g). Moreover liver and kidney were prepared and weighed. Samples of tissues were stored at -20°C until biochemical analyses. The experiment was approved by the First Local Ethics Committee at the Medical University in Lublin, Poland (No. 9/2011)
Enzyme assay

Blood plasma and tissue homogenates (10 % w/v) were assayed spectrophotometrically for the activity of three acid hydrolases: N-acetyl-β-D-glucosaminidase (NAG) and β-D-mannosidase (β-MAN) using appropriate 4-nitrophenyl glycosides as substrates and arylsulfatase (ARYL) with nitrocatechol sulfate (Barrett and Heath, 1977). All substrates were from Sigma Chemical Co. (St. Louis, MO, USA). The enzyme activity unit [U] was defined as the enzyme activity hydrolysing 1 µmol of substrate at 37 °C at optimum pH, expressed per mg of protein determined according to the method of Lowry et al. (1951).

Metals examination

Tissue sample (0.5 g of liver and 0.1 g of kidney) were accurately weighed in a 120 ml Teflon digestion vessel. Then 8 ml of nitric acid was added and dissolved using microwave mineralization (UltraWAVE; Milestone S.r.l., Italy). The acid clear solution was transformed to 25 ml volumetric flasks and diluted with deionized water. Elements were analyzed by inducted coupled plasma optical emission spectrometer (ICP-OES), Thermo iCAP Dual 6500 (USA). All elements were blanked by blind sample which contained only nitric acid and was mineralized in the same time and conditions. In the calibration step, standard solutions for all elements were prepared from a spectroscopic grade reagent. Elemental accumulation and high exposure to cadmium can limit zinc intake (Munga et al., 2000), resulting in increased activity of lysosomal enzymes in body fluids, b suggests that cadmium administered into egg albumen was accumulated in liver and kidney to the same extent (P<0.05; Table 1).

The combined administration of Cd with Zn reduced cadmium toxicity and improved hatching results, for higher used zinc dose (500 nmol/egg) to the level observed in control group. Based on Cd-Zn antagonism, protective effect of zinc against cadmium toxicity have been intensively studied in laboratory animals (Jihen et al., 2009; Rogalska et al., 2009; Saemahayak et al., 2010; Johnson et al., 2011). Our results indicated that Cd administered into egg albumen was accumulated in liver and kidney to the same extent (P<0.05; Table 1). Concomitant treatment with the two elements reduced Cd accumulation in both tissues in comparison with the effect of Cd alone (P<0.05), by 43 and 29% in liver and kidney, respectively. In the opposite, high cadmium exposure decreased the level of zinc in kidney (P<0.05), whereas in Cd-Zn treated chicks liver the Zn level was significantly increased in comparison with the control groups (P<0.05).

Statistical Analysis

The statistical analyses were performed with the SigmaStat 2.03 computer program (SPSS Inc., USA). One-way ANOVA and Tukey test were used to evaluate the differences between experimental groups. Because there were no significant differences in enzyme activities in blood plasma as well as tested tissues homogenates between male and female chicks the data from both sexes were combined. They were presented as means ± S.E. and considered significant at P<0.05.

RESULTS AND DISCUSSION

In our study in ovo injection of 50 nmol Cd per egg reduced hatchability by 2-fold as compared to control group (P<0.05, Fig.1). Similarly, such adverse effect was observed by other authors for chicken embryo (Bridge et al., 1976; Thompson et al., 2010). Moreover, Dżugan et al. (2011) established that cadmium ions administered in ovo on day 4th of incubation at doses exceeding 1 µg/egg gradually decreased hatchability, with LD₅₀ of 3.9 µg Cd/egg. However, zinc in ovo injection in the same experiment condition also reduced hatchability by 1.4- (P<0.05) and 1.3-fold (P>0.05) for doses of 100 nmol and 500 nmol per egg, respectively. Bridge et al. (1976) investigated toxic effect of zinc on chick embryo, at the dose of 0.001 to 10 ppm but it was weaker than the same cadmium dose.

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Table 1 Cd and Zn distribution in the tissues of studied 1-day old chicks

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Cd</th>
<th>Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (0:0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>bs</td>
<td>20.82 ± 2.44*</td>
</tr>
<tr>
<td>Kidneys</td>
<td>bs</td>
<td>20.82 ± 2.44*</td>
</tr>
<tr>
<td>Cd (50:0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>0.07 ± 0.02*</td>
<td>17.74 ± 2.10*</td>
</tr>
<tr>
<td>Kidneys</td>
<td>0.07 ± 0.03*</td>
<td>20.02 ± 2.15*</td>
</tr>
<tr>
<td>Cd+10 Zn (50:500)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>0.04 ± 0.01*</td>
<td>26.60 ± 8.90*</td>
</tr>
<tr>
<td>Kidneys</td>
<td>0.05 ± 0.02*</td>
<td>22.76 ± 3.45*</td>
</tr>
</tbody>
</table>

a,b - values in columns marked by various letters differ statistically (P<0.05) bs - below the sensitivity of the method

Distribution of cadmium in animal after oral exposure is similar to that in humans with the greatest accumulation in the liver and kidneys and lower accumulation in the other parts of the organism (Shaikh and Smith, 1980; Munga et al., 2010). Concentrations of cadmium in the liver and kidneys are comparable after short-term exposure, but the cadmium concentration in kidneys surpasses that of the liver after long-term exposure (Andersen et al., 1988). It has been shown that Zn supply may reduce Cd absorption and, on the other hand, accumulation and high exposure to cadmium can limit zinc intake (Brzóska and Miniuszko-Jakoniuk, 2001). In this point of view our results seem to be in agreement with literature data.

The specific activities of three lysosomal hydrolases in tissues of hatched chicks was determined (Table 2). These enzymes were selected previously (Dżugan et al., 2011) as biomarkers of cadmium toxicity. Accumulation of Cd within the lysosome causes lysosomal damage, i.e. the enlargement of lysosomes and the destabilization of lysosomal membrane (Bradley et al., 1987; Fotakis et al., 2005), resulting in increased activity of lysosomal enzymes in body fluids, blood serum and urine (Nowak et al., 2000).

Generally, cadmium administered into egg, caused the alterations of specific activity of all tested enzymes (Table 2), but they were significant (P<0.05) only in several cases. The most pronounced increase for NAG, as the most active studied enzyme, was observed by 5, 18 and 23% in blood plasma, liver and kidney, respectively. Enhanced level of β-MAN was observed only in plasma (by 50%), whereas cadmium reduced its activity in liver. Similar tendency for ARYL was observed in both tissues.

Meanwhile, in our earlier study (Dżugan et al., 2011) we observed more intensive alterations in studied enzymes activity during cadmium enhancing exposure, at the same cadmium dose the activity increased by 108, 113 and 60% for NAG, β-MAN and ARYL, respectively. The biochemical alterations occur prior to morphological changes in the organs, and the alterations of certain enzyme levels in extracellular fluids may reflect the extent of Cd-induced damage in target organs. However, obtained results indicate that studying enzymatic biomarkers can provide inconsistent results, probably due to the difficulty in keeping repeatability in ovo experiments.
Combined treatment of embryos with cadmium plus higher zinc dose restored the level recorded in the control group (P<0.05). However, zinc supplemented alone caused an increase in all studied biomarkers. Especially, the enhanced levels of β-MAN and ARYL in blood plasma during eggs treatment with 500 nmol Zn dose were observed (P<0.05). Similarly, in the same experiment we confirmed Cd-induced oxidative stress compensated by a co-treatment with 500 nmol zinc dose co-administration, as well as the increase of blood plasma antioxidant capacity when zinc was separately used (Dzúgan et al., 2012). Moreover, during simultaneous using of Zn the negative effect of Cd may be reversed. Various mechanisms have been suggested for this rescuing effect, including competition between Cd and other divalent cations for ionic sites of cell adhesion molecules or enzymes or resistance to Cd-induced oxidative stress (Cullinane et al., 2009; Thompson et al., 2010). It was proposed that the exposure of an organism to zinc on a long-term basis, results in the induction of metallothioneins, the cadmium scavenger proteins, whereas the acute effects involve two mechanisms: protection of sulfhydryls or reduction of ·OH formation from H2O2 through the antagonism of redox-active transition metals, such as iron and copper (Powell, 2000).

**CONCLUSION**

In conclusion, the beneficial effect of zinc on hatching results of Cd-exposed chicken embryos, especially at 10-fold higher molar concentrations compared to cadmium was confirmed. Moreover, the ability of cadmium to damage lysosomal membrane and leakage of lysosomal enzyme was demonstrated once more. The negative cadmium action was completely recoverable in the presence of high zinc dose. These positive effects are based on antagonist interactions between the discussed metals and because when Zn and Cd were administered alone, both were embryotoxic. So we concluded, that adequate zinc supplementation can protect mammals against cadmium toxicity and reduced oxidative stress.

**Acknowledgments:** This work was supported by the research project N N 304 291 140.0

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