JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

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

Regular article

OPEN ACCESS

ABSTRACT

The aim of 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-glucosaminidase (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 ($P \leq 0.05$). 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 (**Czeczot and Skrzycki, 2010**). Chronic exposure to this metal leads to damage of numerous organs and systems, primarily kidneys (**Rogalska *et al.*, 2009; Czeczot and Skrzycki, 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 (**Filipic *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 (**Brzóska 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; Jemai *et al.*, 2007; Cullinane *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; Cullinane *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 perform. The eggs were treated by 50 µL of 0.7% NaCl solution containing: 50 nmol Cd ions (as $CdCl_2 \cdot 2.5H_2O$ Sigma Chemical Co., St. Louis, MO, USA; Cd group), 100 or 500 nmol Zn ions (as $ZnCl_2$, Sigma Chemical Co., St. Louis, MO, USA; 2 Zn and 10 Zn groups), combinations these ions (Cd+2Zn group and Cd+10Zn 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° 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) was 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 (Thermo) with 3 step curve. A curve fit factor for all elements were above 0,99. All analysis has been done in three independent repetitions for each sample. The metal concentrations were expressed on a wet weight basis (µg/g).

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 $P \leq 0.05$.

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 ($P \leq 0.05$, 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 LD_{50} of 3.9 µg Cd/egg. However, zinc *in ovo* injection in the same experiment condition also reduced hatchability by 1.4- ($P \leq 0.05$) 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.

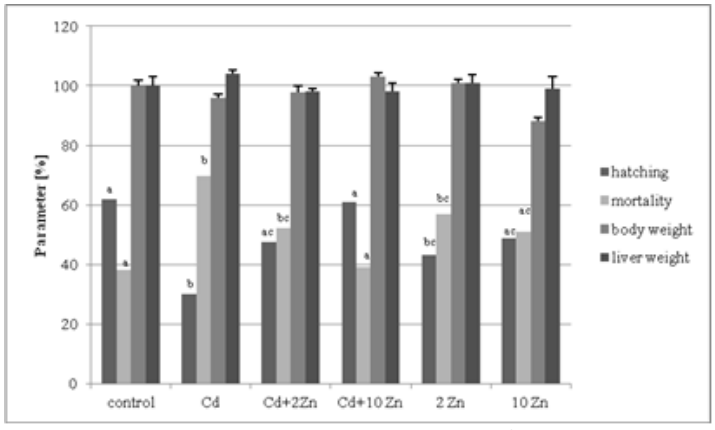

Figure 1 Hatching results and morphometric parameters of newly hatched chicks from experimental group. The weight of body and liver was expressed as % of value observed in control group (a,b,c) values with different letters differ significantly ($P \leq 0.05$). Detailed data on hatching results and quality of chicks have been presented elsewhere (**Dżugan *et al.*, 2012).**

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; Saenmahayak *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 ($P \leq 0.05$; 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 ($P \leq 0.05$).

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

Experimental group Cd:Zn molar ratio	**Cd** **[µg/g]**	**Zn** **[µg/g]**
Control (0:0)		
Liver	bs	20.82 ± 2.44^{a}
Kidneys	bs	20.82 ± 2.44^{a}
Cd (50:0)		
Liver	0.07 ± 0.02^{a}	17.74 ± 2.10^{a}
Kidneys	0.07 ± 0.03^{a}	20.02 ± 2.15^{a}
Cd+10 Zn (50:500)		
Liver	0.04 ± 0.01^{a}	26.60 ± 8.90^{b}
Kidneys	0.05 ± 0.02^{a}	22.76 ± 3.45^{ab}

a,b, , - values in columns marked by various letters differ statistically ($P \leq 0.05$)
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 ($P \leq 0.05$) 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.

Table 2 Specific activity of indicative hydrolases in tissues of newly hatched chicks expose to cadmium and/or zinc during embryogenesis

	Specific activity [mU/mg protein]					
	Experimental group (Cd dose [mmol/egg]: Zn dose [mmol/egg])					
ENZYME	**Control** (0:0)	**Cd** (50:0)	**Cd+ 2Zn** (50:100)	**Cd+10 Zn** (50:500)	**2Zn** (0:100)	**10Zn** (0:500)
NAG						
B. plasma	17.4 ± 4.38^{a}	18.5 ± 4.17^{a}	21.3 ± 8.12^{a}	16.0 ± 5.9^{a}	17.9 ± 10.21^{a}	19.1 ± 6.30^{a}
Liver	182.1 ± 16.75^{b}	214.5 ± 39.79^{c}	211.3 ± 22.16^{bc}	191.5 ± 7.41^{bc}	205.3 ± 21.83^{bc}	210.9 ± 27.39^{bc}
Kidneys	108.9 ± 18.78^{d}	134.0 ± 11.66^{e}	124.9 ± 16.54^{de}	103.6 ± 3.72^{de}	116.1 ± 17.06^{de}	124.3 ± 11.76^{de}
β-MAN						
B. plasma	2.2 ± 0.44^{a}	3.3 ± 0.39^{b}	3.7 ± 0.70^{b}	2.8 ± 0.42^{ab}	2.7 ± 0.35^{ab}	4.1 ± 1.79^{c}
Liver	8.4 ± 0.46^{d}	7.6 ± 1.15^{d}	7.8 ± 1.22^{d}	7.7 ± 0.38^{d}	8.1 ± 0.86^{d}	7.8 ± 1.21^{d}
Kidneys	5.7 ± 0.17^{e}	5.8 ± 0.85^{e}	5.9 ± 0.43^{e}	5.8 ± 0.72^{e}	5.2 ± 0.11^{e}	6.3 ± 0.03^{e}
ARYL						
B. plasma	2.3 ± 0.41^{a}	3.2 ± 0.38^{b}	3.3 ± 0.45^{ab}	$2.9 \pm 0.23a^{b}$	2.7 ± 0.27^{ab}	3.2 ± 1.29^{b}
Liver	4.8 ± 0.30^{c}	4.3 ± 0.63^{c}	4.73 ± 0.53^{c}	4.6 ± 0.56^{c}	5.2 ± 0.66^{c}	4.4 ± 0.40^{c}
Kidneys	8.0 ± 0.99^{d}	8.8 ± 3.47^{d}	10.7 ± 2.07^{d}	8.1 ± 0.59^{dc}	9.1 ± 1.15^{d}	11.2 ± 1.54^{d}

a,b,c,d,e, - values of activity for given enzyme marked by various letters differ statistically ($P \leq 0.05$)

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 ($P \leq 0.05$). 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 (**Dżugan *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 and 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 H_2O_2 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 recover in the presence of high zinc dose. These positive effects are based on antagonist interactions between the discussed metal ions, 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.

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