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Effect of in ovo injection of cadmium on chicken embryo heart

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Due to the ease of absorption, accumulation in tissues, and extremely long biological half-life in the body, cadmium is considered one of the most hazardous heavy metals. The aim of the study was to investigate the effect of in ovo injection of cadmium on chicken embryo heart. A total of 160 chicken hatching eggs were used in the study. On day 4 of incubation, eggs from the experimental groups were injected with cadmium at a dose of 1 and 5 µg/egg and the incubation was prolonged to 21 day until hatching. Cadmium was found to slow the heart rate and reduce heart weight. In embryos exposed to 5 µg of cadmium, the histological analysis and aminotransferases concentration confirmed the occurrence of inflammatory processes in the heart muscle.

Key words: Cadmium, heart, chicken embryo, cardiotoxicity.

INTRODUCTION

Cadmium (Cd) is a heavy metal whose divalent cations are easily absorbed and accumulated in plant and animal tissues (Satarug et al., 2003). Exposure to cadmium occurs as a result of atmospheric emission during Cd production and processing, from combustion of fossil energy sources, waste and sludge, phosphate fertilizers, and deposition of waste and slag in at disposal sites (Satarug et al., 2003).

The main source of cadmium exposure for non-occupational population is food and tobacco smoking (EFSA, 2009). Meat, fish, and fruits generally contain up to 50 µg Cd/kg on fresh weight basis, whereas vegetables, potatoes, and grain products may contain up to 150 µg Cd/kg fresh weight. Higher concentrations are found in the kidneys of animals slaughtered for food, in wild mushrooms, and in seafood such as mussels and oysters (EFSA, 2009). The average cadmium intake from food generally varies between 8 and 25 µg per day (Jarüp and Åkesson, 2009).

Due to the exceptionally long biological half-life (10-30 years) in the body, the toxicity of cadmium ions increases with advancing age and may persist after the end of exposure (Nordberg, 2009). In animals exposed to cadmium compounds, the ions of this metal are particularly abundant in kidneys, heart and liver, and, to a lesser degree, in pancreas and brain (Jarüp and Åkesson, 2009). This can induce irreversible damage to these organs in addition to anemia, osteoporosis and carcinogenic (for kidney, lung, prostate, testis and breast) (Jarüp and Åkesson, 2009) and teratogenic lesions (Järup et al., 1998). The effects at the cellular level are increased oxidative stress, damage to mitochondria,
disturbances in trace element (e.g. calcium and zinc) and vitamin metabolism, and disruption of cell signaling pathways (Czeczot and Skrzyzki, 2010). Cadmium also destabilizes lysosomal membranes, which is possibly associated with the secretion of lysosomal enzymes into cellular fluids, blood and urine (Fotakis et al., 2005; Nordberg, 2009). Induction of an inflammatory reaction is probably one of the mechanisms for the toxic effect of cadmium.

The proinflammatory activity of cadmium was observed in kidneys, liver and the respiratory system (Mlynek and Skoczynska, 2005). Research also shows that cadmium may cause an inflammatory reaction in the cardiovascular system (Yazihan et al., 2011). In experimental chronic cadmium poisoning, a greater incidence of lipid infiltration in the aorta as well as arteriosclerosis and arteriolosclerosis in rat heart, lungs, kidneys and adrenals were observed (Mlynek and Skoczynska, 2005).

It is known that juvenile organisms are particularly sensitive to the adverse effects of heavy metals (Wonga et al., 1980; Yasuda et al., 2012). What is more, research concerning the effect of Cd on embryos is all the more important because cadmium inhaled by females crosses the placenta and accumulates in the fetal body (Trottier et al., 2002).

Chick embryo provides a useful model for studying the effect of various factors on the developing embryos. Development outside the mother’s body, the high rate and peculiar characteristics of embryogenesis, and the relatively well known process of organogenesis make chick embryos a frequent subject of such studies (Tadahiro et al., 1998; Davison, 2003; Rajendra et al., 2004; Mitta and Weber, 2005; Lahijani et al., 2009; Dżugan et al., 2011; Pawlak et al., 2011). Treating avian embryos with Cd has always resulted in dorsally growing upper limbs, malposition of predominantly the lower limbs, and primary ventral body wall defect (Thompson and Bannigan, 2008; Cullinane et al., 2009). The heart is one of the earliest developing embryo organs, the activity of which can be recorded. Therefore, it is apparent that studies concerning the effect of cadmium on the developing embryo heart may be of great importance, especially since cadmium is cardiotoxic in small doses that have no negative effect on other organs (Limaye and Shaikh, 1999).

The aim of the study was to investigate the effect of in ovo injection of cadmium on the heart rate, histological appearance and morphometric parameters of chick embryo heart.

**MATERIALS AND METHODS**

Hatching eggs (62.0 ± 5.4 g) from a Ross 308 broiler breeder flock were incubated in a Masalles 65 DIGIT incubator under standard conditions (1to 18 day of incubation: T = 37.8°C, RH = 50%; 19–21 day: T = 37.2 ± 0.1°C, RH = 70%). On day 4 of incubation the eggs were candled, and eggs with live embryos were randomly divided into 4 groups (n=40 eggs per group). Embryos in Group I were incubated without interference (control group). On day 4 of incubation in Groups II, III and IV, a hole (approximately 5 mm in diameter) was made in the air cell end of each egg to inject 50 μl of 0.7% natrium chloride solution (NaCl; Sigma USA) containing Cd ions (as cadmium chloride; CdCl₂, Sigma USA) into ovalbumin, just under the chorioallantoic membrane. The used cadmium dose were: Group II - 0 µg Cd/egg - (sham); Group III - 1 µg Cd/egg ; and Group IV - 5 µg Cd/egg. The dose was based on our earlier study, where under the study conditions; the lethal dose (LD₅₀) was 3.9 µg Cd/egg (Dżugan et al., 2011). After injection, the hole in the shell was sealed with Parafilm® (area approximately 150 mm²) and the incubation was continued until hatching. On days 6 and 18 eggs were recandled to remove eggs with dead embryos.

The experiment was approved by the First Local Ethics Committee at the Medical University in Lublin, Poland (No. 9/2011).

**Heart rate measurements**

Cardiac work of chick embryo was measured from day 9 to 21 day of incubation at the same hour, using contactless ballistocardiography (Pawlak and Niedziółka, 2004). In the contactless ballistocardiography of the chick embryo, an egg shell bearing electric charges is one capacitor plate, the other being a receiving antenna of the measuring equipment. The heart work of embryo induces micro-movements of the whole egg, resulting in changes of the distances between the plates and thus in the difference of potentials between the shell and the receiving antenna, which is registered by the measuring equipment (Szymański et al., 2002).

Once the measurements were completed the signal from the embryo heart work was analysed by a computer. In the computer analysis performed to determine the heart rate, envelope signal was calculated using a moving average (11 points) from the modulus of the recorded signal in accordance with the following formula:

\[ Y_i = \frac{1}{11} \sum_{j=i-5}^{i+5} |y_j| \]

where: \( y_k \) is kth sample of the recorded signal, and \( Y_i \) is ith sample of the newly formed time series (envelope).

Next, the signal was analysed by dividing the measurement series into 15 second fragments. The signal power spectrum with a resolution of 0.06 Hz was calculated from each fragment of measurement data. Power spectra were calculated using fast Fourier transform (Brigham, 2002). These spectra were summed to determine mean power spectrum of the whole measurement, which finally made it possible to determine the heart rate of the developing embryo on successive days of growth.

**Morphometric measurements**

Immediately after hatching (21 day of incubation) 10 chicks were randomly selected from each group. Chicks were weighed and decapitated. Bleeding out was followed by collection of their hearts, which were weighed and fixed in 4% paraformaldehyde (PFA), dehydrated in increased concentrations of alcohol, and embedded in paraplast. Paraffin blocks were cut using a Leica RM2145 rotary microtome into sections 5 μm thick, which were stained with haematoxylin and eosin. So prepared histological preparations were measured for thickness of left and right heart ventricles using Multi Scan Base 98 software. Fifty measurements of left and right ventricular wall thickness was taken in each preparation, starting from auriculoventricular valves toward the apex of the heart.
Table 1. Effect of in ovo injection of cadmium ions on the cardiac work of chick embryos on successive days of incubation; (n=40); means ± SD.

<table>
<thead>
<tr>
<th>Injection day of incubation</th>
<th>Group I non-injected (control)</th>
<th>Group II 50 µl of 0.7% NaCl (sham)</th>
<th>Group III 1 µg Cd in 50 µl of 0.7% NaCl /egg</th>
<th>Group IV 5 µg Cd in 50 µl of 0.7% NaCl /egg</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>258±11.2^a</td>
<td>258±14.3^b</td>
<td>253±12.9</td>
<td>248±17.5^ab</td>
</tr>
<tr>
<td>11</td>
<td>246±10.8^a</td>
<td>250±15.1^b</td>
<td>254±14.7</td>
<td>236±14.8^ab</td>
</tr>
<tr>
<td>12</td>
<td>237±7.6^a</td>
<td>237±9.6^b</td>
<td>232±12.4</td>
<td>223±16.7^ab</td>
</tr>
<tr>
<td>13</td>
<td>231±8.3^a</td>
<td>230±10.0^b</td>
<td>225±15.6</td>
<td>216±16.0^ab</td>
</tr>
<tr>
<td>14</td>
<td>232±9.1^a</td>
<td>229±18.4^b</td>
<td>224±10.0</td>
<td>217±12.7^ab</td>
</tr>
<tr>
<td>15</td>
<td>231±11.2^a</td>
<td>230±12.6^b</td>
<td>225±15.1</td>
<td>217±11.0^ab</td>
</tr>
<tr>
<td>16</td>
<td>231±7.9^a</td>
<td>230±8.9^b</td>
<td>224±18.2</td>
<td>206±18.6</td>
</tr>
<tr>
<td>17</td>
<td>220±12.3^Ac</td>
<td>220±11.4^Bd</td>
<td>207±11.0^cde</td>
<td>190±20.5^Abe</td>
</tr>
<tr>
<td>18</td>
<td>214±11.7^Ac</td>
<td>213±14.2^Bd</td>
<td>200±15.7^cde</td>
<td>183±20.2^Abe</td>
</tr>
<tr>
<td>19</td>
<td>205±14.6^Ac</td>
<td>202±15.1^Bd</td>
<td>190±14.2^cde</td>
<td>170±19.5^Abe</td>
</tr>
<tr>
<td>20</td>
<td>197±13.9^Ac</td>
<td>193±19.5^Bd</td>
<td>183±20.6^cde</td>
<td>163±18.5^Abe</td>
</tr>
<tr>
<td>21</td>
<td>179±22.4^Ac</td>
<td>181±24.9^Bd</td>
<td>166±22.9^cde</td>
<td>149±24.1^Abe</td>
</tr>
</tbody>
</table>

Values in rows with the same small letters differ significantly at p ≤ 0.05; those with the same capital letters differ significantly at p ≤ 0.01.

Aminotransferase measurement

Concentrations of alanine aminotransferase (ALT; AlaAT) and aspartate transferase (AST; AspAT) were measured in plasma samples collected from chick embryos on day 20 of incubation. The enzymes were determined by means of the automatic kinetic method recommended by IFCC (1986) using kits produced by Alpha Diagnostics (Poland).

Statistical analysis

The results were analysed statistically by two-way analysis of variance for repeated measures, and significant differences between the means were evaluated using Student’s t-test. Prior to the analysis, normality of distribution was assessed using Shapiro-Wilk’s test. Probability of 0.05 was considered an important indicator of statistical differences between the means.

RESULTS AND DISCUSSION

The heart rate of avian embryos is one of the most often reported parameters of cardiac work (Ono et al., 1997; Tazawa et al., 2001). In our research, comparison of the heart rate in different groups showed that it was always lower in embryos exposed to cadmium than in embryos from the control and sham groups. Differences between the group injected with 5 µg of cadmium and Groups I and II were statistically significant from 10 to 15 days (p≤0.05) and from 16 to 21 days of incubation (p≤0.01). Differences in the heart rate between the group exposed to 1 µg of Cd and Groups I and II were statistically significant (p≤0.05) between 17 and 21 days of incubation (Table 1).

Starting from day 16 of incubation, the heart rate in Group IV was significantly lower than in Group III (p ≤ 0.01) (Table 1). The slowing of heart rate in the final stage of incubation was probably due to the embryo absorbing and accumulating cadmium injected into ovalbumin. Similar findings were obtained by Nishiyama et al. (1990), who observed a slowed heart rate in rats receiving cadmium-supplemented feed. Also, the experiments with fish embryos support the thesis that cadmium has a slowing effect on heart rate (Westernhagen et al., 1975; Jezierska et al., 2002).

Our experiment also revealed that cadmium has a negative effect on morphometric elements of the heart. Measurements of heart weight showed a significant (p≤0.05) decrease in this parameter in embryos exposed to 5 µg of cadmium (Group IV) compared to the control and sham groups (Table 2). When comparing the relative heart weight, it was found that in chickens originating from cadmium-injected eggs, the value of this parameter was always lower than in embryos unexposed to cadmium. Statistical analysis revealed that differences between the group of embryos exposed to 1 µg of cadmium and the control and sham groups was significant at 0.05, while differences between the control and sham groups and the group injected with 5 µg of cadmium were significant at 0.01 (Table 2). Similar changes in heart weight in female Pekin ducks exposed to cadmium were observed by Hughes et al. (2000). A decrease in relative heart weight in broiler chickens receiving dietary cadmium was also described by Bokori et al. (1996). Mikhaleva et al. (1991) report that chronic oral administration of cadmium chloride in rats decreased the weight of heart ventricles. On the other hand, Jamall and Smith (1985) demonstrated increased heart weight in cadmium-exposed rats.

Our study, for the first time, showed that in embryos exposed to cadmium, the thickness of the right ventricle wall decreased considerably. Differences between the
control (Group I) and sham groups (Group II) and the group exposed to 1 µg of cadmium were statistically significant at 0.05, and those between the group injected with a higher dose of cadmium ions and Group I and II were significant at P<0.01 (Table 2). Measurements of the left ventricle wall did not show any significant differences in the value of this parameter (Table 2). The available literature provides no data regarding this issue. So, we suggest that observed decrease of ventricle wall thickness could be associated with cadmium-induced apoptosis (Yazihan et al., 2011).

Our study also showed a negative effect of a higher cadmium dose (5 µg) on cardiac muscle structure. In embryos from this group, the microscopic images for the first time showed inflammatory changes indicative of interstitial myocarditis. Muscle fibres were spaced apart, with a large inflammatory infiltration of mononuclear cells (mainly lymphocytes) in between. Lesions of this type were observed in all birds exposed to the higher cadmium dose, both in the muscle fibres of the ventricles and in the interventricular septum (Figure 1d). In embryos from the control and sham groups and in those exposed to 1 µg cadmium, no pathological changes in heart muscle structure were observed (Figure 1a to c). Meanwhile, Mikhaleva et al. (1991) showed the effect of Cd on left ventricular cardiomyocyte hypertrophy. Kolakowski et al. (1983) observed distinct alterations of the intercalated disc structure. The damage to intercalated discs varied from the enlargement of the fissure between membranes (within unspecialized segments) to disruption of the complex junctions.

The results of histological examination correspond with the results of biochemical analysis. The injection of 5 µg of Cd/egg caused a significant increase in the concentrations of aspartate (p ≤ 0.01) and alanine aminotransferase (p ≤ 0.05) in relation to both the control and sham groups (Table 3). The injection of 1 µg of Cd/egg caused no significant changes in the concentration of these enzymes (Table 3).

Aspartate and alanine aminotransferases are two of the enzymes most frequently measured by the clinical laboratory (Rej, 1989). They are most commonly used in the differential diagnosis of various liver diseases where the ratio of the two enzymes provides additional clinical insight. AST is much less liver-specific, therefore is also useful in many cases for diagnosis, or estimating severity of myocardial infarction (Rej, 1989). In plasma of chicks treated with cadmium during embryogenesis the most intensive increase in AST versus ALT levels was observed. In control and sham group the relation between AST and ALT was 1:2, whereas in Cd-group this relation was 1:1. Although used Cd dose caused the chick liver damage, observed the more intensive increase in AST level (by 4 times as compared to control) than in ALT level may indicate other non-liver cadmium toxic action. This observation is confirmed by the study of the effect of local heart irradiation in a rat model which showed, among other myocardial enzymes, an increase in plasma AST activity whereas plasma ALT levels remained unchanged (Franken et al., 2000). Chronic Cd administration (15 ppm per 8 weeks) induces inflammation and apoptosis in rat hearts, which was evaluated using much more specific biomarkers TNF-α and IL-6 (Yazihan et al., 2011).

In conclusion, the present study showed a clear effect of cadmium on the heart rate, and on the morphometric and histological results of chick embryo hearts, with the intensity of these changes being positively correlated.

### Table 2. Effect of in ovo injection of cadmium ions on morphometric parameters of the heart of newly-hatched chicks (n=10); means ± SD.

<table>
<thead>
<tr>
<th>Item</th>
<th>Group I non-injected</th>
<th>Group II 50 µl of 0.7% NaCl (sham)</th>
<th>Group III 1 µg Cd in 50 µl of 0.7% NaCl/egg</th>
<th>Group IV 5 µg Cd in 50 µl of 0.7% NaCl/egg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart weight (g)</td>
<td>0.258±0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.262±0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.252±0.06</td>
<td>0.247±0.07&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Heart weight/body weight</td>
<td>0.588±0.04&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.590±0.06&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>0.574±0.08&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>0.561±0.05&lt;sup&gt;cd&lt;/sup&gt;</td>
</tr>
<tr>
<td>Thickness of the right ventricle (µm)</td>
<td>682.7±50.1&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>685.0±56.2&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>670.4±55.3&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>665.3±66.2&lt;sup&gt;cd&lt;/sup&gt;</td>
</tr>
<tr>
<td>Thickness of the left ventricle (µm)</td>
<td>861±48.2</td>
<td>863.2±52.5</td>
<td>858.3±47.2</td>
<td>853.2±42.4</td>
</tr>
</tbody>
</table>

Values in rows with the same small letters differ significantly at p ≤ 0.05, those with the same capital letters differ significantly at p ≤ 0.01.

### Table 3. Effect of in ovo injection of cadmium (CdCl<sub>2</sub>) on aspartate (AST) and alanine aminotransferase (ALT) levels in the blood of 20-day-old chick embryos (n=10); means ± SD.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Group I Non-injected</th>
<th>Group II 50 µl of 0.7% NaCl (sham)</th>
<th>Group III 1 µg Cd in 50 µl of 0.7% NaCl/egg</th>
<th>Group IV 5 µg Cd in 50 µl of 0.7% NaCl/egg</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST (U/L)</td>
<td>6.72 ± 0.25&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.43± 0.46&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.99 ± 0.53&lt;sup&gt;c&lt;/sup&gt;</td>
<td>24.7 ± 0.51&lt;sup&gt;ABC&lt;/sup&gt;</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>14.04 ± 1.75&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.60 ± 4.83&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16.58 ± 7.12&lt;sup&gt;c&lt;/sup&gt;</td>
<td>29.6 ± 9.47&lt;sup&gt;abc&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values in rows with the same small letters differ significantly at p ≤ 0.05; those with the same capital letters differ significantly at p ≤ 0.01.
The mechanism of cadmium cardiotoxicity needs explanation and probably is connected with calcium, one of the elements responsible for normal heart work (Dhalla et al., 1982). It is known that cadmium may interfere with calcium metabolism by removing it from the body (Czeczot and Skrzycki, 2010). In addition, cadmium is a blocker of calcium channels (Bridges and Zalups, 2005; Martelli et al., 2006). By acting through various metabolic pathways, cadmium affects the activity of the renin-angiotensin system (Martynowicz and Skoczyńska, 2003). Cadmium may reduce concentration of angiotensin II, which is produced as a result of the action of dipeptide carboxylase on angiotensin. The lower concentration of this compound inhibits stimulation of the AT-1 receptor, which is responsible for increased influx of calcium into the heart muscle cells and releases calcium from the sarcoplasmic reticulum (Martynowicz and Skoczyńska, 2003).

It is now a real challenge to understand the precise nature of processes associated with the cadmium exposure and intracellular calcium deficiency in order to achieve proper management of cardiac disorders. It is generally known that cadmium from cigarette smoke, penetrating through the placenta may reach the embryos and accumulates in the fetal body (Trottier et al., 2002).

Assumption than can be made, based on our research is that the cadmium contained in the cigarette smoke can damage the heart of the human embryo. Thus further study of cadmium influence on heart with particular regard to its effects on calcium intracellular balance using in ovo model are planned.

ACKNOWLEDGEMENTS

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REFERENCES


