Electrochemical Analysis of Metallothionein in Chicken Exposed to Cadmium Ions

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Metallothionein as a protective protein has been studied in relationship to cancer diseases, toxicity of anticancer drugs and environmental pollution. Its electrochemical detection is based on Brdicka´s reaction suitable for the protein detection. The original reaction was invented by Rudolf Brdicka in 1930s and adapted protocol of the reaction was utilized in this study. We focused on the effect of cadmium ions contained in CdTe quantum dots in comparison to cadmium ions in a standard solution applied to chicken embryos. One day after application, chicken embryos were euthanized and liver, kidney, brain and muscle tissue were analyzed. The highest metallothionein levels were detected in the liver and kidney, which corresponded with cadmium accumulation determined by atomic absorption spectroscopy (68 µg/g liver tissue and 125 µg/g kidney tissue after application of quantum dots). The shape of electrochemical voltammograms was mathematically processed. In conclusion, metallothionein seems to be significantly involved in Cd detoxification mechanism.

1. INTRODUCTION

Metallothionein (MT) is a protective protein, impacting the development of cancer, chemoresistance of cells towards anticancer drugs and protection of organism against harmful effect of environmental pollution. MTs are named after its competence to interact with divalent heavy metal cations such as cadmium, zinc, mercury and copper using the free thiol groups [1].

MTs are low molecular weight, intracellular proteins that contain an array of 20 cysteines and polynuclear metal–sulfur coordination sites, which can be saturated by metal ions [2]. Most of MTs bind multiple metals in two well-defined metal-thiolate clusters [3], because of the -SH moiety which is sensitive to oxidation [4]. There exist four basic isoforms of MT (MT-1, MT-2, MT-3, MT-4). These have highly homologous structure and are quite conservative over long evolutionary distances [1,5]. MT-1 and MT-2, are induced in the liver by wide range of metals, drugs, and pro-inflammatory mediators shortly after exposure [6]. The MTs accomplish the cellular regulation of essential metals (Cu and Zn), as well as reduction the toxicity of non-essential heavy metals (Cd and Pb) [7]. MTs are participating in many pathophysiological and physiological mechanisms [8] and the most important are detoxification of heavy metals, ion homeostasis and protection against the oxidative stress [9]. Besides, MTs are important in essential metal cellular metabolism providing a non-toxic storing reservoir for these elements. There exists a number of various MT induction factors in response to metal stress and it seems to be also affected by non-metal endogenous and exogenous effectors [10]. Many types of agents can influence the synthesis of the proteins, including the reactive oxygen species, divalent heavy metals, calcium ionophores, several cytokines, and glucocorticoids [1].

The existence of MTs in all kinds of mammalian cells assumes that they play an essential function as an intracellular protein as well as extracellularly active molecules [11]. The extracellular MT can occur in the many body fluids: milk, urine, plasma, pancreatic or amniotic fluid. Inside cells, MTs have been discovered in the mitochondria, cytoplasm, nucleus, and lysosome. MT, in relation to the cell life cycle and oxidative stress, is capable to enter the cell nucleus and act as a transcription factor [9].

Cadmium (Cd) as a selective toxicant is preferentially accumulated in the kidneys where it induces cellular injury [12]. During chronic poisoning, Cd ions stimulate *de novo* synthesis of MTs and it is expected that toxicity starts when loading with Cd ions exceeds the buffering intracellular MTs capacity [11]. MT can be determined by electrochemical detection in Brdicka solution and three characteristic signals can be obtained as follows: RS_2Co (about -1.3 V), Cat1 (-1.4 V) and Cat2 (-1.5 V). The profile of achieved voltammograms can be easily analyzed by mathematical model [13,14] and evaluated to find similarities according to the illness type, treatment, etc. to obtain the electrochemical fingerprints of MT signal.

Quantum dots (QDs) are members of a new class of fluorescent nanoparticles and they show wide utilization in biological and biomedical studies [15,16]. QDs based on cadmium or other metals represent a danger for organisms [17]. The QDs cytotoxicity is closely related to their surface properties and constituent elements as well [18]. The health effect of QDs can be studied on cell cultures or on different organisms. Beside the named models, chicken embryo has interesting properties for cytotoxicity experiments; it represents an accessible and economical *in vivo* model without ethical issues. Chicken embryos have been used in many studies, for example gene expression analysis, developmental biology, stem cell and cancer research and toxicity of different compounds [19].

CdTe QDs could have impacts on Cd bioaccumulation, MTs production, oxidative stress, genotoxicity, and the immune system of mammalian. Toxicity of CdTe QDs is caused by different mechanisms, including free cadmium ions present in particle solution, the elimination of Cd^{2+} ions

from QDs core [20] and formation of reactive oxygen species [21]. In this work, we determined a link between QDs application and MT production in chosen organs (liver, kidney, brain, heart) of the chicken embryonic model.

2. EXPERIMENTAL PART

2.1. Chemicals

All chemicals for the preparation of QDs were purchased from Sigma-Aldrich (St. Louis, MO, USA) in ACS purity. To pipette volumes down to micro, pipettes were purchased from Eppendorf Research (Eppendorf, Hamburg, Germany) with the highest certified deviation (±12%). The deionized water was prepared using reverse osmosis equipment Aqual 25 (Brno, Czech Republic). The deionized water was further purified by using apparatus MiliQ Direct QUV equipped with the UV lamp. The resistance of the deionized water was 18 MΩ. The pH was measured using pH meter WTW inoLab (Weilheim, Germany). Other chemicals for isolation of MT and electrochemical measurements were also purchased from Sigma Aldrich.

2.2. Synthesis of quantum dots CdTe capped mercaptosuccinic acid (MSA)

CdTe QDs were prepared with a slightly modified method published in the following study [22]. Cadmium acetate dihydrate $(Cd(OAc)_2 \cdot 2H_2O$ (0.027 g, 0.1 mM) was dissolved in deionized water (44 mL) and 100 mg of trisodium citrate dihydrate was added with stirring. Solution of 5.5 mg (0.025 mM) Na_2TeO_3 in 1.25 mL of water was poured into the first solution followed by MSA (100 µL, 1.14 mM). Afterwards, solid $NaBH_4$ (50 mg) was added with vigorous stirring and hydrogen evolution was observed, followed by color change of solution to slightly yellow. After 30 min of stirring, 2 mL of solution was heated in glass vial in Multiwave 3000 Microwave Reaction System (Anton Paar, Graz, Austria) using rotor 64MG5. Reaction conditions were as follows – power 300 W, ramp 10 min, hold 10 min, temperature 50 – 130 ºC [23].

2.3. Model organisms

The fertilized eggs of Lankenvelder roosters and ISA Brown hens (Integra, a.s., Zabcice, Czech Republic) were used for the experiment. The experiments were performed with embryos in 16th day of development. The distribution of Cd was analyzed after 24 hours of incubation with 500 µL cadmium solution of $Cd(NO_3)_2 \cdot 4H_2O$ (group CdL with 1 mg/mL applied cadmium standard, $n = 5$, or group CdH with 4.5 mg/mL applied cadmium standard, $n = 5$, both solutions in deionized water) or with 500 µL of 1mg/mL Cd in the CdTe quantum dots solution (group CdTe, $n = 5$). Cadmium was applied through a small hole in a shell directly to chorioallantoic membrane. Embryo was incubated at 37 °C and relative humidity of 45% in incubator (RCom 50 MAX, Gyeongman, Korea). Chicken embryo controls were exposed to 500 µL of deionized water ($n = 5$) or without exposure ($n = 2$). After organs

(liver, kidney, brain and heart) extraction, samples were frozen and kept for other analysis. The samples were stored in -80 °C until assayed.

2.4. Sample preparation for MT electrochemical detection

100 mg of thawed embryo tissue samples (liver, kidney, brain and heart muscle tissue) were used for MT analysis following our protocol: samples were mixed with 1 mL of 0.1 M phosphate buffer (p*H* 7.0) and homogenized by ultrasonic needle for 2 minutes. After homogenization, samples were shaken at 8 °C for 20 minutes and centrifuged for 10 minutes at 4 °C. 10 µL of supernatant was mixed with 990 µL of the phosphate buffer and samples were further denatured at 99 °C for 20 minutes in a thermomixer (Eppendorf 5430, Hamburg, Germany) and centrifuged for 10 minutes (Eppendorf 5402, Hamburg, Germany) to remove ballast proteins and peptides which could interfere in the electrochemical response.

2.5. Metallothionein determination

Levels of MT in all tissues were determined by the differential pulse voltammetry with Brdicka electrolyte (1 mM $Co(NH_3)_6Cl_3$, and 1 M ammonia buffer ($NH_3(aq)$ and NH_4Cl, p*H* = 9.6) using the quoted protocol [24]. Differential pulse voltammetric measurements were performed using the 747 VA Stand instrument connected to a 693 VA Processor and 695 Autosampler (Metrohm, Switzerland), using a standard reaction cuvette with three electrodes (working electrode was a hanging mercury drop electrode with a drop area of 0.4 mm^2 as working electrode Ag/AgCl/3M KCl electrode and as auxiliary platinum electrode) and a cooled sample holder and measurement cell to 4 °C (Julabo F25, Julabo, Germany). The analyzed samples were deoxygenated prior to measurements by purging with argon (99.999%) saturated with water for 120 s. The parameters of the measurement were as follows: initial potential of -0.7 V, end potential of -1.75 V, modulation time 0.057 s, time interval 0.2 s, step potential 2 mV, modulation amplitude -250 mV, E_{ads} = 0 V, volume of injected sample 5 µL. Measurements were done in electrochemical cell with total volume 2 mL (5 µL of sample and 1995 µL Brdicka solution).

2.6. Determination of total protein content by the pyrogallol red

Total protein was determined by using the SKALAB CBT 600T kit (Skalab, Svitavy, Czech Republic) according to manufacturer´s instructions.

2.7. Atomic absorption spectroscopy (AAS)

Cadmium was determined using 280Z Agilent Technologies atomic absorption spectrometer (Agilent, USA) with electrothermal atomization. Cadmium ultrasensitive hollow cathode lamp (Agilent, USA) was used as the radiation source (lamp current 4 mA). The spectrometer was operated

at 228.8 nm resonance line with spectral bandwidth of 0.5 nm. The sample volume 20 µL was injected into the graphite tube. The flow of argon inert gas was 300 mL min^{-1}. Zeeman background correction was used with field strength 0.8 Tesla. Cadmium was determined in the presence of palladium chemical modifier.

2.8. Statistical evaluation

Multivariate ANOVA was employed for statistical evaluation. Tissue type and treatment were used as a categorical predictor. This analysis was followed by Tukey's post-hoc testing to reveal differences between individual variables. Unless noted otherwise, probability levels p 0.05 were considered as significant. Software Statistica 12 (StatSoft, USA) was used for purpose of the analysis.

2.9. Brdicka curves shape analysis

The methodology of Brdicka curves analysis with discussion is published in the quoted paper [14]. The methodology is composed of three main parts: data pre-processing, transformation curves into 32 intervals by wavelet transformation and the analysis the intervals by decision tree method, respectively. The goal of our analysis was to find areas on the curves, where significant differences between our four groups, namely CdH (4.5 mg/mL), CdL (1 mg/mL), CdTe (QDs) and control (water) can be evaluated.

3. RESULTS AND DISCUSSION

Due to the fact that MT is accepted as an indicator of environmental pollution [25] or organism exposure to metals [26,27], this protein was chosen as a biomarker of various forms of cadmium exposure on chicken organism. The detection of MT by using electrochemical approach in Brdicka electrolyte was previously published as a very sensitive and robust method [28-31]. Also the description and accurate evaluation of electrochemical signals was published [32,33]. Electrochemical measurement gives voltammogram with three typical peaks: RS$_2$Co at the potential of -1.30 V, which represents current response of MT complex with components in Brdicka's electrolyte, Cat2 signal (potential about -1.55 V) indicating catalytic signal of hydrogen evolution generated from the supporting electrolyte by the presence of MT. The last mentioned peak is tightly connected with metallothionein concentration within the analyzed sample [34]. Firstly, the treatment agents (CdH, CdL, CdTe and water) were applied on a chorioallantoic membrane of embryo in 16th developmental day (Fig. 1A). After short time exposure (24 hours), the organs were extracted and analyzed. Chosen voltammograms of liver (due to the significant hepatotoxicity of cadmium) are shown in Fig. 1B with inserted details of metallothionein peak. The highest peak at potential of -1.55 V, indicating the highest metallothionein level was detected in CdH group (the highest concentration of applied Cd). In Fig. 1C

are summarized MT levels determined in heart, liver, kidney and brain after application of all treatment agents. The obtained data demonstrated the highest MT level in liver and kidney in comparison to brain and heart. The trend of MT content in tissues after application of different treatment agents was as follows: control < water < CdTe < CdL < CdH. The greatest response was obtained in CdH group in kidney and liver (Fig. 1C). Our data correspond with Sant'Ana's and colleagues results [35], where the most toxic effects after cadmium exposure were found in liver, however kidney functions and cellular immune response were not affected by the cadmium exposure. It should be highlighted that much lower toxicity of CdTe was observed compared to other forms of cadmium. This phenomenon is probably related to the applied Cd form (nanocrystalline particles, which are moreover stabilized with MSA). To verify, whether the effect of nanoparticles on MT level is identical in all analyzed tissues, a combined effect of the tested compound to the tissues were analyzed resulting in proved significance $F_{(12, 60)} = 6.11$, $p < 0.001$ (Fig. 1D). Compared to previous testing, this analysis revealed, that CdH induces significantly higher MT level in liver and kidney tissue comparing to the other treatment agents. In the heart tissue, no significant difference between treatment agents and even between control and water was observed. Statistical statement is described in Table 1 and 2.
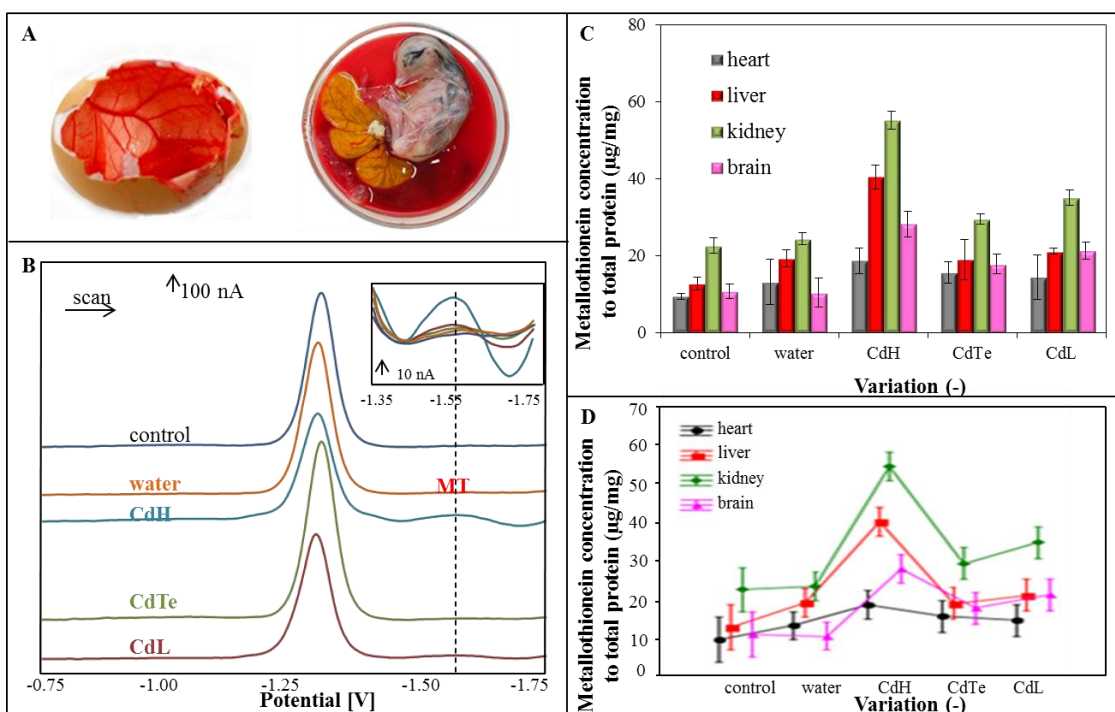

Figure 1. MT content within the analyzed tissue. (**A**) The picture of chorioallantoic membrane, where Cd ions were applied and the picture of embryo in 16th developmental day. (**B**) Electrochemical voltammograms measured for liver in individual groups. Inset: detail of MT peak. (**C**) The level of MT measured in heart, liver, kidney and brain. Error was calculated as a standard deviation. CdH and CdL (application Cd of ions in concentration 4.5 and 1.0 mg/mL), CdTe (1.0 mg/mL of metal in QDs), control is the group without any addition, water group – applied distilled water. (**D**) Stat analysis: the effect of treatment agents on MT level.

Further, the levels of MT were correlated with the amount of cadmium in organs determined by using AAS and the obtained concentrations of Cd (microgram of Cd per gram of tissue) are presented in Table 3. It is obvious that cadmium willingly accumulates mostly in liver and kidney, where highest amount was determined after CdH application (106 µg/g liver tissue and 165 µg/g kidney tissue). Interestingly, also CdTe application caused relatively high accumulation of Cd in organs (68 µg/g liver tissue and 125 µg/g kidney tissue), while much lower toxicity was determined. This phenomenon is probably connected with acute toxicity of free cadmium ions, while in case of CdTe QDs toxicity is decreased due to cadmium form bound within the nanoparticle structure. Such structure is metabolized slowly and release of free cadmium ions is hampered.

Table 1. Univariate results of the ANOVA (Fischer's F and *p* level). Both effects and their combination significantly affect MT concentration.

Effect	Degree of freedom	F	*p*
Tissue	3	68.79	< 0.01
Variation	4	67.75	< 0.01
Tissue*Variation	12	107.54	< 0.01
Error	60	17.59	< 0.01

Table 2. Results of Tukey's post-hoc testing, displayed as homogenous groups. Sorted by mean metallothionein concentration, from lower to higher concentration. Asterisk denote participation to specific "homogenous group", different to each other at *p* < 0.05, e.g. CdH-treated liver metallothionein is significantly higher compared to CdH-treated kidney (asterisks in different columns), conversely, e.g., no significant difference in CdL-treated metallothionein heart and brain (asterisks in same columns).

Tissue	Variation	MT (µg/mg) Mean	1	2	3	4	5	6	7	8
Heart	Control	9.4	*	*						
Brain	Water	10.4	*							
Brain	Control	10.8	*	*	*					
Liver	Control	12.8	*	*	*					
Heart	Water	13.1	*	*						
Heart	CdL	14.5	*	*	*					
Heart	CdTe	15.6	*	*	*					
Brain	CdTe	17.8	*	*	*	*				
Heart	CdH	18.7	*	*	*	*				
Liver	CdTe	19.0	*	*	*	*	*			

Liver	Water	19.2	*	*	*	*	*				
Liver	CdL	21.2		*	*	*	*				
Brain	CdL	21.3		*	*	*	*				
Kidney	Control	22.6	*	*	*	*	*	*			
Kidney	Water	23.6		*	*	*					
Brain	CdH	28.2			*	*	*				
Kidney	CdTe	29.5				*	*				
Kidney	CdL	35.0						*	*		
Liver	CdH	40.4							*		
Kidney	CdH	55.1								*	

Table 3. The level of cadmium in tissue measured by AAS.

Variation	Liver (μg Cd/g sample)	Heart (μg Cd/g sample)	Kidney (μg Cd/g sample)	Brain (μg Cd/g sample)
Control	0.01	0	0	0.2
Water	0.1	0	0	0.4
CdH	106.0	24	165	13.0
CdTe	68.0	9	125	1.0
CdL	55.0	3	65	0.3

The analysis of electrochemical curves was performed using the set of curves measured using liver as a bioaccumulator of heavy metals comparison to heart tissue without detected significant heavy metals accumulation. The main goal of data analysis was to find a pre-processing method that would lead to the definition of a small number of derived attributes characterized achieved voltammograms. The first phase of our analysis was data pre-processing. All curves were measured three times and in ideal case all these three curves had same shape or were only slightly shifted (chosen voltammogram of MT shown in Fig. 2A). If one curve from the triplet had a distinctly different shape or was flat as a consequence of measurement error, it was not included to the evaluation and curve averaging. This was a case few heart curves. The curves for liver were without any problems. The whole set of average curves for liver and heart are depicted in the Fig. 2B and Fig. 2C, respectively. The last step of data pre-processing was discretization of curves by wavelet transformation to 32 intervals. From this moment each curve is represented as 32 values $W_0 \dots W_{31}$, the corresponded areas between value W_x and place on curves are shown in the Fig. 2D.

The analysis of relevant areas which divides data to four groups namely CdH, CdL, CdTe or control was performed by decision tree algorithm [36]. This algorithm recursively construct a tree. It takes a data as input and looking for the best split decision in format $W_x >$ value, *i.e.* the split with the highest information gain is chosen to partition the data into two subsets and the method is called recursively to the newly created subset. The construction stops when the stop conditions are met. In

our case, there are two conditions: the subset contains data only from one group or the subset contains only 4 curves.

The resulting decision tree for liver curves is depicted in the Fig. 3A. It shows that groups named CdH, CdL differ from the rest groups in area W_{12} and W_{17}, which correspond to Co1 and RS$_2$CO area on the Brdicka curve, see Fig. 2A. The group CdTe is surprisingly very similar in shape to control and not to cadmium group. The resulting decision tree for heart curves is shown in the Fig. 3B. The relevant areas are W_0, W_{10} and W_{19}. The difference between control group and Cd groups are in areas W_0 and W_{10} which corresponds to different base line of curves. The difference in area W_{19} corresponds to width of RS$_2$CO area on the Brdicka curve, see Fig. 2A.
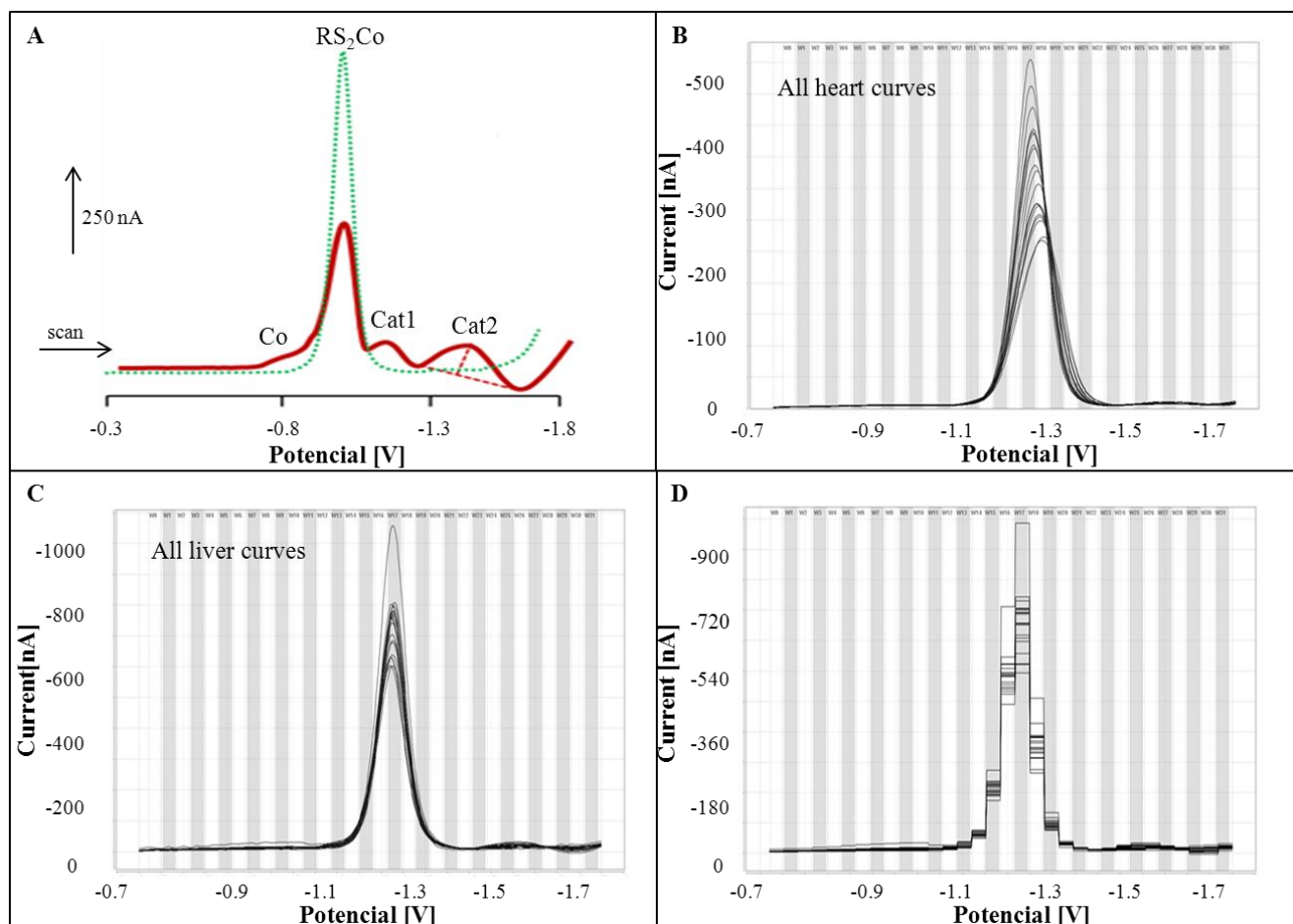

Figure 2. The illustration data for mathematical analysis. (**A**) A typical DPV voltammogram of MT measured in the presence of a supporting electrolyte containing 1 mM Co(NH$_3$)$_6$Cl$_3$ and 1 M NH$_3$(*aq*) and NH$_4$Cl, p*H* = 9.6; dotted line: voltammogram of a supporting electrolyte without MT. During MT analysis four peaks, Co1, RS$_2$Co, Cat1 and Cat2 that correspond to the MT level can be observed. The transformation of DPV voltammogram. (**B**) The whole set of average heart curves for all treatment variations. (**C**) The whole set of average liver curves for all treatment variations. (**D**) The liver curves after discretization to 32 intervals for all treatment variations. The names of intervals are $W_0 – W_{31}$ and position of these intervals is shown in the top of picture
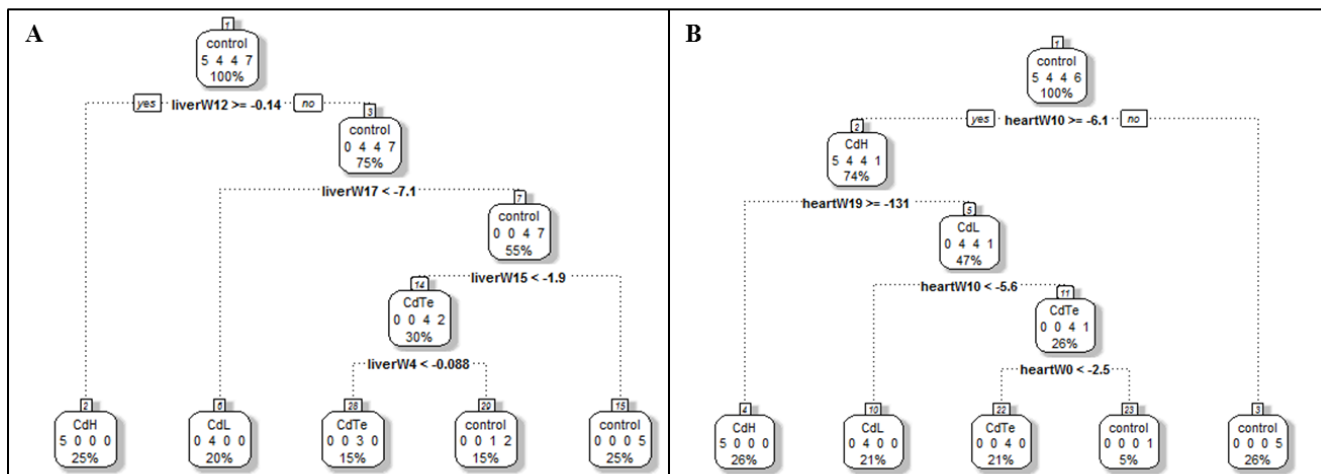
Figure 3. The result of mathematical analysis – decision trees. (**A**) The decision tree is composed of nodes (depicted as squares) and conditions with two branches (depicted as dotted line). The nodes of tree contains three lines of useful information - the name of most represented group in node, number of curves for each group in order CdH, CdL, CdTe and control and the percentage of curves in node. The left branch from condition always means condition is satisfied, the right branch from condition always means condition is not met. The start node is marked by number 1. This node contains all 20 curves; the most represented group is control group. The curves from this node are divided by condition $liverW_{12} >= -0.14$ (the value of interval W_{12} is greater or equal to -0.14 nA). If this condition is satisfied we continue to node marked by number 2, in opposite case if the condition is not met we continue to node marked by number 3. The node marked by number 2 contains all curves from group CdH, therefore we can stop division. The node marked by number 3 contains 75% of all curves, 4 in group CdL, 4 in group CdTe and 7 in control group. These curves are divided by condition $liverW_{17} < -7.1$ (value of interval W_{17} is less than -7.1 nA) etc. The relevant interval where is difference between our four groups are $liverW_4$, $liverW_{12}$, $liverW_{15}$ and $liverW_{17}$. These intervals correspond to Co1 and RS$_2$Co areas on the Brdicka curves; see Figure 2A for more details. (**B**) Division heart curves by decision tree. The relevant interval where is the difference between our four groups are $heartW_0$, $heartW_{10}$, $heartW_{19}$. These intervals correspond to basal level of curves ($heartW_0$, $heartW_{10}$). The interval $heartW_{19}$ corresponds to width of the RS$_2$Co areas; the area is wider in the group CdH.

4. CONCLUSION

It was observed from the current study that supplementation of different concentrations and forms of cadmium led in chicken embryos (16 days old) to higher MT levels after 24 h incubation when compared to control. The level of MT was detected by electrochemical method in four different tissues - liver, kidney, brain and heart. The highest accumulation of cadmium and MT presence was found in liver and kidney, less in heart and brain tissue. The trend in MT level corresponds also with measured content metals detected by AAS. Obtained electrochemical signals were analyzed by mathematical model and the method suitable for characterization of voltammograms was found. The analysis of relevant areas was performed by decision tree algorithm and data were separated to four

groups (CdH, CdL, CdTe or control). The model was able to differentiate the source of toxicity in the individual tissue. MTs seem to be significantly involved in Cd detoxification mechanism.

Conflict of interest:
The authors have declared no conflict of interest.

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