MALDI-TOF MSI and electrochemical detection of metallothionein in chicken liver after cadmium exposure

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Metallomics is an area that studies the interactions of metal ions with proteins and other biomolecules and their function in living organisms. Metallothioneins (MTs) belong to a large family of metalloproteins in this field. As it is known, the presence of Cd^{2+} ions causes higher expression of MTs. Therefore we focused on determination of MT and Cd content in liver from chicken embryos (model organism) by electrochemistry and atomic absorption spectroscopy (AAS) after the exposure of chicken embryos to different concentrations of $Cd(NO_3)_2$. We also determined the spatial distribution of MT in chicken liver tissue slices by matrix assisted laser desorption/ionization time-of-flight mass spectrometry imaging (MALDI-TOF MSI) and evaluated the toxicity of $Cd(NO_3)_2$ to chicken embryos. Finally, we attempted to find the correlation between 2D MSI maps of MT and concentration of MT in chicken liver.

Keywords: metallothionein; Cd toxicity; chicken liver; MALDI MSI

1. Introduction

Metallothioneins (MTs) are intracellular, low--molecular weight, proteins where cysteines form at least one third of all protein amino acids and thiol groups serve as pockets for coordination with divalent metal ions, especially Zn and Cu [1]. Metallothioneins are connected with cancer development, protection of organism against environmental pollution effects and also with chemoresistance of cells. Their main functions include detoxification of heavy metals, maintenance of ion homeostasis and protection against oxidative stress. MTs exist in all kind of mammalian cells. Four isoforms of human MT (MT-1, MT-2, MT-3, MT 4) were found so far [2] and according to UniProt database two chicken MTs (MT1 and MT3) have been reported. The expression of MTs in the liver can be induced by several metals and drugs.

MTs serve as cellular regulators by coordination of essential metals (Zn, Cu) and reducing the toxicity of heavy metals (Cd, Pb) [3].

MTs can be detected electrochemically in Brdicka solution. Here, three characteristic signals are measured for construction of electrochemical fingerprint of MT: RS_2Co (about -1.3 V), Cat1 (-1.4 V) and Cat2 (-1.5 V). The obtained voltammograms are then analyzed by mathematical model [4,5].

Cadmium (Cd), a non-essential heavy metal and a selective toxicant, is accumulated mainly in the kidneys where it causes cellular injury [6]. Cd ions stimulate expression of MTs and toxicity occurs when the concentration of Cd ions exceeds the buffering capacity of intracellular MTs [7]. Cd naturally occurs in the environment as a pollutant from agriculture and industry [8-10]. Cadmium is a known teratogen

and can cause damage to chicken embryos [11]. Cadmium levels can be determined by different methods. In this study, atomic absorption spectroscopy (AAS) method was used as described in our previous work [12].

Matrix assisted laser desorption/ionization time-of-flight mass spectrometry imaging (MALDI TOF MSI) is an imaging technique used for determining spatial distribution of various molecules (biomarkers) in a variety of tissue samples. MALDI is "soft" ionization technique, which does not completely fragment the molecules of analytes and therefore a molecular weight of whole analyte can be measured [13]. MALDI-TOF has been used for detection of cancer biomarkers of different tumors, namely; gastrointestinal tumors, tumors of respiratory system, renal and bladder tumors, prostate, breast and ovarian tumors [14]. In this study we have used MALDI-TOF MSI for imaging MT in chicken liver for the first time.

The purpose of this study was the detection of MT in liver from chicken embryos exposed to different concentrations of cadmium by electrochemistry and MALDI-TOF MSI. Also, we attempted to find some correlation between measured MT concentrations and administered concentrations of cadmium.

2. Results and Discussion

Chicken embryos were divided into control group and five groups exposed to the 100 µl solutions of $Cd(NO_3)_2$ with total cadmium amount: 3.75, 7.5, 15, 30 and 60 µg/egg. A control group was administered with the same volume of pure water. Each group contained ten chicken embryos/fertilized eggs.

Cadmium toxicity was evaluated using Kaplan-Meier survival analysis [15]. It showed (Fig. 1C) that 5 hours after application of $Cd(NO_3)_2$ solutions or pure water into chicken embryos the overall survival of chicken embryos will
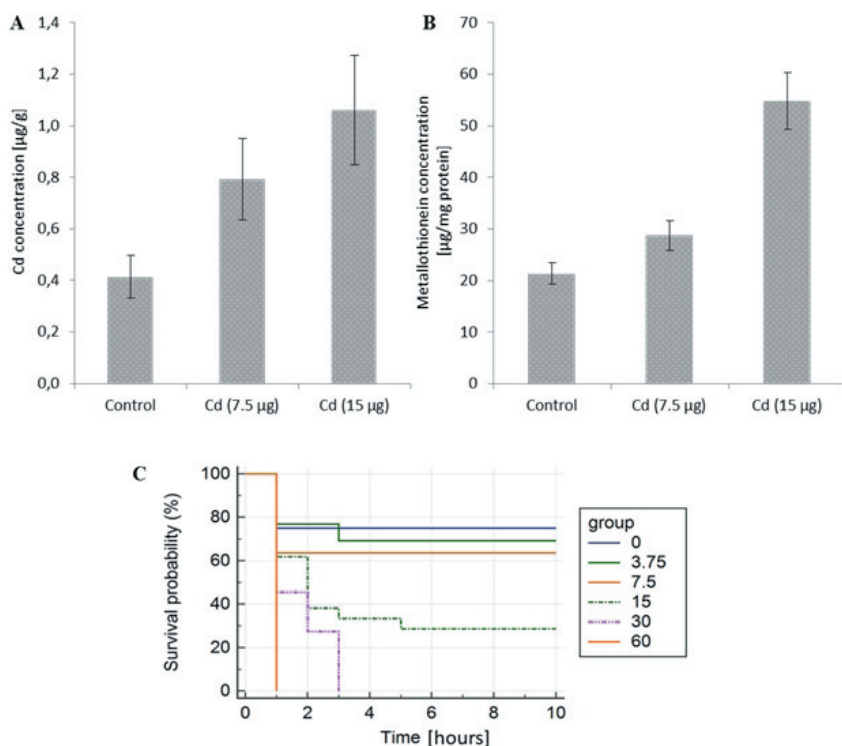

Figure 1: (A) Determination of concentration of Cd relative to the weight of liver from chicken embryos. Cd was measured by AAS (paragraph 3.6.). (B) Determination of concentration of metallothionein (MT) relative to the total protein amount measured in liver from chicken embryos. MT was measured by differential pulse voltammetry (DPV) using Brdicka solution (paragraph 3.4.). (C) Kaplan-Meier survival analysis of chicken embryos after the application of 0, 3.75, 7.5, 15, 30 and 60 µg/egg of cadmium in the form of Cd^{2+} ions – these concentrations are shown as groups with different color according to the legend.

be 0 % for group 60 and 30, cca 30 % for group 15, cca 62 % for group 7.5, cca 70 % for group 3.75 and cca 75 % for control group (group 0). The number of group means total amount of administered Cd in µg/egg. For other analyses were used samples only from groups 15, 7.5 and 0 because chicken embryos from groups 60 and 30 were dead and the difference of group 3.75 from group 0 is not so significant as in the case of group 7.5 and 15.

Liver samples were extracted from chicken embryos in the 17th day of their growth and were further processed for determining cadmium and metallothionein concentrations and total protein content. The AAS analysis of Cd content and differential pulse voltammetric (DPV) analysis of MT content gave similar results (Fig. 1A, 1B). The determined concentrations of Cd in liver samples had the same trend as determined concentrations of MT.

This highlights a fact that MTs are expressed more in the presence of heavy metals and that
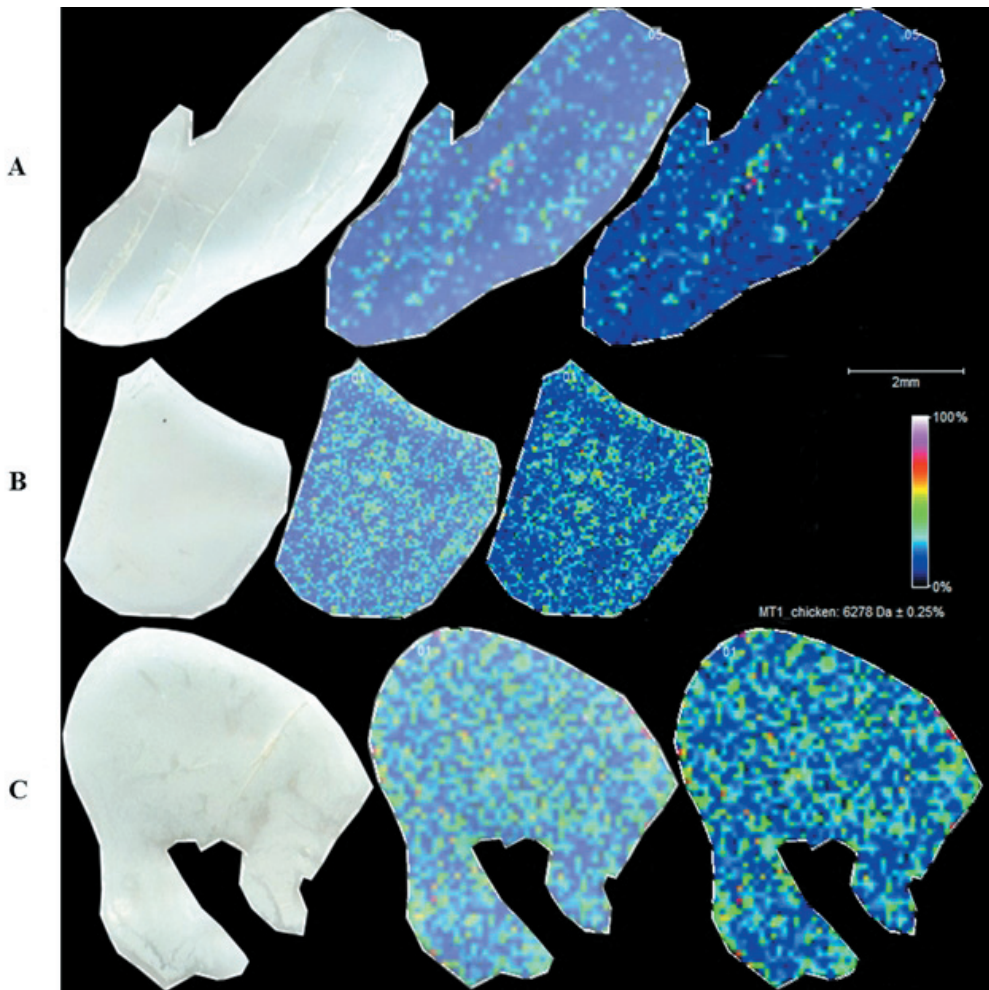
Figure 2. MALDI mass spectrometry images of spatial distribution of chicken metallothionein MT1 (6277 Da) in liver from chicken embryo after exposure to Cd: (A) control sample (0 µg of Cd), (B) 7.5 µg of Cd and (C) 15 µg of Cd. Pictures on the left represent scanned images of liver slices, pictures on the right represent results from MSI, and pictures in the middle represent scanned images of liver slices merged with results from MSI and.

they bind these metals [16,17].

To support these results further a MALDI-TOF mass spectrometry imaging was used to obtain spatial distribution of MT in liver from chicken embryos. Typically, cryo sectioned frozen tissue samples are used for MALDI MSI, but formalin-fixed and paraffin-embedded (FFPE) tissue samples can be used too [18]. In this work, FFPE liver from chicken embryos was chosen because of optimized method of deparaffinization and antigen retrieval [19].

Results from MALDI TOF MSI are shown in Fig. 2. Chicken metallothionein MT1 with molecular weight 6277 Da was detected by MALDI-TOF MSI, and was verified in UniProt database. Liver from chicken embryos exposed to 0 (Fig. 2A), 7.5 (Fig. 2B) and 15 (Fig. 2C) µg/egg of Cd were analyzed. Obtained results indicate that with higher concentration of Cd in liver, higher amounts of chicken MT1 were detected. This was in correlation with results from DPV and AAS analyses. A spatial distribution of MT1 showed that MT1 is uniformly expressed in whole liver tissue and the expression sites cannot be determined precisely. However, these data have shown that MALDI-TOF MSI can be used for determination of MT in FFPE chicken liver tissue which can be used in future experiments.

3. Experimental Section
3.1 Chemicals and material

All chemicals used in this study were purchased from Sigma Aldrich (St. Louis, MO, USA) in ACS purity unless noted otherwise. Acetate buffer of pH 5 was prepared by titrating 0.2 M acetic acid with 0.2 M sodium acetate then diluted with water and used as a supporting electrolyte. The pH was measured using pH meter WTW inoLab (Weilheim, Germany). High purity deionized water (Milli-Q Millipore 18.2 MΩ.cm^{-1}, MA, USA) was used throughout the study.

3.2 Model organism

The fertilized eggs of Lenghorn hen (Integra, a.s., Zabcice, Czech Republic) were incubated at 37 °C and relative humidity of 55% in the incubator (RCom 50 MAX, Gyeongnam, Ko-

rea). The experiments were performed with embryos in 7th developmental day. The toxicity of Cd^{2+} was analyzed after the application of 100 µl of $Cd(NO_3)_2 \cdot 4H_2O$ (37.5, 75, 150, 300 or 600 µg.ml^{-1} Cd^{2+}). This solution was applied through a small hole in a shell on the chorio-allantoic membrane. Chicken embryo controls were exposed to 100 µl of deionized water (n = 5). 17th developmental day, the embryos were removed from the shell and after the extraction of liver, a few samples were used for MALDI-TOF MSI (paragraph 3.7.) and other samples were frozen and kept for other analysis. The samples were stored in -80 °C until assayed.

3.3 Sample preparation for MT electrochemical detection

100 mg of thawed embryo's liver samples were used for MT analysis according to protocol [20]: samples were mixed with 1 ml of 0.1 M phosphate buffer (pH 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 bulk proteins and peptides that interfere in the electrochemical response.

3.4 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, pH = 9.6)) using previous protocol [21]. Differential pulse voltammetric measurements were performed using the 747 VA Stand instrument connected to a 693 VA Processor and 695 Autosampler (Metrohm, Herisau, Switzerland), and a standard reaction cuvette with three electrodes (working electrode was a hanging mercury drop electrode with a drop area of 0.4 mm^2, Ag/AgCl/3M KCl electrode was used as reference electrode and platinum electrode was used as auxiliary electrode) and also a

cooled sample holder and measurement cell to 4 ºC (Julabo F25, Julabo, Seelbach, 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, Eads = 0 V, volume of injected sample 5 µl. Measurements were done in electrochemical cell with total volume of 2 ml (5 µl of sample and 1995 µl Brdicka solution).

3.5 Determination of total protein content

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

3.6 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.

3.7 MALDI-TOF mass spectrometry imaging

Preparation of tissue samples

The extracted embryo's liver samples were paraffinized according to [22]. Then they were cut into 10 µm thin slices using microtome Leica SM2010 R (Baria s.r.o., Prague, Czech Republic) and they were mounted onto ITO (indium-tin oxide) glass slides (Bruker Daltonik GmbH, Bremen, Germany). The conductivity of surface was checked by ohmmeter. Deparaffinization and antigen retrieval were performed according to protocol by Casadonte et al. [19]. Position of tissue slices was marked by at least

three marks by white pencil corrector. Then the glass slides with samples were scanned by Epson Perfection V500 Office (Epson Europe B.V., Amsterdam, Netherlands) with resolution 2400 DPI. MALDI matrix was sprayed onto glass slides with samples by Bruker ImagePrep (Bruker Daltonik GmbH, Bremen, Germany). As MALDI matrix, 2,5-dihydroxybenzoic acid (DHB) (Sigma-Aldrich, St. Louis, MO, USA) was used. DHB was prepared in concentration of 30 mg.ml^{-1} in 50% methanol and 0.2% TFA. MALDI matrix mixtures were thoroughly vortexed and ultrasonicated using Bandelin 152 Sonorex Digital 10P ultrasonic bath (Bandelin electronic GmbH, Berlin, Germany) for 2 minutes at 50 % of intensity at room temperature. The samples were ready for analysis after drying.

Mass spectrometry imaging

The mass spectrometry experiments were performed on a MALDI-TOF mass spectrometer Bruker ultrafleXtreme (Bruker Daltonik GmbH, Bremen, Germany). Data acquisition and processing of mass spectra was performed on flexControl 3.4 and flexAnalysis 2.2 softwares, while analysis of MSI data was done on flexImaging 3.0 software. Firstly, scanned images of tissue slices were loaded into flexImaging 3.0 while MALDI adapter with glass slides was loaded into mass spectrometer. Then, the position of MALDI adapter was changed according to white marks on glass slides in the way, that MALDI adapter was moved in flexControl to a position of white marks and on each mark the position was pointed manually in flexImaging by mouse pointer − thus the position of tissue slices was programmed into the mass spectrometer. Next, regions of acquisition were highlighted by mouse pointer in flexImaging and raster width of approximately 100 µm was chosen. Before MALDI MSI, the measuring method was standardized and mass spectrometer was calibrated on a mixture of peptide and protein calibration standards (Bruker Daltonik GmbH, Bremen, Germany). The laser power was set to 65 %. MALDI MSI was performed in linear positive mode in the m/z range 2−20 kDa. The MS spectra were acquired by averaging 1600 sub spectra from a total of 1600 laser shots

per raster spot. Automatic method of MALDI MSI was then initiated. The mass spectra were automatically loaded into flexAnalysis, where they were processed (baseline substraction was performed), and finally the processed spectra were automatically loaded into flexImaging.

Preparation of MALDI MSI images

Final preparation of MSI images was made in flexImaging by selecting peak of chicken metallothionein 1 (MT1) – the molecular weight of chicken MT1 was chosen according to UniProt database (www.uniprot.org). From a peak molecular weight a mass filter was made in the format "(molecular weight + atomic weight of hydrogen) ± 0.25 %". Finally, images of tissue slices with used mass filters of selected peaks were used for preparation of final MALDI MSI images, which were made in GIMP 2.8 (www.gimp.org).

3.8 Statistical analysis

Data were processed using MICROSOFT EXCEL (Microsoft, WA, USA). The results are expressed as an average ± standard deviation (SD) unless otherwise noted.

4. Conclusions

In our work, cadmium toxicity was connected with expression of metallothionein. With higher amounts of cadmium injected to chicken embryos, higher amounts of metallothionein were detected. This was further supported by MALDI-TOF mass spectrometry imaging of metallothionein in liver from chicken embryos. In liver tissue with higher amount of cadmium, higher metallothionein peak intensities were detected corresponding to higher concentration of metallothionein. To our current knowledge this was the first use of MALDI-TOF MSI for detection of metallothionein from formalin--fixed and paraffin-embedded tissue sample.

Acknowledgments

Conflicts of Interest

The authors declare no conflict of interest.

The authors declare they have no potential conflicts of interests concerning drugs, products, services or another research outputs in this study. The Editorial Board declares that the manuscript met the ICMJE „uniform reguirements" for biomedical papers.

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