Int. J. Electrochem. Sci., 10 **(2015)** 3623 - 3634

International Journal of ELECTROCHEMICAL SCIENCE
www.electrochemsci.org

The Effect of Cadmium Ions and Cadmium Nanoparticles on Chicken Embryos and Evaluation of Organ Accumulation

Renata Kensova[1,2], Iva Blazkova[1,2], Marketa Vaculovicova[1,2], Vedran Milosavljevic[1,2], Lucie Blazkova[1,2], David Hynek[1,2], Pavel Kopel[1,2], Marie Novotna[1,2], Josef Zehnalek[1,2], Miroslav Pohanka[2,3], Libuse Trnkova[2,4] , Vojtech Adam[1,2], Rene Kizek[1, 2]*

[1] Department of Chemistry and Biochemistry, Faculty of Agronomy, Mendel University in Brno, Zemedelska 1, CZ-613 00 Brno, Czech Republic - European Union
[2] Central European Institute of Technology, Brno University of Technology, Technicka 3058/10, CZ-616 00 Brno, Czech Republic - European Union
[3] Karel Englis College, Sujanovo nam, 356/1, CZ-602 00 Brno, Czech Republic – European Union
[4] Department of Chemistry, Faculty of Science, Masaryk University, Kamenice 5, CZ-625 00 Brno, Czech Republic – European Union
[*]E-mail: kizek@sci.muni.cz

Received: 5 January 2015 / *Accepted:* 9 February 2015 / *Published:* 24 February 2015

The presence of metals in the organism has both positive and negative influence on the functions of the body, depending on the concentration. Cadmium is one of the metals that have irrefutable negative effect. It is highly toxic metal with long biological half-life causing damage of kidneys, bones and has carcinogenic effect. The aim of this work was the study of the effect and distribution of the cadmium in the body and its comparison with cadmium nanoparticles – CdTe quantum dots (QDs). Model organism for these experiments was chicken embryo in 16th developmental day. The distribution of cadmium was analyzed after the application of 500 µL of cadmium solution (Cd ions) in concentration 1 mg/mL or 4.5 mg/mL). This solution was applied through the small hole in the egg shell onto the chorioallantoic membrane. After 24 hours of the incubation (37.5 °C, 45% rH) liver, kidney, brain and hearth were extracted. The concentration of cadmium was analyzed after the mineralization of the samples by atomic absorption spectrometry and differential pulse voltammetry. The toxicity of cadmium solution was significantly higher compared to the solution of QDs. Embryos exposed to the cadmium ions exhibited 100% mortality after 24 hours. On the contrary, the mortality of embryos exposed to QDs was 0%. The highest levels of cadmium were detected in the kidneys (125 µg per g) and in the liver (107 µg per g).

Keywords: Cadmium; Quantum Dots; Chicken embryos; Tissue; Distribution; Atomic absorption spectroscopy; Differential pulse voltammetry

1. INTRODUCTION

Cadmium (Cd) is a non-essential heavy metal naturally occurring in the environment as a pollutant emanating from agriculture and industry [1-3]. It has extremely long biological half-life and therefore has been implicated as the cause of severe deleterious effects on wildlife including developmental defects in a variety of vertebrate species [4]. Cadmium is a known teratogen in avian, rodent and embryos of *Xenopus laevis* [5]. The damage caused include facial, eye and ear defects, limb abnormalities, body wall defects, neural tube defects, heart, lung and kidney anomalies, developmental delay, and death. [6]

On the other hand, chicken embryos are a useful model to investigate the development of early stages in both birds as well as mammals, including embryotoxicity influences of dangerous chemicals [7]. Besides the heavy metal ions [8] also the effects of various nanoparticles including copper [9], platinum [10], silver [11] and/or gold [12] on the chicken embryogenesis is investigated to characterize in details the potential toxic effects of these new materials. Special attention is paid to the highly fluorescent nanoparticles – quantum dots [13,14], which are commonly synthesized from Cd ions, which may result in potential *in vitro* toxicity that hampers their practical applications. Even though advances in synthetic and surface ligand chemistry have provided materials with an almost unrivalled photostability in aqueous solution, problems such as the unsuitability of the capping agents, the retention of particles over a certain size, biological magnification, and specifically, the breakdown and decomposition products of these inorganic materials have to be addressed. On the other hand, certain compounds such as zinc [15-18], selenium [19,20] and/or ascorbic acid [21] exhibit the abilities to protect organisms against Cd embryotoxicity.

The aim of this study was to investigate the distribution of cadmium in the chicken embryo after short time exposition to the both solution of Cd ions and solution of CdTe QDs. The metal content was determined by two analytical methods – atomic absorption spectroscopy (AAS) and differential pulse voltammetry (DPV).

2. EXPERIMENTAL PART

2.1 Chemicals and material

All chemicals used in this study were purchased from Sigma Aldrich (St. Louis, MO, USA) in ACS purity unless noted otherwise. Pipetting was performed by pipettes from Eppendorf (Hamburg, Germany). Acetate buffer of pH 5 was prepared with 0.2 M acetic acid and 0.2 M sodium acetate and diluted with water and used as a supporting electrolyte. High purity deionized water (Milli-Q Millipore 18.2 MΩ/cm, MA, USA) was used throughout the study.

2.2. Preparation of deionized water and pH measurement

The deionized water was prepared using reverse osmosis equipment Aqual 25 (Czech Republic). The deionized water was further purified by using apparatus MiliQ Direct QUV equipped

with the UV lamp. The resistance was 18 MΩ. The pH was measured using pH meter (WTW inoLab, Germany). Deionized water was used for dilution, rinsing, washing, and buffer preparation.

2.3. In Vivo distribution of cadmium - preparation of samples

The fertilized eggs of Lankenfeld roosters and ISA Brown hens (Integra, a.s., Zabcice, Czech Republic) were incubated in the incubator RCom 50 MAX (Gimhae, Korea) with temperature (37.5°C) and humidity control (45% rH). After the sixteen days of the incubation the vitality of embryos was checked by digital egg monitoring system Avitronics (Vetronic services, Devon, England) and then the solution of $Cd(NO_3)_2 \cdot 4H_2O$ (1 mg/mL or 4.5 mg/mL in ACS water) or solution of CdTe quantum dots (1 mg/mL of cadmium) was applied (500 µL) by injection Chirana T. injecta (maximal volume: 1 mL, size: 0.33 x 12 mm) through small hole in egg shell into the air cell on the chorioallantoic membrane. After that the hole was covered by the plaster. Chicken embryos were incubated 24 hours and then the liver, heart, kidney and brain were extracted and analyzed. The samples were stored in -80°C until assayed.

2.4. Preparation of QDs

All chemicals were purchased from Sigma-Aldrich and used without further purification. QDs were prepared according to Duan [22] with some modifications. Cadmium (II) acetate $Cd(OAc)_2$ (5 mL; 5.32 g/L) was dissolved in miliQ water (43 mL). Mercaptopropionic acid (MPA) (1 mL; 60 mg/mL) was slowly added to stirred solution. Afterwards, 1.25 mL Na_2TeO_3 (4.432 g/L) was added. $NaBH_4$ (50 mg) was poured into the solution under vigorous stirring. Subsequently the ACS water was added to the final volume of 100 mL, than the solution was pipetted (2 mL) into the vials, which were closed and put into the Microwave Reaction System (Multiwave 3000, Anton Paar, Graz, Austria). Microwave heating conditions: max. 300 W, temperature: 100°C, 10 minutes rising of temperature, 10 minutes continuance and then cooling. Synthetized QDs were stored in dark at 4ºC.

2.5. Microwave digestion for electrochemical and spectrometric determination of cadmium

10 mg of tissue was weighed out into digestion vials. Nitric acid (65 %, *v*/*v*) and hydrogen peroxide (30 %, *v*/*v*) were used as the digestion mixture. Volume of 500 µL of this mixture was used, while the volume ratio between nitric acid and hydrogen peroxide was always 7:3 (350 µL HNO_3 and 150 µL H_2O_2). Samples were digested by Microwave 3000 (Anton Paar, Austria) using rotor MG-65. The program begins and ends with the same ten-minute-long-step, beginning with the power of 50 W and ending with the power 0 W (cooling). Microwave power was set to 100 W in the main part of the programs (30 minutes) at temperature of 140°C (Fig. 1). After mineralization, the samples were diluted as necessary and using electrochemical methods and atomic absorption spectrometry the cadmium content was determined [23-25].

2.6. Sample preparation for electrochemical determination of free cadmium

0.1 g of the tissue was transferred into a test tube and then deep froze by liquid nitrogen to disrupt cells. The frozen tissues were mixed with extraction buffer (100 mM potassium phosphate, pH 7) to a final volume of 1 mL and homogenised using hand-operated homogenizer ULTRA-TURRAX T8 (IKA, Königswinter, Germany) placed in an ice bath for 3 min at 25 000 rpm. The homogenate was centrifuged at 10 000 g for 15 min and at 4°C (Eppendorf 5402, Hamburg, Germany). The resulting supernatant was used for analysis of free cadmium.

2.7. Electrochemical determination of cadmium

Determination of cadmium by differential pulse voltammetry were performed with 797 VA Computrace instrument connected to 813 Compact Autosampler (Metrohm, Switzerland), using a standard cell with three electrodes. The three electrode system consisted of a hanging mercury drop electrode with a drop area of 0.4 mm^2 as the working electrode, an Ag/AgCl/3 M KCl reference electrode and platinum as the auxiliary electrode. 797 VA Computrace software by Metrohm CH was employed for data processing. The analyzed samples were deoxygenated prior to measurements by purging with argon (99.999%). Acetate buffer (0.2 M CH_3COONa and 0.2 M CH_3COOH, pH 5) was used as a supporting electrolyte. The supporting electrolyte was replaced after each analysis. The parameters of the measurement were as follows: purging time 90 s, deposition potential -1.15 V, accumulation time 240 s, equilibration time 5 s, modulation time 0.057 s, interval time 0.04 s, initial potential of -1.3 V, end potential 0.2 V, step potential 0.005 V, modulation amplitude 0.025 V, volume of injected sample: 15 μL, volume of measurement cell 2 ml (15 μL of sample and 1985 μL acetate buffer) [26].

2.8. Atomic absorption spectrometry (AAS)

Cadmium was determined on 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 (1 g/L) chemical modifier.

2.9. Mathematical treatment of data and estimation of detection limits

Data were processed using MICROSOFT EXCEL (Microsoft, WA, USA). The results are expressed as an average ± standard deviation (SD) unless otherwise noted. The detection limits (3 signal/noise, S/N) were calculated to Long and Winefordner [27], whereas N was expressed as a

standard deviation of noise determined in the signal domain unless otherwise stated. AAS was compared with DPV by means of linear regression analysis. Possible systematic errors were revealed by tests of intercept and slope of the regression line. The intercept and slope were tested for significant difference against 0 and 1, respectively, by Student's t-test. The intercept was tested for significant difference against 0, which relates to a systematic constant error, while the slope was tested for significant difference against 1, which relates to systematic proportional error. When the intercept and slope are equaled to 0 and 1, respectively, the methods give same results. Significant error of intercept from 0 indicates systematic constant error. In case of significant error of slope from 1 indicates systematic proportional error.

3. RESULTS AND DISCUSSION

Twenty chicken embryos were divided into four groups of five and exposed to the cadmium. Two groups were exposed to Cd ions (2.25 mg and 0.5 mg) and third group was exposed to the solution of CdTe QDs (0.5 mg of Cd ions). Simultaneously, a control group was administered the same volume of pure water. The tested solution was applied on the chorioallantoic membrane and after 24-hour exposition the embryos were taken out from the shell and selected organs were extracted, mineralization was performed and the cadmium content was determined electrochemically and by AAS. The scheme of the work flow is shown in Fig. 1.

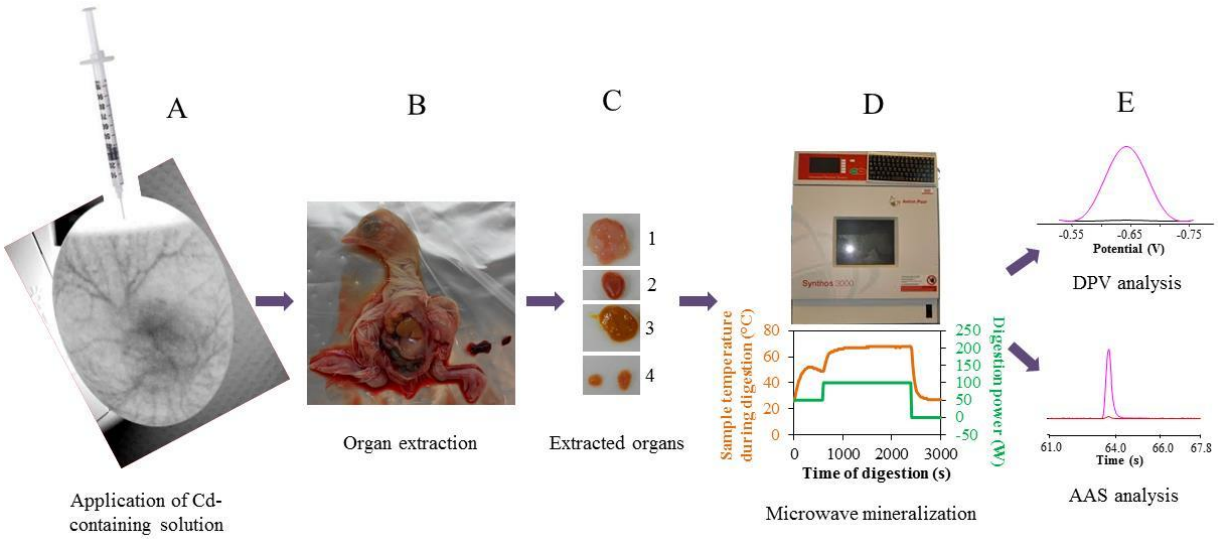

Figure 1. Workflow diagram of the experiment. **(A)** Application of Cd-containing solution, **(B)** Organ extraction, **(C)** Extracted organs – 1: brain, 2: heart, 3: liver, 4: kidneys, **(D)** tissue mineralization, microwave time program (for conditions see *Experimental part*), **(E)** determination of Cd content by DPV and AAS (for measurement parameters see *Experimental part*).

The lethal dose LD_{50} of Cd presented by Dzugan et al. [28] (3.9 μg/egg) is significantly lower compared to the dose administered in this study (2 250 μg/egg and 500 μg/egg in the form of Cd ions and 500 μg/egg in the form of QDs). Therefore the embryos exposed to the solution of free Cd ions (both concentrations) led to the 100% mortality. The survival analysis by Kaplan-Meier of chicken embryos after the application of 500 μg/egg of cadmium in the form of Cd ions and QDs is presented in Fig. 2. On the contrary, the mortality of embryos exposed to QDs was 0%. This result has to be verified on the larger group of embryos; however this was not the aim of this study. Obtained results however suggest, that the bioavailability of Cd in the QDs is significantly lower compared to free Cd ions and therefore the toxicity is lowered probably due to the crystalic structure of QDs and due to the capping of the CdTe core with the MPA. Generally the higher doses of Cd tolerated compared to the literature [14,28] may be caused due to the exposition of relatively well-developed embryos (16^{th} developmental day) with more powerful defense mechanisms in comparison to early-stage embryos. And the way of application also influenced the distribution; the distribution of Cd ions from chorioallantoic membrane can be more regulated than direct application of Cd ions into the egg yolk [29].

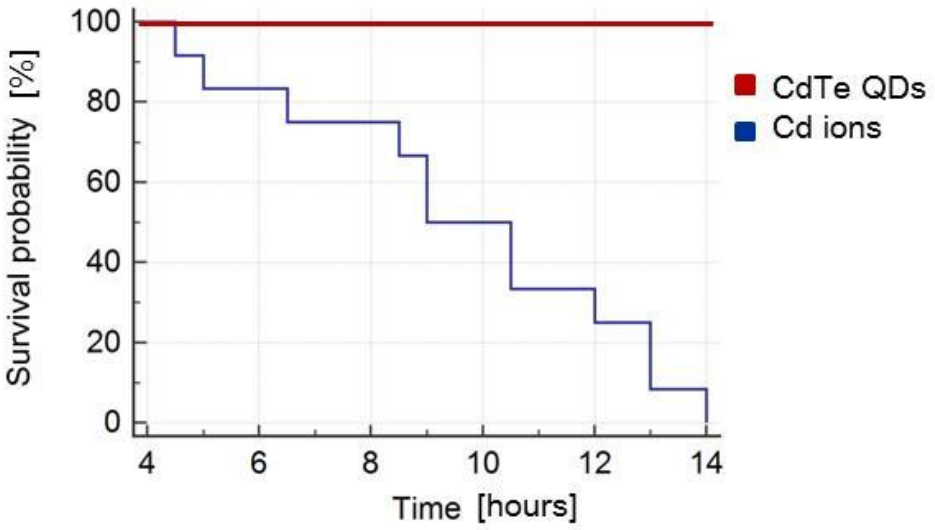

Figure 2. Kaplan-Meier survival analysis of chicken embryos after the application of 500 μg/egg of cadmium in the form of Cd ions and CdTe QDs.

Analytical methods selected for analysis of the organ distribution of cadmium, AAS and DPV, are standard analytical techniques for metal ions determination [30-32]. To demonstrate their suitability for the purpose of this study, standard analytical figures of merit were determined and are summarized in Tab. 1. Even though the AAS can reach lower limits of detection (0.1 μg/L of Cd ions) the electrochemical determination by DPV exhibit linear response up to higher concentrations of the metal (linear range: 1-100 μg/L). The calibration solutions were dissolved in the mineralization mixture to take in to account its potential influences on the determination. The interceptions (constant systematic error) of both calibration curves were evaluated as statistically insignificant on a confidence

level alpha = 0.05 and the determination coefficients R^2 were determined as 0.9995 and 0.9979 for DPV and AAS, respectively (Fig. 3A,B).

Table 1. Figures of merit of AAS and DPV

Substance	Regression equation	Linear dynamic range (µM)	Linear dynamic range (µg/L)	R^2	LOD (µM)	LOD (µg/L)	LOQ (µM)	LOQ (µg/L)
Cd - DPV	y = 0.8182x	0.01 – 0.9	1. - 100	0.9995	0.004	0.4	0.01	1
Cd - AAS	y = 0.0204x	0.001 - 0.2	0.1 - 20	0.9979	0.0003	0.03	0.001	0.1

limits of detection - 3 S/N), limits of quantification - 10 S/N

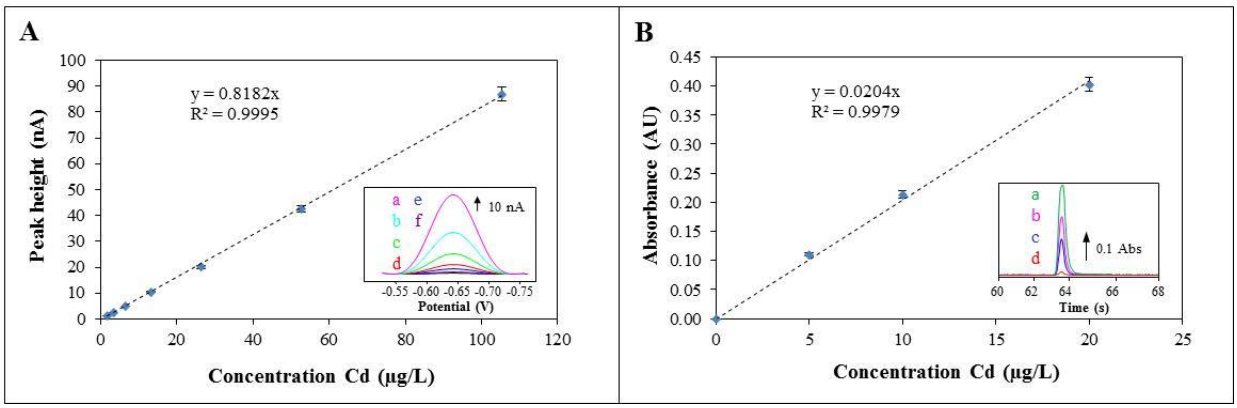

Figure 3. Calibration curves of cadmium determined by **(A)** DPV method. 0.2 M acetate buffer (pH = 5) was used as supporting electrolyte. The parameters were chosen as follows: initial potential -1.3 V, end potential 0.2 V, deposition potential -1.15 V, accumulation time 240 s, pulse amplitude 25 mV, pulse time 0.04 s, voltage step 5.035 mV, voltage step time 0.3 s, sweep rate 0.0168 V/s. Characteristic peak for cadmium was at potential -0.645 V. Different concentrations of cadmium were used: a) 105 µg/L, b) 52.5 µg/L, c) 26.3 µg/L, d) 13.1 µg/L, e) 6.6 µg/L, f) 3.3 µg/L. **(B)** Calibration curves of cadmium determined by AAS. Parameters of the method were as follows: lamp current 4 mA, wavelength 228.8 nm, spectral bandwidth 0.5 nm, sample volume 20 µl. Different concentrations of cadmium were used: a) 20 µg/L, b) 10 µg/L, c) 5 µg/L, d) 2.5 µg/L.

The results for each organ are summarized in Fig. 4. As shown, the lowest amount of Cd was determined in the brain (Fig. 4A), followed by heart (Fig. 4B), liver (Fig 4C) and kidneys (Fig. 4D). As expected, in cases of all analyzed organs the 0.5 mg of cadmium ions led to the lower amount of Cd determined by both DPV and AAS compared to the solution containing 2.25 mg of Cd. Our accumulation results are in accordance to study by Marettova et al., where the highest concentrations of Cd ions were also determined in kidneys and liver. They observed this accumulation in the cocks and hens exposed to the Cd ions and as well as in organs of chicks hatched from Cd-treated hens [33].

Interestingly, application of the solution of QDs containing 0.5 mg of Cd resulted in lower amounts of Cd determined in all organs with exception of kidneys compared to the solution of Cd ions at the same concentration. This can lead to the conclusion that in brain, heart and liver the QDs are less accumulated compared to free Cd ions and therefore are less toxic for these organs. Another explanation can be seen in the size of QDs which are not transported through the haematoencephalic barrier. In case of kidneys, the amount of Cd determined after application of QDs was at the same level compared to the solution of 0.5 mg free Cd(II) ions. However, it has to be noted that the standard deviations of both determination were very high and therefore the comparison between QDs and Cd solution is inconclusive.

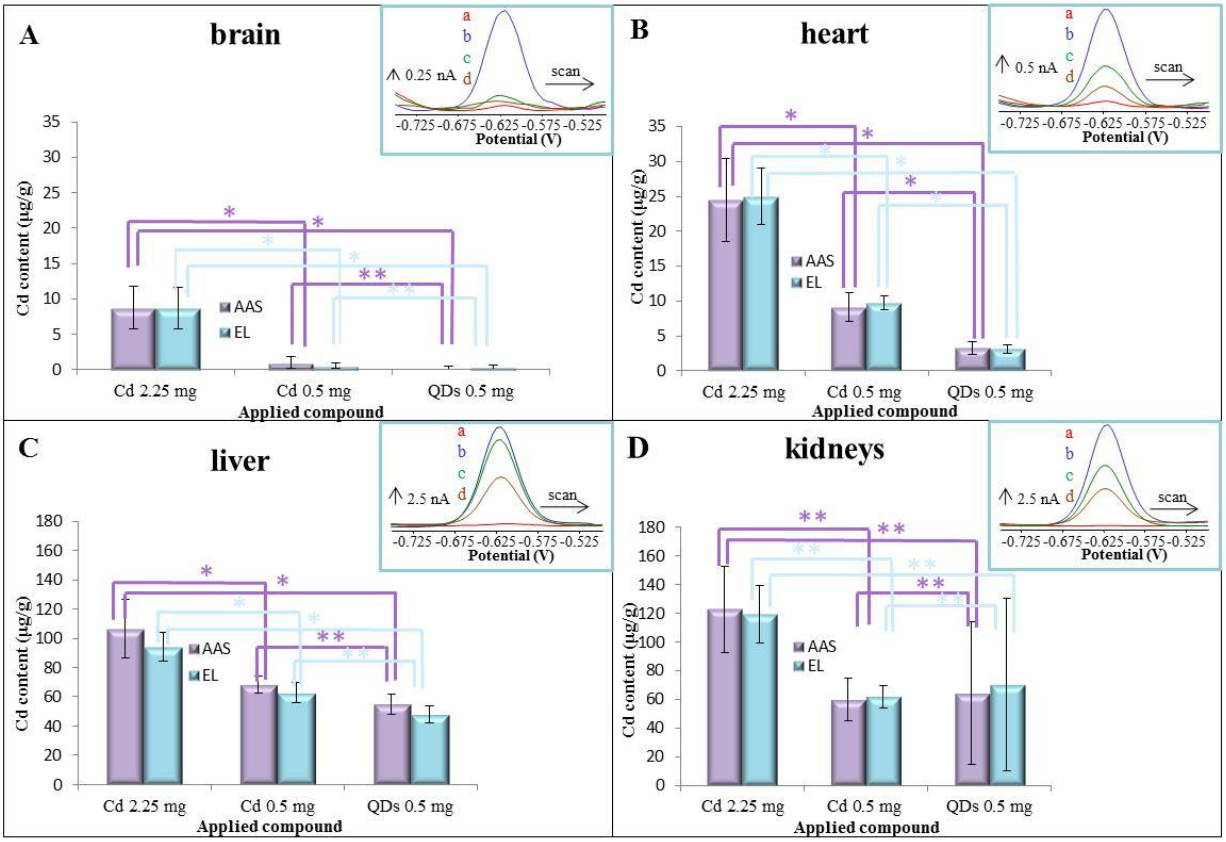

Figure 4. Cadmium distribution in analyzed organs (mineralized samples). **(A)** brain, **(B)** heart, **(C)** liver, and **(D)** kidneys determined by AAS and DPV after 24-hour exposure to the Cd (2.25 mg and 0.5 mg) and CdTe QDs (0.5 mg Cd). Inserted pictures show an electrochemical signal of cadmium applied compounds in various tissues analyzed (a) control, b) Cd 2.25 mg, c) Cd 0.5 mg, d) QDs 0.5 mg). The asterisk indicates that *p* is less than 0.05, and the double asterisk denotes that *p* is less than 0.005. Statistical significance between groups was assessed by the paired Student's t-test (*n*=3).

The correlation between AAS and DPV is shown in Fig. 5. No significant differences between AAS and DPV were found based on Youden plots of AAS versus DPV in case of all organs. The

interception (constant systematic error) and the difference of the slope from 1 (systematic proportional error) were evaluated as statistically insignificant on a confidence level alpha = 0.05.

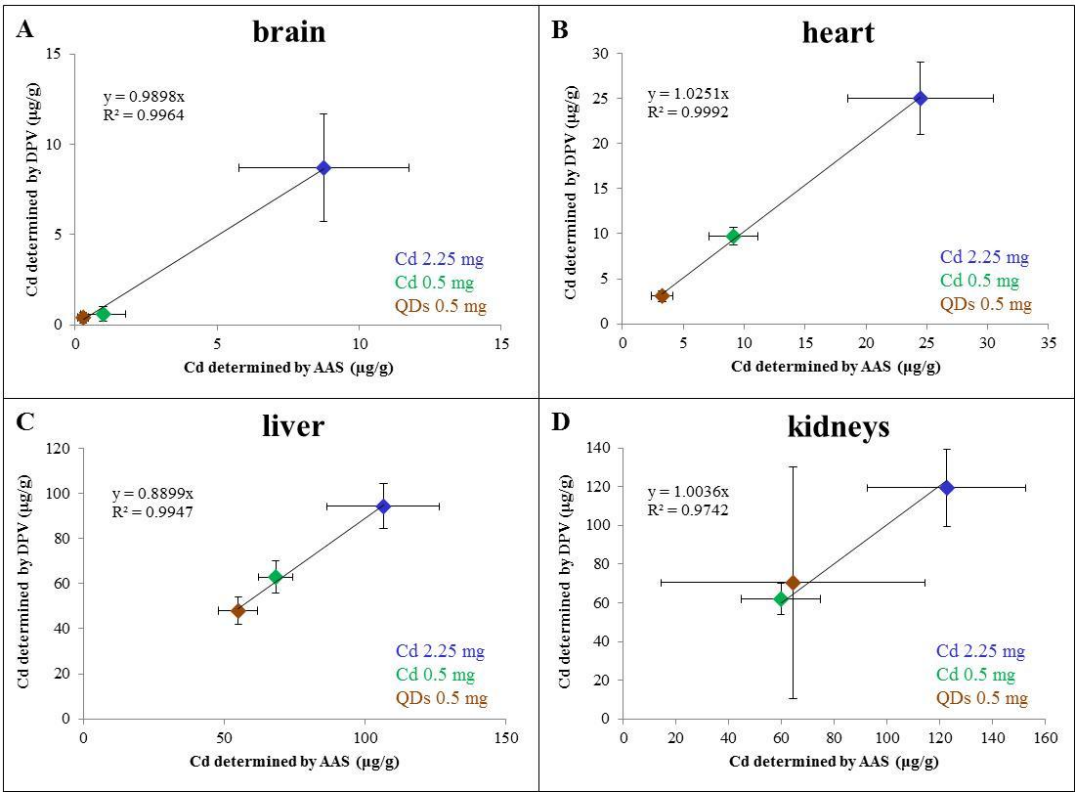

Figure 5. Youden plots representing the correlation between AAS and DPV results for each particular organ: **(A)** brain, **(B)** heart, **(C)** liver, and **(D)** kidneys.

The concentration of free cadmium in individual organs was also analyzed (Fig. 6). The metal was extracted just by homogenization of the tissue in phosphate buffer followed by centrifugation (without mineralization of the solid portion of the tissue). It has to be noted that these results are taking to the account the matrix effect. The concentration of cadmium determined in the supernatant was significantly lower in all organs compared to the mineralized samples. This suggests that most of the metal is transported into the cells and interacts with intracellular components and therefore mineralization is needed to extract the total metal content. The lowest concentration was detected in brain (Fig. 6A), similarly as in case of mineralized samples which means that the metal did not reach this tissue due to the haematoencephalic barrier. In case of heart (Fig. 6B) the highest concentration of metal was determined after exposition to Cd ions (2.25 mg) followed by Cd ions (0.5 mg) and QDs. The significantly higher signal of QDs compared to Cd ions was observed in liver tissue (Fig. 6C) which can be explained by weaker interaction of QDs with the tissue and therefor the nanoparticles are released more easily compared to Cd ions. The lowest metal content obtained without the mineralization was determined in kidneys (Fig. 6D), which is in agreement with the fact that the

maximum of the metal was determined in the mineralized tissue (Fig. 5D) and apparently the mineralization process is required to release the metal from the tissue.

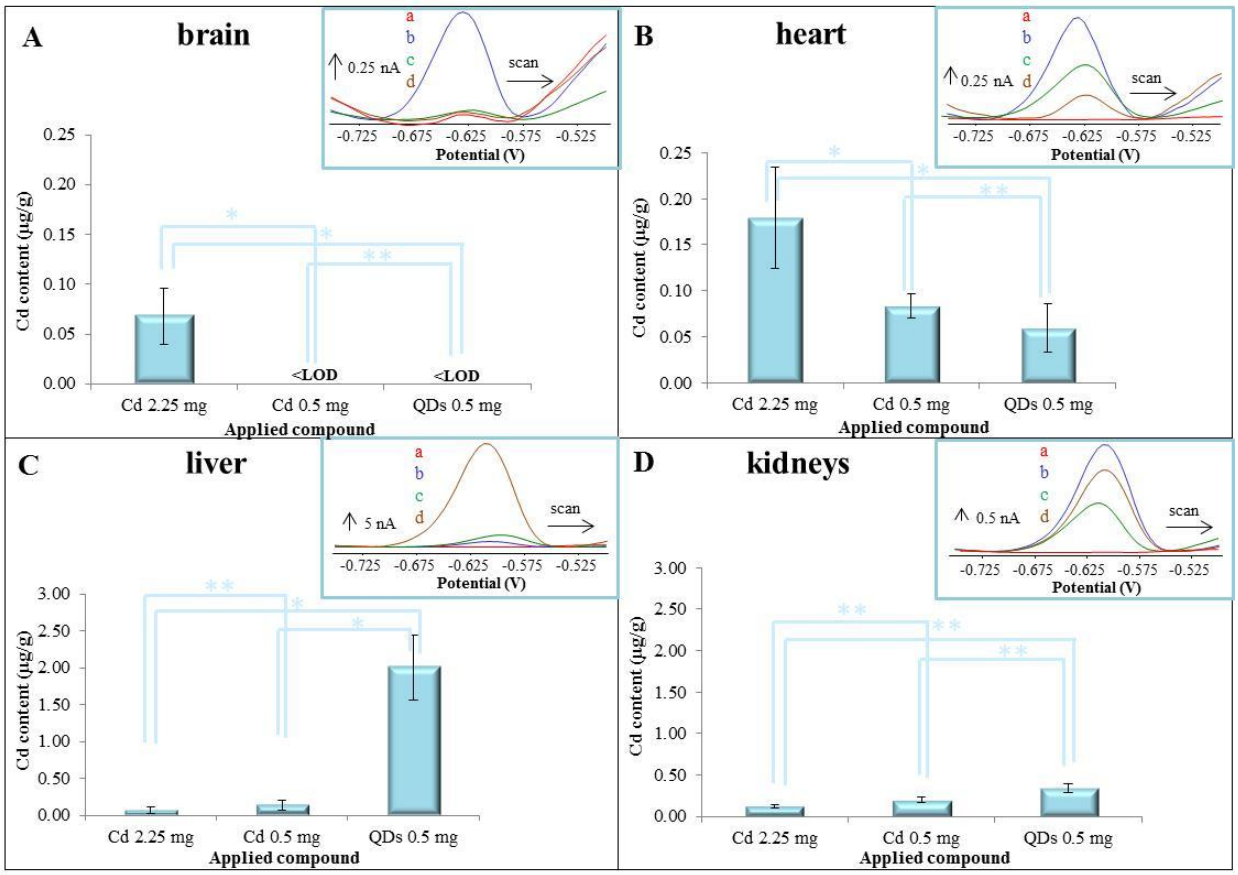

Figure 6. Cd distribution in analyzed organs obtained by homogenization and centrifugation (without mineralization). **(A)** Brain, **(B)** heart, **(C)** liver, and **(D)** kidneys determined by DPV after 24-hour exposure to the Cd (2.25 mg and 0.5 mg) and CdTe QDs (0.5 mg Cd). Inserted pictures show an electrochemical signal of cadmium applied compounds in various tissues analyzed (a) control, (b) Cd 2.25 mg, (c) Cd 0.5 mg, (d) QDs 0.5 mg. The asterisk indicates that *p* is less than 0.05, and the double asterisk denotes that p is less than 0.005. Statistical significance between groups was assessed by the paired Student's t-test (*n*=3).

4. CONCLUSIONS

From the presented results can be concluded that even a short-time exposition (24 hours) to cadmium led to the accumulation of the metal in the tested organs. As expected liver and kidneys were the most affected as they primary task is the detoxification of the organism. Moreover, it was found out that the exposure to the QDs solution led to the lower acute toxicity compared to the free Cd ions of the same concentration.

ACKNOWLEDGEMENTS
Financial support from NANOLABSYS CZ.1.07/2.3.00/20.0148 is highly acknowledged. Moreover, the authors wish to express their thanks to Tomas Vaculovic for consultations in the area of statistical analysis.

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

References

1. O. Krystofova, L. Trnkova, V. Adam, J. Zehnalek, J. Hubalek, P. Babula and R. Kizek, *Sensors*, 10 (2010) 5308.
2. V. Supalkova, J. Petrek, J. Baloun, V. Adam, K. Bartusek, L. Trnkova, M. Beklova, V. Diopan, L. Havel and R. Kizek, *Sensors*, 7 (2007) 743.
3. J. Kovarova, R. Kizek, V. Adam, D. Harustiakova, O. Celechovska and Z. Svobodova, *Sensors*, 9 (2009) 4789.
4. P. K. Chan and S. H. Cheng, *Arch. Toxicol*., 77 (2003) 69.
5. J. Thompson and J. Bannigan, *Reprod. Toxicol*., 25 (2008) 304.
6. J. Thompson, E. Hipwell, H. V. Loo and J. Bannigan, *Reprod. Toxicol.*, 20 (2005) 539.
7. F. Y. Yamamoto, F. F. Neto, P. F. Freitas, C. A. O. Ribeiro and C. F. Ortolani-Machado, *Environ. Toxicol. Pharmacol*., 34 (2012) 548.
8. S. H. Gilani and Y. Alibai, *Anat. Rec.*, 211 (1985) A68.
9. L. Pineda, E. Sawosz, K. P. Vadalasetty and A. Chwalibog, *Anim. Feed Sci. Technol*., 186 (2013) 125.
10. M. Prasek, E. Sawosz, S. Jaworski, M. Grodzik, T. Ostaszewska, M. Kamaszewski, M. Wierzbicki and A. Chwalibog, *Nanoscale Res. Lett*., 8 (2013) 1.
11. J. Sikorska, M. Szmidt, E. Sawosz, T. Niemiec, M. Grodzik and A. Chwalibog, *J. Anim. Feed Sci*., 19 (2010) 286.
12. M. Zielinska, E. Sawosz, M. Grodzik, M. Balcerak, M. Wierzbicki, J. Skomial, F. Sawosz and A. Chwalibog, *Arch. Anim. Nutr*., 66 (2012) 1.
13. A. A. Clancy, Y. Gregoriou, K. Yaehne and D. T. Cramb, *Chem. Phys. Lett.*, 488 (2010) 99.
14. P. Rodriguez-Fragoso, J. Reyes-Esparza, A. Leon-Buitimea and L. Rodriguez-Fragoso, *J. Nanobiotechnol.*, 10 (2012) 1.
15. M. Dzugan, M. W. Lis, M. Droba and J. W. Niedziolka, *J. Environ. Sci. Health Part A-Toxic/Hazard. Subst. Environ. Eng.*, 47 (2012) 1288.
16. J. Thompson and J. Bannigan, *Teratology*, 64 (2001) 87.
17. P. deForest and S. Chopin, *Dev. Biol*., 186 (1997) B166.
18. P. A. deForest and S. F. Chopin, *Faseb J*., 11 (1997) 2608.
19. W. Zhao, W. Liu, X. Chen, Y. Zhu, Z. Zhang, H. Yao and S. Xu, *Biol. Trace Elem. Res*., 161 (2014) 328.
20. I. Saidi, Y. Chtourou and W. Djebali, *J. Plant Physiol*., 171 (2014) 85.
21. B. V. Somashekharaiah and A. R. K. Prasad, *Ind J Med Res Sec B*, 94 (1991) 387.
22. J. L. Duan, L. X. Song and J. H. Zhan, *Nano Res*., 2 (2009) 61.
23. D. Hynek, J. Prasek, J. Pikula, V. Adam, P. Hajkova, L. Krejcova, L. Trnkova, J. Sochor, M. Pohanka, J. Hubalek, M. Beklova, R. Vrba and R. Kizek, *Int. J. Electrochem. Sci*., 6 (2011) 5980.
24. A. Kleckerova, P. Sobrova, O. Krystofova, J. Sochor, O. Zitka, P. Babula, V. Adam, H. Docekalova and R. Kizek, *Int. J. Electrochem. Sci*., 6 (2011) 6011.
25. P. Majzlik, A. Strasky, V. Adam, M. Nemec, L. Trnkova, J. Zehnalek, J. Hubalek, I. Provaznik and R. Kizek, *Int. J. Electrochem. Sc*i., 6 (2011) 2171.
26. R. Kensova, D. Hynek, J. Kynicky, M. Konecna, T. Eckschlager, V. Adam, J. Hubalek and R. Kizek, *Int. J. Electrochem. Sci*., 9 (2014) 4675.
27. G. L. Long and J. D. Winefordner, *Anal. Chem*., 55 (1983) A712.
28. M. Dzugan, M. Lis, M. Droba and J. W. Niedziółka, *Acta Vet Hung*, 59 (2011) 337.

29. M. S. Rahman, M. Mochizuki and M. Mori, *J. Poult. Sci.*, 46 (2009) 334.
30. M. Strouhal, R. Kizek, J. Vacek, L. Trnkova and M. Nemec, *Bioelectrochemistry*, 60 (2003) 29.
31. J. Kovarova, O. Celechovska, R. Kizek, V. Adam, D. Harustiakova and Z. Svobodova, *Neuroendocrinol. Lett*., 30 (2009) 169.
32. J. Kaiser, M. Galiova, K. Novotny, R. Cervenka, L. Reale, J. Novotny, M. Liska, O. Samek, V. Kanicky, A. Hrdlicka, K. Stejskal, V. Adam and R. Kizek, *Spectroc. Acta Pt. B-Atom. Spectr*., 64 (2009) 67.
33. E. Marettova, M. Maretta, J. Legath and E. Kosutzka, *Biol. Trace Elem. Res*., 147 (2012) 130.