UNIVERSITY OF PARDUBICE

FACULTY OF CHEMICAL TECHNOLOGY

Department of Biological and Biochemical Sciences

Jiří Handl

Nephrotoxicity Testing *in vitro*

Theses of the Doctoral Dissertation

Pardubice 2020

Study program: **Analytical chemistry**

Study field: **Analytical chemistry**

Author: **Mgr. Jiří Handl**

Supervisor: **doc. RNDr. Tomáš Roušar, Ph.D.**

Year of the defence: **2020**

References

HANDL, Jiří. *Nephrotoxicity Testing in vitro*. Pardubice, 2020. 129 pages. Dissertation thesis (PhD.). University of Pardubice, Faculty of Chemical Technology, Department of Biological and Biochemical Sciences. Supervisor: doc. RNDr. Tomáš Roušar, Ph.D.

Abstract

The doctoral thesis deals with *in vitro* testing of nephrotoxic effects of substances in immortalized human proximal tubular cells HK-2. The basic aim of the thesis was to introduce and optimize the cultivation of HK-2 cells under different culture conditions, to characterize the cultivation and growth stability of HK-2 cells and last but not least to characterize the nephrotoxic effect of cadmium in HK-2 cell line using bioanalytical and biochemical methods. The introduction summarizes the basic rules and conditions for long-term cultivation of cell lines. Furthermore, HK-2 cells and their importance in nephrotoxicity testing are characterized in detail. The thesis also mentions methods for monitoring toxic effects of substances with focus on measuring cell viability and oxidative stress. The outcomes of the thesis are characterizing nephrotoxic effects of $CdCl_2$ in HK-2 cells. The results part describes obtained findings and the discussios follows.

Abstrakt

Disertační práce pojednává o charakterizaci nefrotoxického působení látek *in vitro* u imortalizovaných lidských proximálních tubulárních buněk HK-2. Základním cílem práce bylo zavést a optimalizovat kultivaci HK-2 buněk za různých kultivačních podmínek, charakterizovat kultivační a růstovou stabilitu HK-2 buněk a v neposlední řadě charakterizovat nefrotoxické působení kadmia u HK-2 buněčné linie pomocí bioanalytických a biochemických metod. V úvodní části práce jsou shrnuta základní pravidla a podmínky pro dlouhodobou kultivaci buněčných linií. Dále jsou podrobně charakterizovány buňky HK-2 a jejich význam v testování nefrotoxického působení. V práci jsou také zmíněny způsoby monitorování a analýzy toxického působení látek se zaměřením na stanovení buněčné viability a míry oxidačního stresu.
Ve výsledkové části disertační práce jsou uvedeny výsledky získané při testování nefrotoxického působení $CdCl_2$ u HK-2 buněk. V závěrečné části práce jsou pak naměřené výsledky shrnuty a diskutovány.

Keywords

Nephrotoxicity; *In vitro* cultivation; HK-2 cell line; Cell viability; Cadmium.

Klíčová slova

Nefrotoxicita; *in vitro* kultivace; HK-2 buněčná linie; buněčná viabilita; kadmium.

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1 Introduction

1.1 *In vitro* cell cutlivation

Although *in vivo* studies likely provide the most valuable preclinical results for testing the efficacy and applicability of chemical compounds (Bhatia et al., 2016; Palumbo and Pellegrini, 2017), the basic mechanisms and acting principles of both medicinal and toxic agents can be clearly described by well-defined and controlled *in vitro* studies (Schmidt et al., 1998). *In vitro* cell models including primary cell cultures and cell lines are used in cytotoxicity screening (Tong et al., 2017; Xia et al., 2013). Use of the cell lines is very popular because of their unlimited growth potential. Cell lines can be isolated from tumor tissues or can be prepared by spontaneous or induced immortalization of cell cultures (Boukamp et al., 1988; Soule et al., 1990; Zeng et al., 2018). While working with cell lines, it is necessary to follow basic cultivation techniques and elementary principles. Passage number is one of the important factors that informs about the cell line's age and that is essential for evaluating a cell line's growth integrity (Clynes, 1998; Freshney, 2005; Kwist et al., 2015). It is well established that cell phenotype can change during the process of passaging, and this may affect reproducibility of the results from *in vitro* experiments (Hughes et al., 2007; Reeves et al., 2018; Vasilevsky et al., 2013). Most studies assume that the number of passages in a cell line does not affect its phenotype and therefore the passage number is not often mentioned. Some results have shown, that some cell lines exhibit genetic heterogeneity and instability (Ben-David et al., 2018).

1.2 *Cell lines and nephrotoxicity testing*

The kidneys constitute a very common target of toxic agents (Boogaard et al., 1990). The nephrotoxic effect of a compound can be monitored using several *in vitro* models. There are a number of animal and human cell lines that can be used to assess nephrotoxic properties (Vrbová et al., 2016). The animal cell lines include JTC-12, LLC-PK1, MDCK, and NRK-52E cells (De Larco and Todaro, 1978; Gaush et al., 1966; Hull et al., 1976; Takuwa and Ogata, 1985; Thomasina et al., 1990). The human cell lines are HEK293, IP-15, and HK-2 cells (Graham et al., 1977; L'Azou et al., 2006; Ryan et al., 1994). The human proximal tubular HK-2 (Human Kidney-2) cell line is one of the immortalized cell lines. The HK-2 cell line was originally prepared by transduction with human papilloma virus 16 (HPV-16) E6/E7 genes (Ryan et al., 1994). HK-2 cells grow in a monolayer and are suited to studying proximal tubular toxicity of a variety of compounds (Racusen et al., 1997). The main advantage of HK-2 cells is that they retain the basic morphological and functional properties of proximal tubular epithelial cells (Ryan et al., 1994). Therefore, HK-2 cells have been used in a number of recent studies focused on estimating proximal tubular injury (Du et al., 2010; Gao et al., 2013; García-Pastor et al., 2019; Schmidt et al., 2019; Wu et al., 2009; Yang et al., 2019b; Zaza et al., 2015). Only a limited number of studies have addressed the possibility that characteristic properties of cell lines could change during the passaging process. Those studies focusing on this issue have shown that biological characteristics can be changed not only in stem cell cultures (Kwist et al., 2015) but also in transfected cell lines (O'Driscoll et al., 2006) and definitely in tumor cell lines (Jin et al., 2017; Roberts et al., 2018; Zeng et al., 2018). Despite the rising number of studies using HK-

2 cells, there unfortunately have been no previous studies focused on characterizing potential changes in susceptibility to toxic compounds associated with the passaging of HK-2 cells. Therefore, one of the goals of our study, was to evaluate the functional stability of HK-2 cells after treatment with toxic compounds under conditions of continual cultivation.

1.3 HK-2 cells and cadmium toxicity

Cadmium is a widely occurring, highly toxic heavy metal. It can be toxic even at low concentrations (Tobwala et al., 2014). The toxic effect of cadmium is most commonly detected in kidney, liver, and neuronal cells (Linhartová et al., 2016; Wang et al., 2007). In addition, the toxicity can be found in bone and blood cells (Fahim et al., 2012; Fongsupa et al., 2015; Klaassen et al., 2009; Li et al., 2016; Madden et al., 2002; Zhang et al., 2007). Cadmium (i.e. cadmium ion) causes both acute and chronic toxic effects in the organism. These effects are mostly linked with induction of oxidative stress (Thévenod and Friedmann, 1999; Tobwala et al., 2014). Therefore, Cd is able significantly to decrease the levels of glutathione (GSH), a major intracellular nonprotein thiol (López et al., 2006; Zahir et al., 1999). In addition, some reports have indicated that low Cd concentrations induce mutations through DNA oxidative damage and by diminishing the genetic stability of cells (Valverde et al., 2001). These events increase the probability of mutations and, consequently, initiation of tumor growth (Filipič, 2012).

The HK-2 cells also have been used for testing nephrotoxicity of heavy metals, including Cd (Shrestha et al., 2017; Wilmes et al., 2011). Acute exposure of HK-2 cells to Cd leads to apoptosis of those cells (Mao et al., 2007; Shrestha et al., 2017), as Cd induces the expression and activation of pro-apoptotic proteins, including caspases (Huang et al., 2017). A number of studies have reported that Cd can induce both apoptotic and necrotic cell death (Kondo et al., 2012). Necrosis and apoptosis are linked with lipid peroxidation and increased reactive oxygen species (ROS) production induced by Cd (López et al., 2006). The reports have proven that higher ROS production induces phosphorylation of C-Jun-N-terminal kinase (JNK) in human renal proximal tubular cells (Fongsupa et al., 2015). All these processes can lead to decrease of cell viability and even to cell death.

2 Experimental Part

2.1 Aim of Ph.D study

The main aim of the study was to estimate the effect of continuous passaging on the susceptibility of HK-2 cells to model nephrotoxic compounds. Furthermore, our goal was to use an established HK-2 cell line to study the nephrotoxicity of cadmium.

2.2 Characterization and cultivation of HK-2 cells

Human kidney (HK-2) cells, a proximal tubular epithelial cell line derived from normal adult human kidney cells immortalized by transduction with human papillomavirus (HPV 16) DNA fragment (Ryan et al., 1994), were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured according to a published protocol (Hauschke et al., 2017) in supplemented Dulbecco's modified Eagle's medium (DMEM/F12 = 1:1) with 5% (v/v) fetal bovine serum, 1 mM pyruvate, 10 μg/ml insulin, 5.5 μg/ml transferrin, 5 ng/ml sodium selenite, 50 μg/ml penicillin, 50 μg/ml streptomycin, and 5 ng/ml epidermal growth factor. DMEM/F-12 (with or without phenol red), insulin, transferrin, and sodium selenite were purchased from Sigma-Aldrich (USA). Fetal bovine serum, pyruvate, penicillin, streptomycin, and epidermal growth factor were purchased from Invitrogen-Gibco (USA). Cells were maintained at 37 °C in a sterile humidified atmosphere of 5% CO_2 and the medium was replaced every 3–4 days. The cells were removed by adding trypsin–EDTA and passaged when they reached 75–85% confluence. After every 7 days, cells were passaged, counted, and then the optimal amount (4×10^5 cells) was seeded into 10 ml of cultivation medium on a new 75 cm^2 culture vessel. All experiments were conducted using the HK-2 cells between passages 3 and 15. Microscopic phase contrast pictures were obtained using an Eclipse-Ts2R inverted microscope (Nikon, Japan). The HK-2 cells were tested for mycoplasma contamination using the MycoAlert Mycoplasma Detection Kit (Lonza). All cells used in the experiments were mycoplasma free.

2.3 HK-2 cells – short tandem repeat analysis

Short tandem repeat (STR) analysis (i.e., DNA fingerprinting) is a standard method for cell line authentication. The isolation of genomic DNA from HK-2 cells in passages 3, 8, and 15 was performed using a commercial kit (Generi Biotech a.g., Czech Republic). STR analysis was conducted by Generi Biotech, Ltd. (Czech Republic). Seventeen selected autosomal polymorphisms (CSF1PO, D13S317, D16S539, D18S51, D19S433, D21S11, D2S1338, D3S1358, D5S818, D7S820, D8S1179, FGA, Penta D, Penta E, TH01, TPOX, and vWA) and amelogenin were amplified in a multiplex polymerase chain reaction and separated by capillary electrophoresis. Authentication of the cell line was then completed by comparison to established STR databases.

2.4 HK-2 cells – cell diameter and doubling time

Mean diameter of the HK-2 cells was monitored in each passage. Mean diameter was evaluated using the CASY model TT cell counter and analyzer (Roche, Germany) during the counting of cultured cells prior to transfer of cells into a new culture vessel.

The principle of the measurement is based upon electrical current exclusion. Another characteristic used for characterizing HK-2 cell growth was doubling time (i.e., the time needed to double the cell population). This is calculated from the number of seeded cells, number of viable cells, and time elapsed from cell seeding into the culture vessel until their passaging and counting.

2.5 HK-2 cells – treatment with toxic compounds

HK-2 cells were seeded into 96-well plates at density 3×10^4 cells/well in 100 µl of culture medium. After 24 h, the supernatants were aspirated by pipette and discarded. Then, 100 µl of medium containing the tested compounds was pipetted into 96-well plates to the cultured cells. To induce cell impairment, the HK-2 cells were treated with 10 mM acetaminophen, 100 µM cisplatin, 50 µM *tert*butylhydroperoxide, or 100 µM $CdCl_2$. Cells were treated for 6 and 24 h. The WST-1 test (Roche, Germany) and glutathione assay were then used for characterizing cell damage.

2.5.1 Intracellular dehydrogenase activity

Dehydrogenase activity was evaluated using the WST-1 test. This test measures the activity of intra- and extramitochondrial dehydrogenases. After treatment, the WST-1 reagent was added to the cultured cells (1:10 final dilution). The cells were incubated in a gassed atmosphere (5% CO_2) for 1 h and the absorbance change (0–1 h) was measured spectrophotometrically at wavelength 440 nm using a Tecan Infinite M200 plate reader (Tecan, Austria). The dehydrogenase activity was expressed as the percentage intra- and extramitochondrial dehydrogenases activity relative to that in control cells (=100 %).

2.5.2 Glutathione assay

Glutathione (GSH) levels were measured using an optimized bimane assay (Čapek et al., 2017). The cells were incubated in cell medium (100 µl) on 96-well plates with selected toxic compounds for an appropriate time. After incubation, 20 µl of the bimane solution was added to cells and the measurement was started. The final concentration of monochlorobimane in each well was 40 µM. The fluorescence (Ex/Em = 394/490 nm) was measured for 20 min using a Tecan Infinite M200 fluorescence reader incubated at 37 °C. The fluorescence was expressed as the slope of change in fluorescence over time. The GSH levels were expressed as the percentage relative to those in control cells (=100 %).

2.6 $CdCl_2$ nephrotoxicity testing

The HK-2 cell line was used to test the nephrotoxic effect of $CdCl_2$. Cells were cultivated in DMEM/F12 culture medium with/without phenol red. HK-2 cells were cultured under the same conditions as described in the section *2.2*. All the experiments were performed between 4 and 11 passages. $CdCl_2$ treatment followed after 24 h of seeding into 96-well plates at density of 3×10^4 cells/well and exposure medium containing 0-1 mM $CdCl_2$. The cells were incubated for specific periods of 2, 6, 10, 24 and 48 h. Dehydrogenase activity and intracellular glutathione concentration were

determined in $CdCl_2$ treated HK-2 cells by the WST-1 assay and MCB method. The WST-1 and MCB assay protocols are summarized in the chapters *2.5.1* and *2.5.2*.

2.7 Statistical analysis

All experiments were repeated two or three times independently. The HK-2 stability experiments were performed over a course of 8 months. All values were measured at least in quadruplicate during an experiment. The results on cell viability and GSH levels are expressed as means ± SD. A statistical analysis (p=0.05) estimated influence of cell passage number on cell toxicity as determined using cell viability and GSH assay. That evaluation was by two-way analysis of variance (two-way ANOVA; factor A = passage number; factor B = toxin) and followed by Bonferroni correction (OriginPro 9.0.0, USA).

Experiments associated with $CdCl_2$ nephrotoxicity testing were repeated at least three times independently. All values were measured at least in duplicate. The results are expressed as mean ± SD. Statistical significance was analyzed after normality testing using one-way analysis of variance (ANOVA) followed by Bonferroni correction (OriginPro 9.0.0, USA). In comparing results with control cells without cadmium treatment, the significance level was set at p=0.05 or lower (* p<0.05; ** p<0.01; *** p<0.001)

3 Results

3.1 HK-2 cells – short tandem repeat analysis

We tested the behavior of HK-2 cells among passages 3–15. That range was selected based upon frequently used numbers of passages as found in the literature. To confirm the origin of cells, we analyzed the short tandem repeats in passages 3, 8, and 15. The STR analysis proved 100% conformity of HK-2 cells with the reference standard in all tested passages and also confirmed no shift of STR sequences in the HK-2 cells.

3.2 HK-2 cells – cell diameter and doubling time

Mean data for other cultivation characteristics of the HK-2 cells during passaging (i.e., cellular diameter and doubling time) are presents in Table 1. Mean diameters of viable HK-2 cells were in the range 17.4–18.6 μm. The mean diameter of HK-2 cells calculated across all passages was 18.2 μm. No significant relationship was found between mean cellular diameter and passage number. Doubling time was expressed as mean doubling time of cells in each passage. The values ranged between 47.3 h and 61.7 h (Table 1).

Table 1: Mean diameter and doubling time of the HK-2 cells (passages 3-15, standard deviation <1 %).

HK-2 cell line		
Passage Number	**Mean Diameter [μm]**	**Doubling Time [h]**
3	18.0	57.0
4	18.4	51.4
5	18.4	52.2
6	18.4	61.7
7	18.2	51.1
8	18.3	60.3
9	18.1	53.6
10	18.5	56.2
11	18.6	54.5
12	18.2	51.6
13	18.2	50.2
14	17.7	47.3
15	17.4	51.2

The mean doubling time across all passages was 53.7 h, and we observed no significant relationship between doubling time and cell passage number. In addition, using phase contrast microscopy at passages 3, 7, 12, and 15 (Fig. 1), we confirmed similar morphology and size of HK-2 cells across all passages.

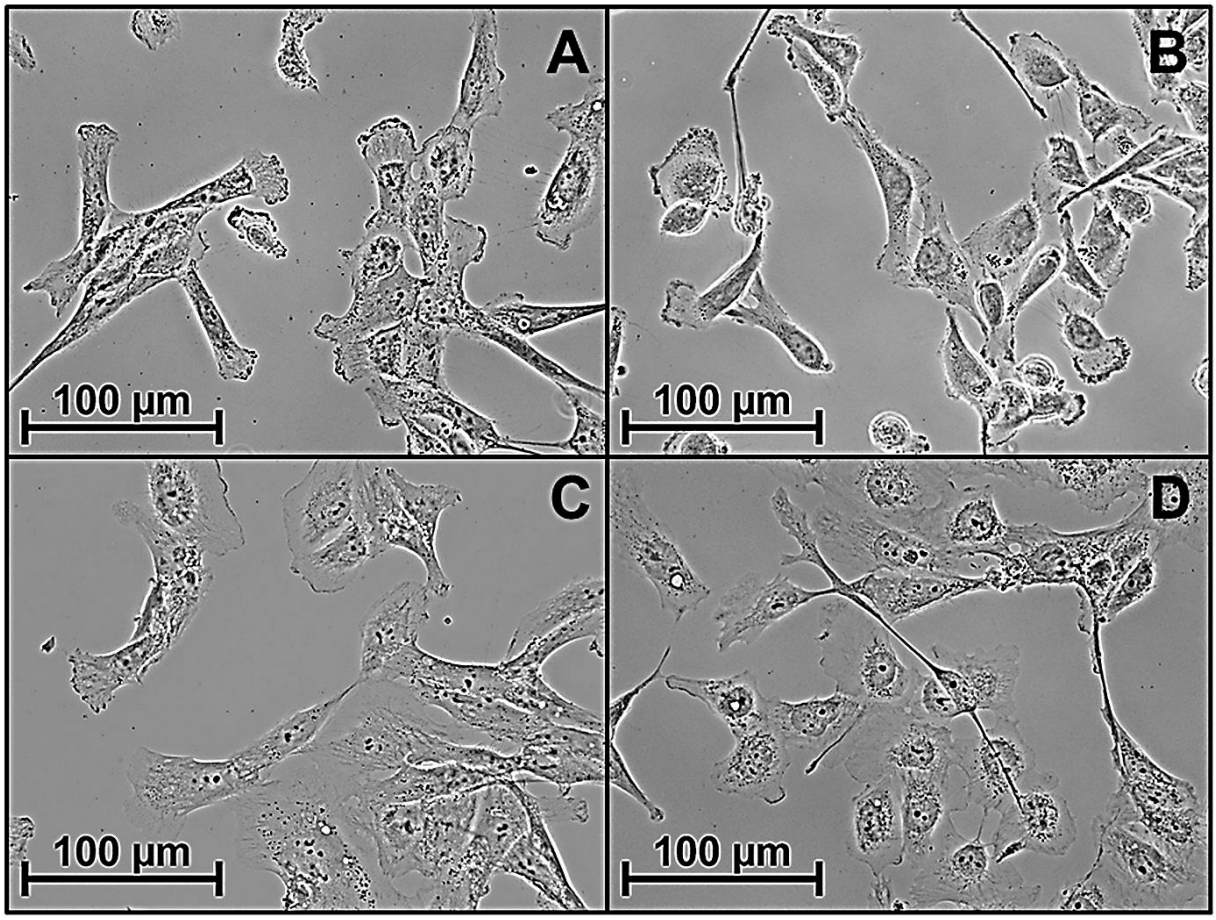

Fig. 1. Human kidney cells (HK-2). HK-2 cells were cultured in FBS supplemented Dulbecco's modified Eagle's medium for passages 3–15 (**A**, passage 3; **B**, passage 7; **C**, passage 12; **D**, passage 15; Phase contrast, 200×).

3.3 HK-2 cells – treatment with toxic compounds

In addition to estimation of the functional parameters in untreated HK-2 cells, we estimated the effect of continuous passaging on the susceptibility of HK-2 cells to model nephrotoxic compounds. To induce the toxicity, we used toxic substances differing in their mechanisms of toxicity, including *tert*-butylhydroperoxide (tBHP) to induce oxidative stress; two drugs, acetaminophen (APAP) and cisplatin (CisPt); and $CdCl_2$ as a heavy metal. Tested concentrations of the compounds were chosen according to previous toxicological studies relating to cisplatin (Genc et al., 2014; Huang et al., 2015; Kim et al., 2014), cadmium (Fujiki et al., 2013; Handl et al., 2019; Kim et al., 2014), acetaminophen (Wu et al., 2009), and tBHP (Hauschke et al., 2017).

HK-2 cells in passages 3, 6, 9, 12 and 15 were treated with toxic compounds for 6 and 24 h. Indeed, we determined the significant effect of a number of passages on decrease of dehydrogenase activity and the extent of glutathione depletion according to Two-way ANOVA test after 6 and 24 h. After 6 h of treatment, the intracellular dehydrogenase activity showed significant decrease in comparison with control cells of the corresponding passage number in toxic compounds, especially in $CdCl_2$ treated cells (Fig. 2A). Based on the WST-1 test results, we found also a significant relation of the number of passage and the extent of decreasing cell viability in HK-2 cells treated with

a toxic compound *(p<0.001)*. In addition to the glutathione reduction caused by 10 mM APAP, 100 μM CisPt, and 100 μM $CdCl_2$ in comparison with controls of the corresponding passage number, the effect of passaging on the extent of glutathione depletion in toxic compounds was found *(p<0.001)* (Fig. 2B).

After 24 h of incubation, deepening of toxic acting of tested compounds was found. In addition, the outcomes of the WST-1 test *(p<0.001)* and glutathione assay *(p<0.001)* also showed a significant effect of the number of passage on the estimated level of toxicity. In Fig. 3 (A, B), the increasing cell impairment was especially found in CisPt and APAP treated HK-2 cells.

According to determined decrease of cell viability and glutathione depletion in HK-2 cells, the extent of cell damage reported in present study is fully comparable with the outcomes and toxin concentrations reported in a number of other studies on APAP (Ruan et al., 2019; Zhang et al., 2007), CisPt (Oh et al., 2017; Yang et al., 2019a), and $CdCl_2$ (Fujiki et al., 2019; Ge et al., 2018; Chou et al., 2019; Kim et al., 2014). On the other hand, our findings on significance of the relation of cell damage and number passage is not allowed to discuss with comparable reports on the HK-2 cell line. Some studies focusing on this issue have shown that biological characteristics can be changed not only in stem cell cultures (Kwist et al., 2015) but also in transfected cell lines (O'Driscoll et al., 2006) and definitely in tumor cell lines (Bušek et al., 2008; Jin et al., 2017; Roberts et al., 2018; Zeng et al., 2018) which outcomes can be supported by our study.

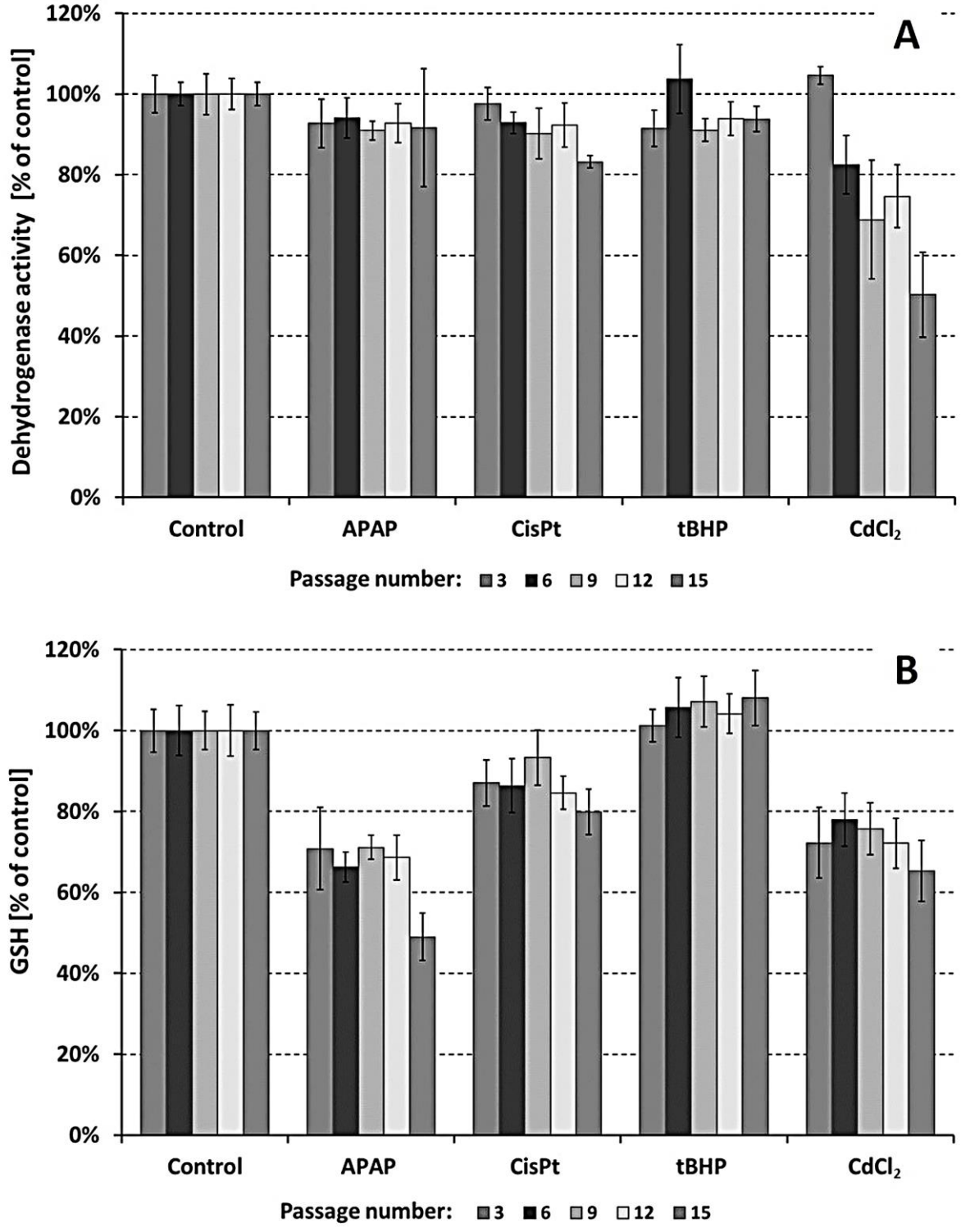

Fig. 2. Estimation of cell impairment in HK-2 cells after 6 h of treatment during repeated passaging. Acetaminophen (APAP, 10 mM), cisplatin (CisPt, 100 μM), *tert*-butylhydroperoxide (tBHP, 50 μM), and $CdCl_2$ (Cd, 100 μM). **A)** Intracellular dehydrogenase activity in HK-2 cells in passages 3-15 was determined using the WST-1 test. **B)** Intracellular GSH levels of HK-2 cells in each of passages 3–15 were determined using monochlorobimane assay. Results are expressed as means ± SD (control = 100%; n = 8–12; 3 independent experiments).

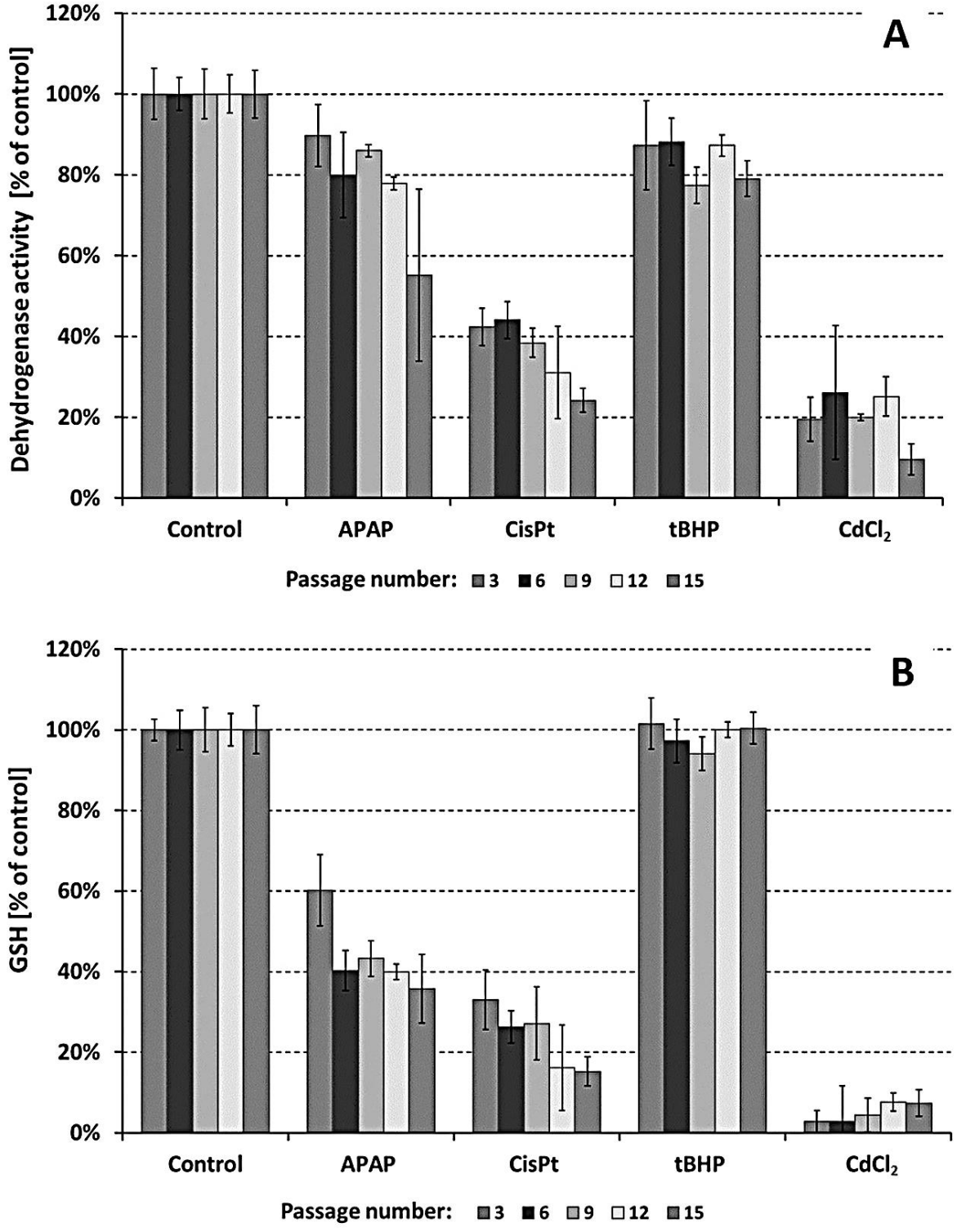

Fig. 3. Estimation of cell impairment in HK-2 cells after 24 h of treatment during repeated passaging. Acetaminophen (APAP, 10 mM), cisplatin (CisPt, 100 μM), *tert*-butylhydroperoxide (tBHP, 50 μM), and $CdCl_2$ (Cd, 100 μM). **A)** Intracellular dehydrogenase activity in HK-2 cells in each of passages 3-15 was determined using the WST-1 test. **B)** Intracellular GSH levels of HK-2 cells in passages 3–15 were determined using monochlorobimane assay. Results are expressed as means ± SD (control = 100%; n = 8–12; 3 independent experiments).

3.4 $CdCl_2$ nephrotoxicity – dehydrogenase activity

Based on the results reported in our previous study (Hauschke et al., 2017), we aimed to characterize the toxic effect of Cd across a broad range of $CdCl_2$ concentrations. The HK-2 cells were treated with $CdCl_2$ (0 μM-1 mM) for 6 and 24 h, and intracellular dehydrogenase activity was measured using the WST-1 test. After 6 h, we detected in cells treated with 200 μM and 1 mM $CdCl_2$ a significant reduction of cellular dehydrogenase activity to 19±2 % (p<0.001) and 80±8 % (p<0.001), respectively, in comparison with controls (=100 %). On the other hand, significant increase in cell viability was found in cells treated with 25 μM (124±8 %; p<0.001), 50 μM (169±7 %; p<0.001), and 100 μM $CdCl_2$ (152±9 %; p<0.001) in comparison to control cells (Fig. 4).

After 24 h, the significant decrease in cell viability was found in cells treated with 100, 200, and 1,000 μM $CdCl_2$ (Fig. 5). The viability of HK-2 cells treated with 25 μM and 50 μM $CdCl_2$ increased significantly to 128±18 % (p<0.001) and 163±9 % (p<0.001), respectively, in comparison with controls.

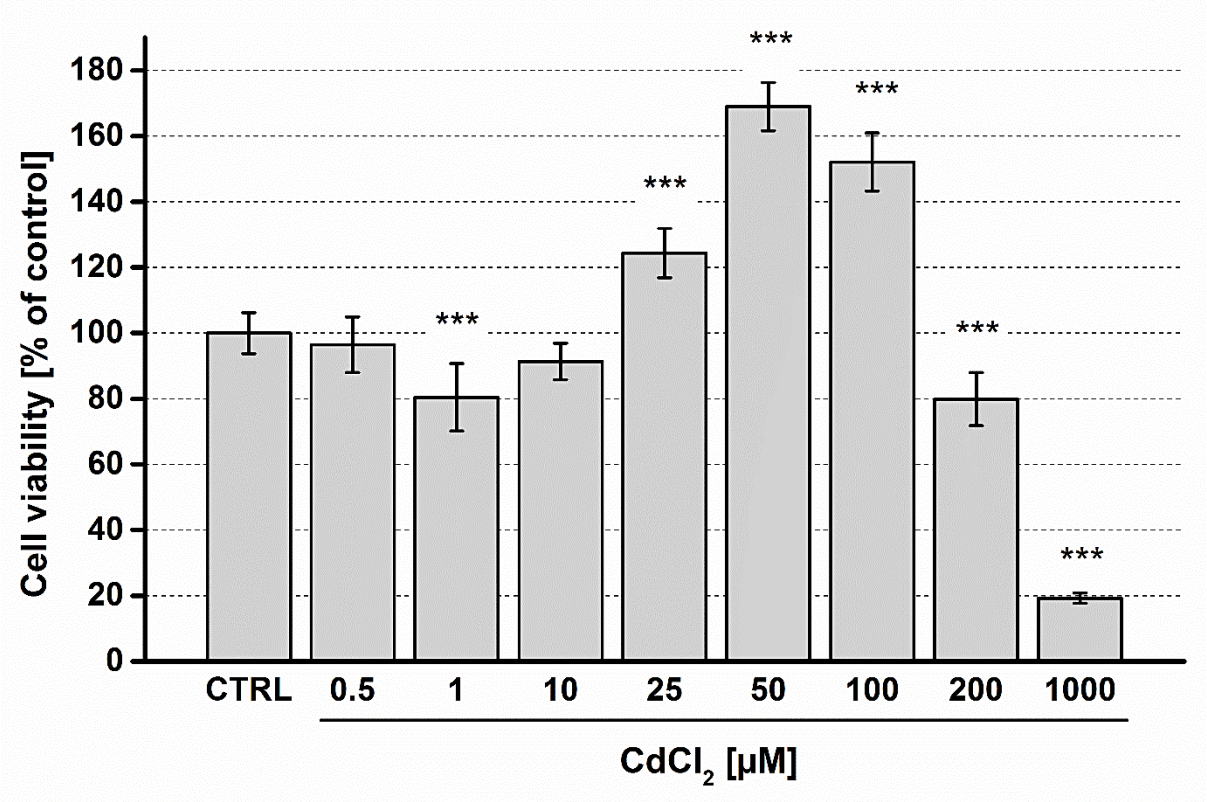

Fig. 4. Dehydrogenase activity measurement – HK-2 cells were assayed using the WST-1 test after 6 h of treatment with $CdCl_2$ at concentrations 0-1,000 μM. The results are expressed as mean ± SD (control=100 %; n=6-10). *** p<0.001 (compared to control).

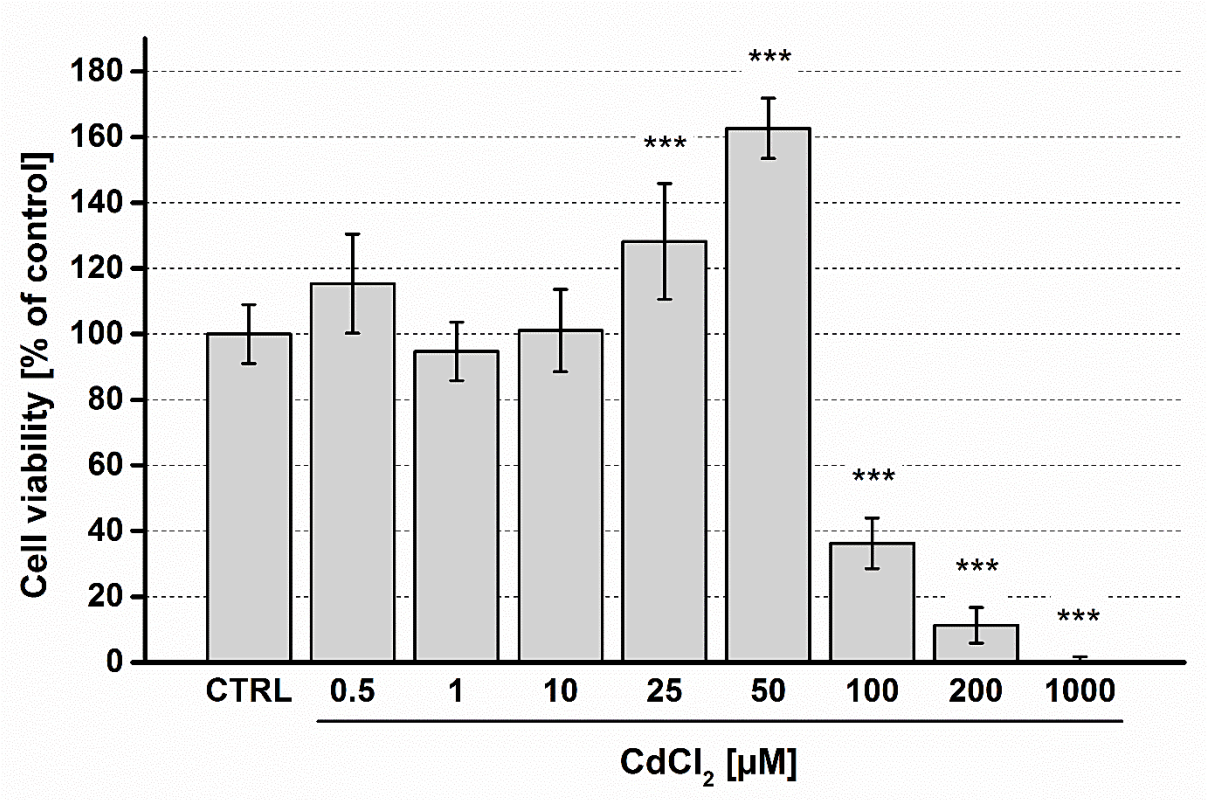

Fig. 5. Dehydrogenase activity measurement – HK-2 cells were assayed using the WST-1 test after 24 h of treatment with $CdCl_2$ at concentrations 0-1,000 µM. The results are expressed as mean ± SD (control=100 %; n=6-10). *** $p<0.001$ (compared to control).

We selected treatments of 5, 25, 100, and 200 µM $CdCl_2$ for the following characterization of changes in dehydrogenase activity of HK-2 cells incubated with $CdCl_2$ for 2, 6, 10, and 24 h. We first tested again intracellular dehydrogenase activity using WST-1 (Table 2). With the exception of the 5 µM treatment, we detected significant increase in intracellular dehydrogenase activity that was dependent on incubation time in all tested $CdCl_2$ concentrations. The increased in dehydrogenase activity was strongly related to both. Based on these results, we have proven that, under the given conditions, the HK-2 cells could exhibit enhanced intracellular dehydrogenase activity after $CdCl_2$ treatment as opposed to the expected diminished intracellular dehydrogenase activity. $CdCl_2$ dose and duration of treatment. In the case of cells treated with 200 µM $CdCl_2$, a significant increase in intracellular dehydrogenase activity was detected only after 2 h. Longer treatment times with 200 µM $CdCl_2$ caused a decrease in dehydrogenase activity of HK-2 cells. Treatment with 100 µM $CdCl_2$ caused a significant increase in intracellular dehydrogenase activity after 2, 6, and 10 h but a significant decrease after 24 h. In cells treated with 25 µM $CdCl_2$, increase in intracellular dehydrogenase activity was detected only after treatment from 6 to 24 h.

Table 2: The activity was assayed using the WST-1 test after 2, 6, 10, 24 and 48 h of treatment with $CdCl_2$ at concentrations 0-200 μM. Gray shading indicates the finding of increased intracellular dehydrogenase activity. The results are expressed as mean ± SD (control=100 %; n=6-10). * $p<0.05$; *** $p<0.001$.

Time	$CdCl_2$ [μM]				
	0	**5**	**25**	**100**	**200**
2 h	100 ± 7%	79 ± 7% ***	96 ± 7%	138 ± 10% ***	114 ± 4% *
6 h	100 ± 6%	80 ± 9% ***	124 ± 8% ***	152 ± 9% ***	80 ± 8% ***
10 h	100 ± 5%	101 ± 5%	139 ± 6% ***	173 ± 7% ***	80 ± 5% ***
24 h	100 ± 9%	92 ± 6%	128 ± 18% ***	36 ± 8% ***	11 ± 5% ***
48 h	100 ± 10%	102 ± 4%	101 ± 3%	2 ± 0% ***	0 % ***

3.5 $CdCl_2$ nephrotoxicity – glutathione levels

Changes in cellular dehydrogenase activity detected using the WST-1 test could be related to changes in oxidative metabolism. One of the main parameters which we decided to measure was the change of the levels of the main intracellular antioxidant, glutathione. Significant changes in cellular GSH levels were detected in treatments using 200 μM $CdCl_2$ for all tested time periods. Similar GSH depletion was detected in cells treated with 100 μM $CdCl_2$. No changes of GSH levels were found in treatments using 5 and 25 μM $CdCl_2$.

Table 3: Glutathione (GSH) levels were measured using monochlorobimane. The results are expressed as mean ± SD (control=100%; n=6-10). *** $p<0.001$.

Time	$CdCl_2$ [μM]				
	0	**5**	**25**	**100**	**200**
2 h	100 ± 5 %	102 ± 4 %	99 ± 3 %	93 ± 1 %	87 ± 4 % ***
6 h	100 ± 4 %	97 ± 4 %	97 ± 3 %	79 ± 3 % ***	59 ± 3 % ***
10 h	100 ± 4 %	99 ± 3 %	105 ± 2 %	59 ± 3 % ***	39 ± 2 % ***
24 h	100 ± 3 %	102 ± 4 %	102 ± 4 %	7 ± 1 % ***	5 ± 1 % ***

4 Conclusion

In conclusion, we report that HK-2 cell line did not possess stable susceptibility to model toxic compounds during continuous passaging for 13 weeks. Based on presented results, we conclude that the outcomes of experiments obtained using the HK-2 cell line passaged are supposed to be considered with regard to the number of passages. Conclusions of the characterization of nephrotoxic effects of $CdCl_2$ in HK-2 cells. We found that $CdCl_2$ at high concentrations (i.e. 25-200 μM $CdCl_2$) are able to induce a transient increase of cell viability in human kidney cells preceding cell death. That change in intracellular dehydrogenase activity is followed by transiently increased ROS production leading to GSH depletion and other processes progressing the cell death. A number of questions remain about causation and a possible role for this phenomenon associated with $CdCl_2$.

5 List of References

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6 List of Student´s Published Works

Publications associated with the Ph.D. thesis topic in the journals with IF

Handl Jiří, Čapek Jan, Majtnerová Pavlína, Petira Filip, Hauschke Martina, Roušarová Erika, Roušar Tomáš. Transient increase in cellular dehydrogenase activity after cadmium treatment precedes enhanced production of reactive oxygen species in human proximal tubular kidney cells. *Physiological Research*, 2019, 68, pages 481-490. *ISSN 0862-8408, doi.org/10.33549/physiolres.934121.* **(IF = 1.701)**

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Práce vznikla za podpory projektů Posilování mezioborové spolupráce ve výzkumu nanomateriálů a při studiu jejich účinků na živé organismy (CZ.02.1.01/0.0/0.0/17_048/0007421) a GAČR 19-11867S.