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Title: Transient increase in cellular dehydrogenase activity after cadmium treatment precedes enhanced production of reactive oxygen species in human proximal tubular kidney cells

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Short title: Cadmium induces a transient increase of HK-2 cell viability

Summary

Cadmium is a heavy metal causing toxicity especially in kidney cells. The toxicity is linked also with enhanced oxidative stress leading to cell death. On the other hand, our recent experiments have shown that an increase of total intracellular dehydrogenases activity can also occur in kidney cells before declining until cell death. The aim of the present study, therefore, was to evaluate this transient enhancement in cell viability after cadmium treatment. The human kidney HK-2 cell line was treated with $CdCl_2$ at concentrations 0–200 μM for 2–24 h and intracellular dehydrogenase activity was tested. In addition, we measured reactive oxygen species (ROS) production, glutathione levels, mitochondrial membrane potential, and C-Jun-N-terminal kinase (JNK) activation. We found that significantly increased dehydrogenase activity can occur in cells treated with 25, 100, and 200 μM $CdCl_2$. Moreover, the results showed an increase in ROS production linked with JNK activation following the enhancement of dehydrogenase activity. Other tests detected no relationship with the increased in intracellular dehydrogenase activity. Hence, the transient increase in dehydrogenase activity in HK-2 cells preceded the enhancement of ROS production and our finding provides new evidence in cadmium kidney toxicity.

Key words: Cadmium toxicity; Kidney injury; Dehydrogenase activity; Oxidative stress; ROS production

1. Introduction

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. In addition, the toxicity can be found in bone and blood cells (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).

Recently, a human immortalized proximal tubular cell line (HK-2) has been developed for studying nephrotoxicity *in vitro* (Gunness *et al.* 2010, Ryan *et al.* 1994). 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.

The goal of the present study is directly linked to the results of our previous study (Hauschke *et al.* 2017), whereby we recently found that Cd treatment, surprisingly, can also lead to temporary increase in the viability of HK-2 cells. Indications of increase in cell viability after Cd treatment can be found also in reports from other authors (Iwatsuki *et al.* 2011, Lee *et al.* 2015, Somji *et al.* 2006), but none of the previous studies had given much attention to this finding. Therefore, the aim of the present study was to examine whether the increase in HK-2 cell viability after Cd exposure is related to Cd concentration and/or duration of Cd treatment, as well as whether the increase of total intracellular dehydrogenases activity (further referred as dehydrogenase activity) can be linked to any other changes in oxidative metabolism.

2. Materials and Methods

2.1. Chemicals

Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (with/without phenol red), insulin, transferrin, and sodium selenite were purchased from Sigma-Aldrich (USA). Fetal bovine serum, pyruvate, penicillin, streptomycin, epidermal growth factor, and all other chemicals, if not otherwise specified, were purchased from Invitrogen-Gibco (USA).

2.2. Cell culture

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 in keeping with our previous studies (Hauschke *et al.* 2017). All the experiments were conducted using the HK-2 cells (passages 4–11). HK-2 cells were seeded 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.

2.3. Dehydrogenase activity measurement

Dehydrogenase activity was evaluated by WST-1 test (Roche, Germany). The WST-1 test measures the activity of intra- and extramitochondrial dehydrogenases. At the required time, 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 60 min and the absorbance change (0–1 h) was measured spectrophotometrically at wavelength

of 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%).

2.4. Measuring glutathione levels

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 $CdCl_2$ for an appropriate time. After incubation, 20 µl of the bimane solution was added to cells and measurement was started. The final concentration of monochlorobimane in a 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%).

2.5. Measuring ROS production

We used chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA; Thermo, USA) as an intracellular probe to detect ROS production. The working solution was prepared fresh at the time of analysis by dilution in Dulbecco's Modified Eagle's Medium. The cells were incubated with $CdCl_2$ for appropriate periods. After incubation, the CM-H_2DCFDA working solution was added to cells to be loaded with the probe for 90 min. The final concentration of CM-H_2DCFDA in a well was 1 µM. The cells were then washed with phosphate buffered saline (PBS) and the fluorescence measurement was started. The fluorescence (Ex/Em = 485/535 nm) was measured for 60 min using a Tecan Infinite M200 fluorescence reader. The

ROS levels were expressed as the percentage relative to ROS levels in control cells (=100%).

2.6. Detecting mitochondrial membrane potential

Mitochondrial membrane potential was measured using a JC-1 intracellular probe. The working solution of JC-1 was prepared fresh at the time of analysis by dilution in Dulbecco's phosphate buffer. After Cd treatment, 20 µl of the JC-1 solution was added to cells. The final concentration of JC-1 in a well was 10 µg/ml. The HK-2 cells were loaded for 20 min and then washed with PBS. The fluorescence (red: Ex/Em = 485/595 nm; green: Ex/Em = 485/535 nm) was measured using a Tecan Infinite M200 fluorescence reader. The rate of mitochondrial membrane potential was expressed as the red/green ratio.

2.7. Measuring nuclear condensation

We used Hoechst 33258 dye for detecting DNA fragmentation in cells. The working solution of Hoechst 33258 was prepared fresh at the time of analysis. After Cd treatment, 10 µl of the Hoechst 33258 solution was added to cells and the fluorometric measurement was started after 20 min of loading. The final concentration of Hoechst 33258 in a well was 2 µg/ml. The fluorescence (Ex/Em = 352/461 nm) was measured using a Tecan Infinite M200 fluorescence reader. The fluorescence signal was expressed as the percentage relative to fluorescence in control cells (=100%).

2.8. Measuring caspase-3/7 activity

Caspase-3/7 activation was measured by Apo-ONE® Homogeneous Caspase-3/7 Assay (Promega, USA). The working solution of caspase-3/7 was prepared fresh

at the time of analysis. The cells were incubated with $CdCl_2$ and cisplatin (100 µM) for appropriate times. Then, 100 µl of the caspase-3/7 working solution was added to cells and the fluorescence (Ex/Em = 485/530 nm) was measured using a Tecan Infinite M200 fluorescence reader. At 14 h of treatment, the fluorescence was expressed as the percentage relative to the fluorescence in control cells (=100%).

2.9. Detecting protein levels

The protein levels of JNK and NFκB were detected using western blot analysis. Briefly, HK-2 cells (1.5×10^6) were washed in PBS, lysed in RIPA Lysis Buffer (30 min; 4 °C), centrifuged (16,000 g; 20 min; 4 °C), and the supernatant was loaded onto SDS-PAGE. Proteins were transferred onto Immun-Blot PVDF Membrane (Bio-Rad, USA). After blocking in TBST buffer (20 mM TRIS; 150 mM NaCl; 0.1% Tween-20; pH 7.5) containing 5% bovine serum albumin, the samples were incubated with Anti-ACTIVE® JNK (anti-54 kDa JNK2; Rabbit; Promega), Anti-NFκB p65 monoclonal antibodies (Mouse; Invitrogen) or Anti-B-Actin (Rabbit; Sigma-Aldrich) according to the manufacturer's instructions. The membrane was then incubated with horseradish peroxidase-conjugated secondary antibodies (Goat; Sigma-Aldrich, Goat anti-mouse; Invitrogen). The proteins were visualized using Clarity™ Western ECL Substrate (Bio-Rad) and ChemiDoc™ MP System (Bio-Rad).

2.10. Statistics

All experiments 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

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 $CdCl_2$ (0.1 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. 1). After 24 h, the significant decrease in cell viability was found in cells treated with 100, 200, and 1000 µM $CdCl_2$ (Fig. 2). 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. 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.

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 1). 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 $CdCl_2$ 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.

Because the increase in dehydrogenase activity detected using the WST-1 test could be related to changes in oxidative metabolism, we assessed mitochondrial membrane potential, GSH levels, and ROS production (Table 2). To assess oxidative stress after $CdCl_2$ treatment, we measured intracellular ROS production. We found that ROS production was considerably related to outcomes of increased dehydrogenase activity. The results showed that ROS production was increased significantly in cells treated with 200 µM $CdCl_2$ after both 2 and 6 h. At 6 and 24 h, ROS production was increased in those cells treated with 100 and 25 µM $CdCl_2$, respectively (Table 2).

We found no significant change of MMP in relation to the observed increase in intracellular dehydrogenase activity after $CdCl_2$ treatment. Our experiments showed that MMP was significantly reduced mostly after treatment with $CdCl_2$ for longer periods. Therefore, the change of MMP in HK-2 cells was rather unrelated to the $CdCl_2$-induced increase in intracellular dehydrogenase activity. 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 $CdCl_2$.

Finally, we examined changes in cell nucleus induced by $CdCl_2$ using three methods: detecting DNA condensation, measuring caspase activity, and assessing JNK activation. We found significantly increased fluorescence signal of DNA condensation in cells treated with 200 µM $CdCl_2$ after both 10 and 24 h and in cells treated with 100 µM $CdCl_2$ after 24 h (Table 2). The examination of caspase activities showed the activity of caspase 3/7 to be non-significantly increased after 200 µM $CdCl_2$ treatment (data not shown). JNK activation was detected using western blot analysis. Increased protein levels of p-JNK were detected after 2, 6 and 10 h in both the 100 and 200 µM treatments. At 24 h, JNK activation was detected at all tested $CdCl_2$ concentrations with the exception 200 µM $CdCl_2$ treated cells (Fig. 3). In addition, we detected NFkB activation in Cd treated HK-2 cells, but the results did not show any changes in the NFkB expression.

4. Discussion

The toxicity of Cd has been tested in renal cell lines of human (i.e., HK-2 cells; (Fujiki *et al.* 2013, Kim *et al.* 2014, Simon *et al.* 2014, Wilmes *et al.* 2011) and animal origin (i.e., canine MDCK (Zimmerhackl *et al.* 1998), pig LLC-PK1 (Fotakis and Timbrell 2006), rat HTC (Gennari *et al.* 2003). HK-2 cells are immortalized proximal tubular cells (Gunness *et al.* 2010) and presently are regarded as providing the most relevant model for studying Cd toxicity (Fongsupa *et al.* 2015, Fujiki *et al.* 2013, Huang *et al.* 2017, Iwatsuki *et al.* 2011, Komoike *et al.* 2011, Shrestha *et al.* 2017, Somji *et al.* 2006). Therefore, HK-2 cells were used for characterizing Cd cytotoxicity in the study we present here.

Our results have shown that, contrary to expected decrease in cell viability, $CdCl_2$ can induce a transient increase of cell viability in relation to $CdCl_2$ dose and incubation time (Hauschke *et al.* 2017). In addition, some outcomes from several studies by other authors also lend support to our findings. Enhanced cell viability after Cd treatment has been reported from studies using both human (Iwatsuki *et al.* 2011, Kondo *et al.* 2012) and animal (Fotakis and Timbrell 2006, Riemschneider *et al.* 2015) kidney cells as well as cells of other tissue origin (Bonham *et al.* 2003, Somji *et al.* 2006). Because those reports provided no information on the finding of increased in intracellular dehydrogenase activity after Cd treatment, we decided to characterize that role of $CdCl_2$.

We tested treatment of HK-2 cells with $CdCl_2$ in a variety of concentrations (5, 25, 100, and 200 µM $CdCl_2$) and times (2–24 h). Intracellular dehydrogenase activity was detected using the WST-1 test, which measures the activity of intracellular dehydrogenases. We found that all tested concentrations of $CdCl_2$ with the exception of 5 µM induced significant transient increase in intracellular dehydrogenase activity

in HK-2 cells and that the time of the occurrence of dehydrogenase activity increase was inversely correlated with CdCl2 concentration. Our results can also be supported by outcomes from two other studies (Iwatsuki *et al.* 2011, Kondo *et al.* 2012) which tested $CdCl_2$-induced changes in HK-2 dehydrogenase activity using the WST-8 test. The induction of increased HK-2 dehydrogenase activity was found in cells treated with 20 µM $CdCl_2$ for 4 h. Unfortunately, those two studies did not use $CdCl_2$ at levels higher than 50 µM. Two other studies on cadmium toxicity reported the increased in intracellular dehydrogenase activity after exposure to 10 µM $CdCl_2$ for 24 and 48 h in an animal MDCK kidney cell line (Bonham *et al.* 2003) and after treatment with 10 µM $CdCl_2$ for 24 and 48 h in the RAW 264.7 macrophage cell line (Riemschneider *et al.* 2015).

Seeking the implication of the cell viability increase in HK-2 cells, we followed the experiments with additional biochemical tests. Because the increase of intracellular dehydrogenase activity could be related to changes in redox metabolism, we assessed ROS production, GSH levels, MMP, and JNK activation. Intracellular ROS production and GSH levels were measured as markers of oxidative stress. We found that induction of ROS production followed the enhancement of dehydrogenase activity as measured by the WST-1 test in $CdCl_2$-treated HK-2 cells. After 2 h, increase in dehydrogenase activity and ROS production were detected in cells treated with 200 µM $CdCl_2$. Inasmuch as the 100 µM treatment induced increased dehydrogenase activity after 2 h and 6 h but ROS production was stimulated significantly only after 6 h, we might conclude that increase in intracellular dehydrogenase activity precludes the increase in ROS production. This can be supported by the finding that intracellular dehydrogenase activity of HK-2 cells incubated in 25 µM $CdCl_2$ increased after 6 and 24 h but ROS production was

stimulated significantly until 24 h. Another conclusion from our results is that significant depletion of intracellular GSH levels appeared in all incubation periods only after increase in ROS production. In contrast, after 24 h of treatment of HK-2 cells with 25 μM $CdCl_2$, the increase in intracellular dehydrogenase activity and ROS production was not linked with GSH depletion. This might be because such short duration of treatment did not allow the GSH depletion to appear.

To characterize other cellular processes related to increased dehydrogenase activity of HK-2 cells after Cd treatment, we used detection of JNK and NFkB activation. We found no changes in NFkB protein expression. On the other hand, our results showed increased p-JNK levels in cells treated with 100 and 200 μM $CdCl_2$ after 2, 6 and 10 h. After 24 h, the p-JNK levels were enhanced at all tested $CdCl_2$ concentrations with the exception of 200 μM in which case the cells likely were dead. The results of other methods (i.e., measurements of MMP, caspase activity, and DNA condensation) provided no relevant findings elucidating any mechanism likely participating in the detected increased intracellular dehydrogenase activity. The significant disappearance of MMP together with increased nuclear condensation were always found at late time periods but never during periods with the detected increased dehydrogenase activity. The detection of caspase 3 activity showed no significant differences for any treatment in comparison to control cells.

Our results proved the presence of increased ROS production after $CdCl_2$ treatment as reported in other studies (Wang *et al.* 2013, Wilmes *et al.* 2011, Zahir *et al.* 1999). Although Cd toxicity is linked with a number of subcellular toxic mechanisms, recent studies have reported that the induction of oxidative stress could play an essential role in Cd-induced toxic effect (Kim and Sharma 2006, Thévenod and Friedmann 1999, Tobwala *et al.* 2014, Zahir *et al.* 1999). According to our

findings on GSH depletion after Cd treatment, the antioxidants play an essential role in the protection against Cd toxicity (López *et al.* 2006, Zahir *et al.* 1999). After cellular oxidative stress becomes heightened, activation of JNK kinases occurs (Fongsupa *et al.* 2015) and the subsequent cellular and mitochondrial signaling can lead to apoptotic or necrotic cell death (Chambers and LoGrasso 2011). The toxic effect of $CdCl_2$ leading to apoptosis or necrosis in kidney cells has been described in other studies (Kondo *et al.* 2012, López *et al.* 2006), but the surprising finding presented here on transient increase in intracellular dehydrogenase activity has not been described in any of those reports. Our results proved a significant relationship between increased dehydrogenase activity followed by stimulated ROS production after treatment with $CdCl_2$ across a wide range of concentrations. It follows that Cd is able significantly to influence the function of mitochondria, as these constitute the main cellular sources of dehydrogenase activity and ROS production in cells. This finding could be supported by a recent study reporting an induction of mitochondrial permeability in rat mitochondria (Belyaeva *et al.* 2011). As a consequence of mitochondrial permeabilization, apoptotic-inducing factor (AIF) is released from the intermembrane space of mitochondria to the cytosol and caspase-independent apoptosis can thus be induced (Mao *et al.* 2007). In addition, AIF induces the expression and activation of other pro-apoptotic proteins, including caspases (Huang *et al.* 2017). A report has also provided evidence that Cd can induce a mitogen potential followed by increased cell proliferation (Templeton and Liu 2010). This phenomenon is brought about by interaction of Cd with mitogen-activated protein kinases (e.g., JNK and ERK), controlling cell growth, differentiation, and apoptosis (Filipič 2012, Levinthal and DeFranco 2005). Low concentrations of Cd can activate JNK transiently, but high doses of Cd induce a permanent JNK activation (Chuang *et*

al. 2000). The activation of JNK is caused by increased ROS levels (Kamata *et al.* 2005). These reports could provide some justification for our finding of increased cell dehydrogenase activity, as this may be caused by a change of signaling between mitochondria and cell nucleus that likely is linked with increased ROS production. Another line of reasoning in support of our data on increased intracellular dehydrogenase activity may be related to a direct role of ROS. Some reports have indicated that increased ROS levels during oxidative stress could induce an increase in intracellular dehydrogenase activity or mitochondrial function (Chen *et al.* 2006, Lee *et al.* 2000). Other reports come up with the statement that cadmium ions affect the role of free radicals and reactive species that are formed during oxidative stress (Ďuračková *et al*. 2010).

In conclusion, 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 transient increased ROS production leading to GSH depletion and other processes progressing to cell death. A number of questions remain, however, about causation and a possible role for this phenomenon associated with $CdCl_2$. Thus, additional work is needed to elucidate this subject further, especially relating to changes in activity of the mitochondrial respiratory chain.

Conflict of interest: There is no conflict of interest.

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Table legends:

Table 1: Dehydrogenase activity of HK-2 cells 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 (i.e., increased intracellular dehydrogenase activity). The results are expressed as mean ± SD (control = 100%; n = 6-10). *, $p < 0.05$; ***, $p < 0.001$

Table 2: Estimation of oxidative metabolism in HK-2 cells after $CdCl_2$ treatment (0–200 µM) for 2, 6, 10, and 24 h. Reactive oxygen species (ROS) levels were assessed using chloromethyl-2',7'-dichlorodihydrofluorescein diacetate probe. Glutathione (GSH) levels were measured using monochlorobimane. The mitochondrial membrane potential (MMP) was measured by fluorometric method using the JC-1 probe and the results were expressed as the red/green (R/G) ratio. Nuclear condensation was measured using Hoechst 33258 probe. The results are expressed as mean ± SD (control = 100%; n = 6–10). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$

Table 1

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% ***

Table 2

Time	$CdCl_2$ [µM]	ROS	GSH	MMP [R/G]	DNA condensation
2 h	0	100 ± 13%	100 ± 5%	2.29 ± 0.11	100 ± 11%
	5	60 ± 6% ***	102 ± 4%	2.17 ± 0.13	94 ± 15%
	25	57 ± 9% ***	99 ± 3%	2.45 ± 0.18	65 ± 13% **
	100	49 ± 12% ***	93 ± 1%	2.07 ± 0.14	59 ± 9% ***
	200	148 ± 13% ***	87 ± 4% ***	1.98 ± 0.11 **	61 ± 17% ***
6 h	0	100 ± 14%	100 ± 4%	2.16 ± 0.31	100 ± 50%
	5	90 ± 9%	97 ± 4%	2.45 ± 0.19	107 ± 28%
	25	69 ± 5% **	97 ± 3%	2.02 ± 0.09	98 ± 5%
	100	189 ± 14% ***	79 ± 3% ***	2.41 ± 0.25	82 ± 31%
	200	311 ± 21% ***	59 ± 3% ***	2.45 ± 0.25	87 ± 44%
10 h	0	N/A	100 ± 4%	2.57 ± 0.33	100 ± 30%
	5	N/A	99 ± 3%	2.40 ± 0.34	69 ± 26%
	25	N/A	105 ± 2%	1.94 ± 0.26 *	70 ± 21%
	100	N/A	59 ± 3% ***	2.46 ± 0.17	54 ± 17%
	200	N/A	39 ± 2% ***	1.49 ± 0.11 ***	563 ± 26% ***
24 h	0	100 ± 14%	100 ± 3%	2.16 ± 0.27	100 ± 13%
	5	86 ± 5%	102 ± 4%	1.80 ± 0.12	74 ± 16%
	25	204 ± 23% ***	102 ± 4%	1.62 ± 0.13 **	86 ± 8%
	100	78 ± 12%	7 ± 1% ***	0.31 ± 0.07 ***	529 ± 64% ***
	200	88 ± 15%	5 ± 1% ***	0.22 ± 0.03 ***	561 ± 36% ***

Figure legends:

Fig. 1: Dehydrogenase activity measurement – of HK-2 cells assayed using the WST-1 test after 6 h of treatment with $CdCl_2$ at concentrations 0–1000 µM. The results are expressed as mean ± SD (control = 100%; n = 6–10). ***$p < 0.001$ (compared to control)

Fig. 2: Dehydrogenase activity measurement – of HK-2 cells assayed using the WST-1 test after 24 h of treatment with $CdCl_2$ at concentrations 0–1000 µM. The results are expressed as mean ± SD (control = 100%; n = 6–10). ***$p < 0.001$ (compared to control)

Fig. 3: Detection of p-JNK and NFkB in HK-2 cells after $CdCl_2$ exposure using western blot analysis. Cells were exposed to different concentrations of $CdCl_2$ (1–200 µM) for 2, 6, 10, and 24 h. B-actin (42 kDa), NFkB (65 kDa), p-JNK (46 kDa, 54 kDa) were determined for each interval and concentration (with comparison to control cells)

Figure 1:

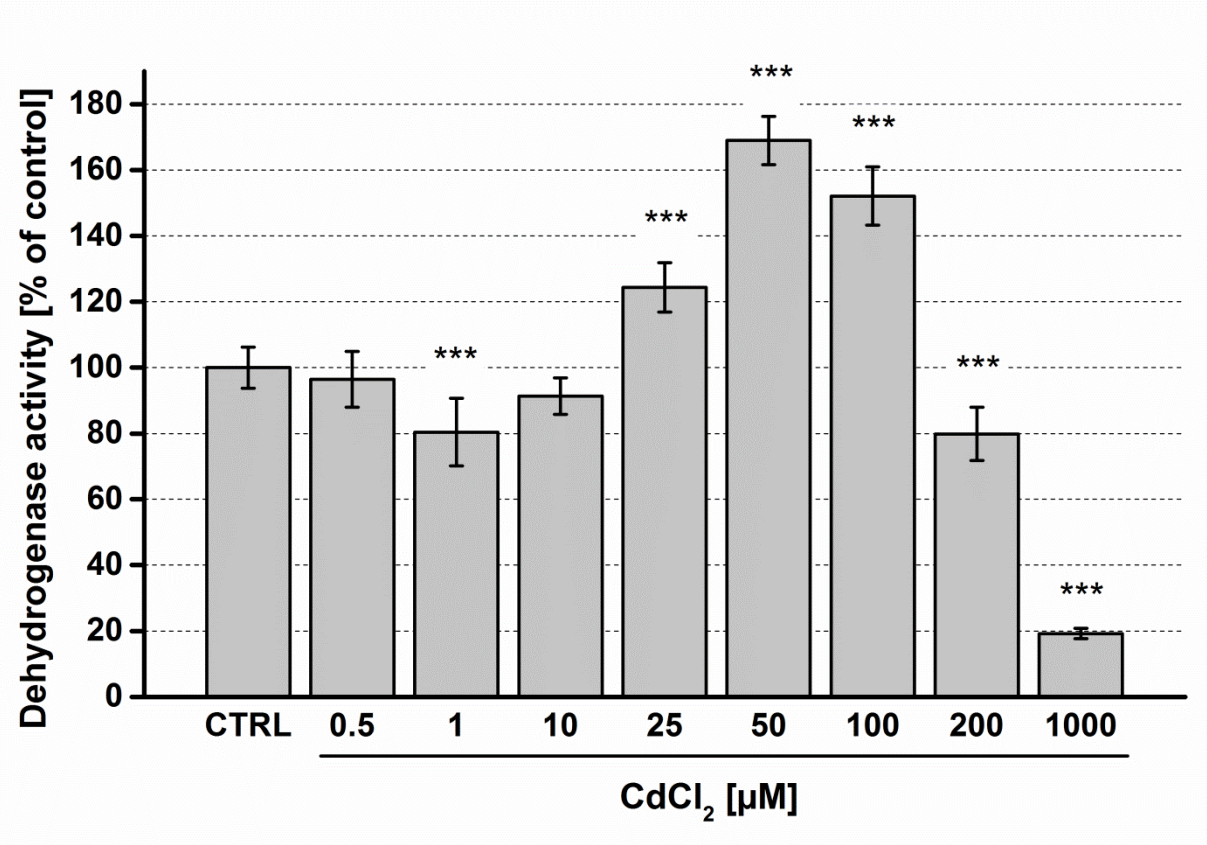
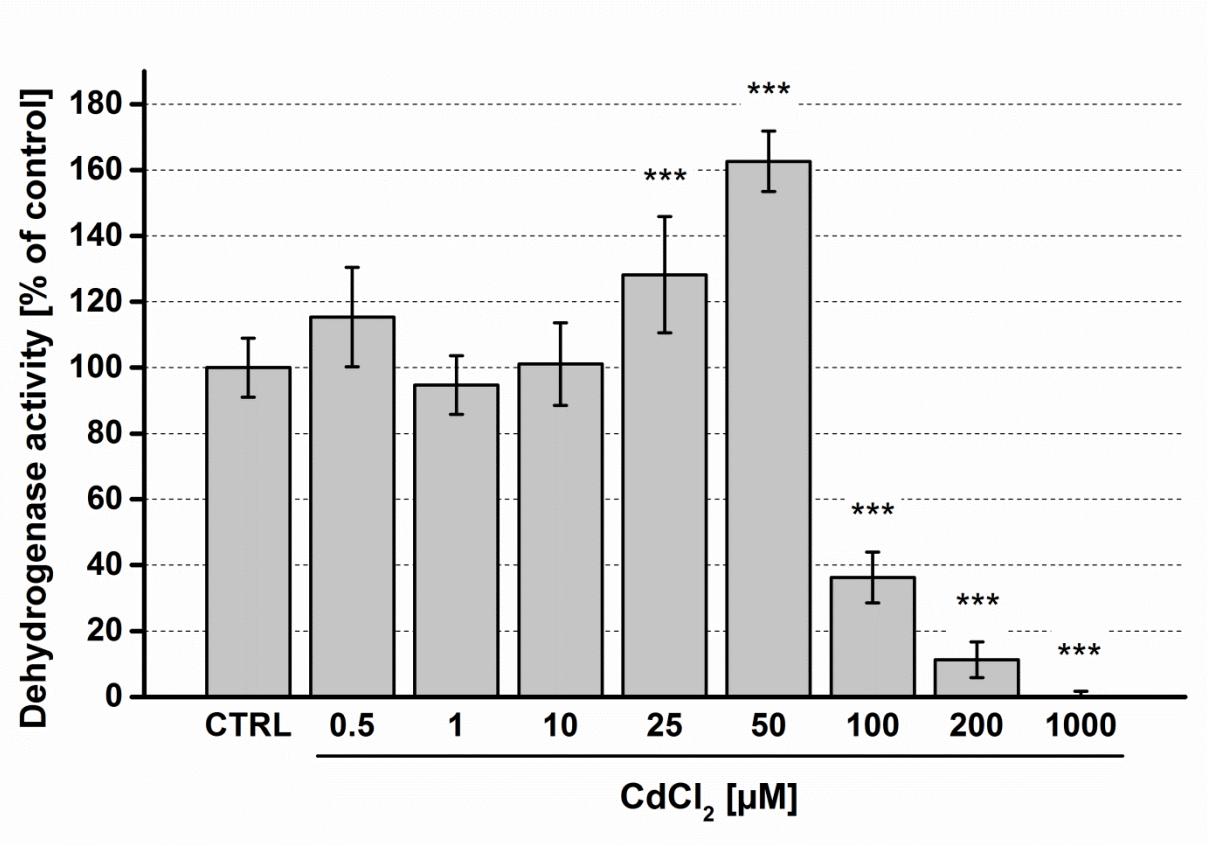

Dehydrogenase activity [% of control]
180
160
140
120
100
80
60
40
20
0
***
***
***
***
***
CTRL
0.5
1
10
25
50
100
200
1000
CdCl2 [µM]

Figure 2:


Dehydrogenase activity [% of control]
180
160
140
120
100
80
60
40
20
0
***
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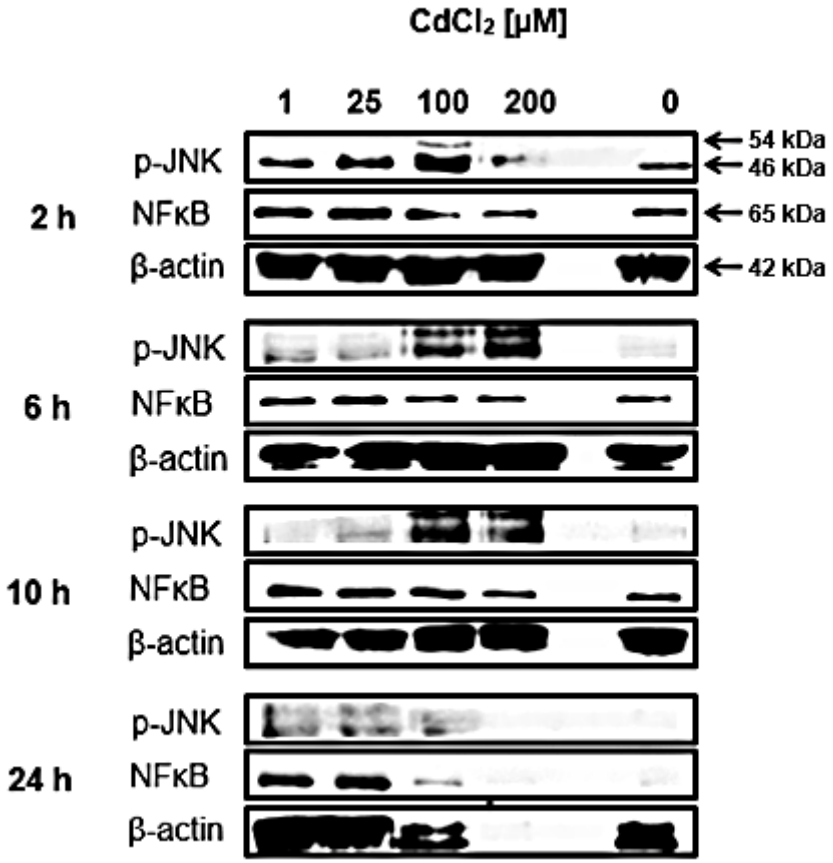
***
***
CTRL
0.5
1
10
25
50
100
200
1000
CdCl2 [µM]

Figure 3:


CdCl2 [μM]
1
25
100
200
0
2 h
6 h
10 h
24 h
p-JNK
NFκB
β-actin
54 kDa
46 kDa
65 kDa
42 kDa