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Title: The effect of repeated passaging on the susceptibility of human proximal tubular HK-2 cells to toxic compounds

Rapid Communication

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Short title: HK-2 cells susceptibility during passaging

Abstract

The human proximal tubular HK-2 cell line is an immortalized cell line commonly used for studying proximal tubular toxicity. Even as their use is presently increasing, there unfortunately are no studies focused on functional changes in HK-2 cells associated with passaging. The aim of the present study, therefore, was to evaluate the functional stability of HK-2 cells during 13 weeks of continuous passaging after 6 and 24 h of treatment with model nephrotoxic compounds (i.e., acetaminophen, cisplatin, $CdCl_2$). Short tandem repeat profile, the doubling time, cell diameter, glutathione concentration, and intracellular dehydrogenase activity were measured in HK-2 cells at each tested passage. The results showed that HK-2 cells exhibit stable morphology, cell size, and cell renewal during passaging. Mean doubling time was determined to be 54 h. On the other hand, we observed a significant effect of passaging on the susceptibility of HK-2 cells to toxic compounds. The largest difference in results was found in both cadmium and cisplatin treated cells across passages. We conclude that the outcomes of scientific studies on HK-2 cells can be affected by the number of passages even after medium-term cultivation and passaging for 13 weeks.

Keywords:

HK-2 cells; nephrotoxicity; cell passaging; in vitro culture; cell lines

In vitro cell models including primary cell cultures and cell lines are used in cytotoxicity screening (Popelová *et al.* 2018; Tong *et al.* 2017; Xia *et al.* 2013). 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 mentioned. Some results have shown, however, that some non-cancer cell lines exhibit genetic heterogeneity and instability (Ben-David *et al.* 2018).

There are a number of animal and human cell lines that can be used to assess nephrotoxic properties (Kim 2016; Vrbová *et al.* 2016). 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; Vošahlíková and Svoboda 2011). The human proximal tubular HK-2 (Human Kidney-2) cell line is an immortalized cell line. 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 suitable 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; Yang *et al.* 2019c; Zaza *et al.* 2015).

According to the PubMed database, as many as about 200 studies a year are now published that used HK-2 cells for *in vitro* testing, and that frequency is increasing. Based upon the very recent scientific papers, only limited numbers of authors have reported the numbers of HK-2 cell passages they have used in their studies. Most of those have reported using passages in the ranges of ≤5 passages (Devocelle *et al.* 2019; Gao *et al.* 2017; Handl *et al.* 2019; Huang *et al.* 2017; Lu *et al.* 2018), ≤20 passages (Devocelle *et al.* 2019; Han *et al.* 2018; Huang *et al.* 2019; Lee *et al.* 2019b; Lu *et al.* 2018; Vrbová *et al.* 2016; Wang *et al.* 2006), or for even longer periods (Chang and Singh 2019; Wang *et al.* 2006). Meanwhile, a large number of presently existing scientific studies on HK-2 cells have not provided any information at all on the passage number (Amaral *et al.* 2017; Campos *et al.* 2018; Lee *et al.* 2019a; Medina-Navarro *et al.* 2019; Nho *et al.* 2018; Song *et al.* 2018; Yang *et al.* 2019b; Yeh *et al.* 2019). Surprisingly, only one study marginally estimated an effect of the number of passages on the results obtained in HK-2 cells (Lu *et al.* 2018) confirming the occurrence of differences in protein expressions between low (6) and high (18) number of passages. Due to the lack of information on probable functional changes in HK-2 cells during continuous passaging, we report here a study on evaluation of functional stability and susceptibility of HK-2 cells to model toxic compounds after 13 weeks of passaging (i.e., in passages 3–15).

Human kidney (HK-2) cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured according to a published protocol (Handl *et al.* 2019; 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. 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. 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.

We analyzed functional parameters of the cells during passaging, i.e. short tandem repeat analysis, cell diameter and doubling time. Short tandem repeat (STR) analysis (i.e., DNA fingerprinting) was used for HK-2 cell line authentication in passages 3, 9, and 15 using a commercial kit (Generi Biotech a.g., Czech Republic). STR analysis was conducted by Generi Biotech, Ltd. (Czech Republic). We used 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 to compare the STR profile with established STR databases. Mean diameter of the HK-2 cells was evaluated using the CASY model TT cell counter and analyzer (Roche, Germany). Doubling time (i.e., the time needed to double the cell population) was 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.

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, to induce cell impairment, the HK-2 cells were treated with 10 mM acetaminophen, 100 µM cisplatin, 50 µM *tert*-butylhydroperoxide, and 100 µM $CdCl_2$. Cells were treated for 6 and 24 h. The WST-1 test to evaluate dehydrogenase activity (Roche, Germany) and glutathione assay were used for

characterizing cell damage. After treatment, the WST-1 reagent was added to the cultured cells (1:10 final dilution) (Hauschke *et al.* 2017). 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 440 nm using a Tecan Infinite M200 plate reader (Tecan, Austria) and expressed as the change of absorbance during 1 h and also relative to controls in percentage terms. Glutathione (GSH) levels were measured using an optimized bimane assay (Čapek *et al.* 2017). After 6 or 24 h of incubation, 20 µl of the bimane solution was added to cells and the measurement was started. 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 percentages relative to those in control cells (=100%). All experiments were repeated two or three times independently 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 (OriginPro 9.0.0, USA). The effect of number of passages and toxic compounds on the cell impairment was tested using Two-way ANOVA ($p = 0.05$; OriginPro 9.0.0, USA).

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. The diameters of viable HK-2 cells ranged 17.4–18.6 µm. The mean diameter of HK-2 cells calculated across all passages was 18.2 µm. Doubling time ranged between 47.3 h and 61.7 h. The mean doubling time across all passages was 53.7 h, and we observed no significant relationship between doubling time and cell passage number. The doubling time is in accordance with characteristics for the HK-2 cell line presented by ATCC.

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 Cd treated cells (Fig. 1A). 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. 1B).

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. 2(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.

In conclusion, we report here 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.

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Conflicts of Interest

The authors declare that there is no conflict of interest.

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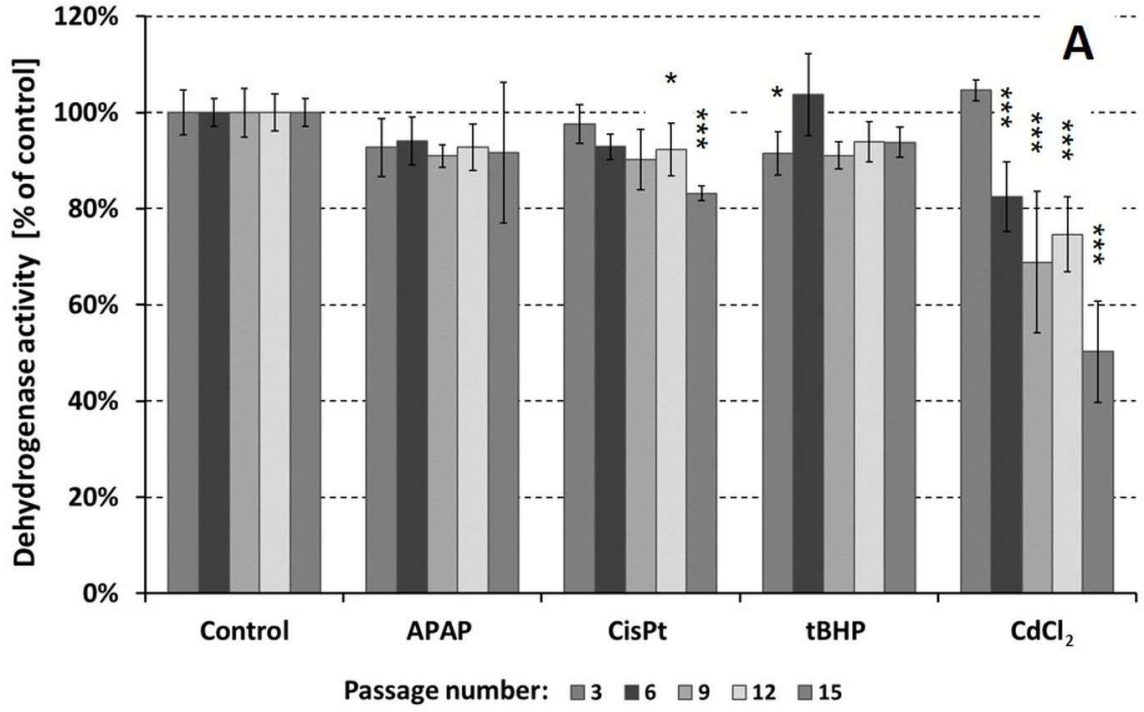
Figure Legends:

Fig. 1. 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). One-way ANOVA with post-hoc test were used for comparison of means with control cells at appropriate number of passages *(*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).*

Fig. 2. 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). One-way ANOVA with post-hoc test were used for comparison of means with control cells at appropriate number of passages *(**, $p < 0.01$; ***, $p < 0.001$).*

Figure 1


A
Dehydrogenase activity [% of control]
120%
100%
80%
60%
40%
20%
0%
Control
APAP
CisPt
tBHP
CdCl2
Passage number: 3 6 9 12 15

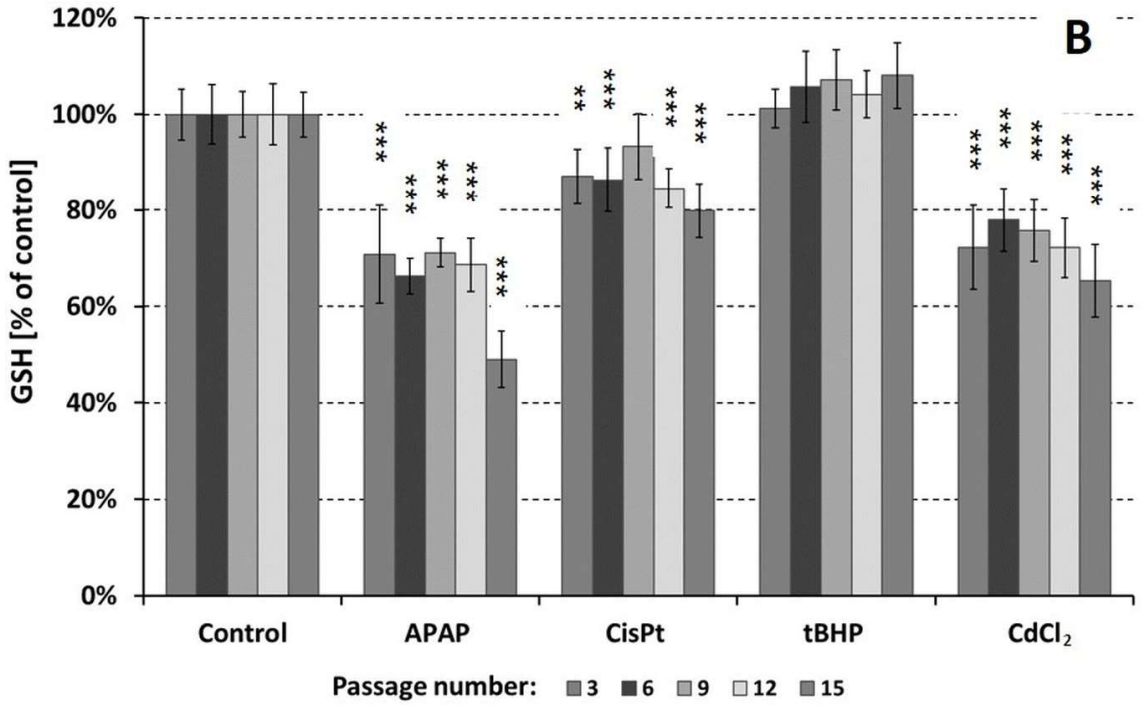
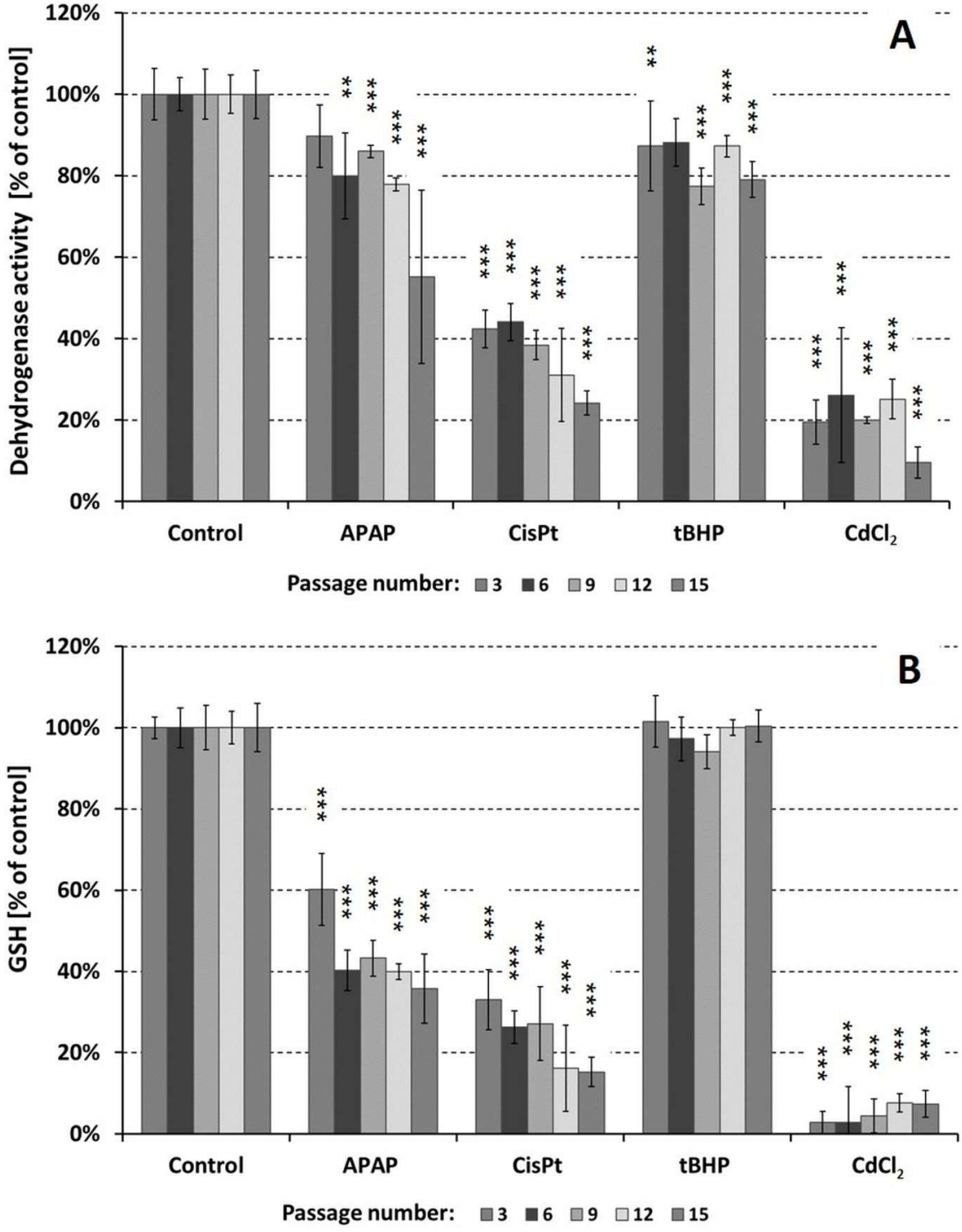

B
GSH [% of control]
120%
100%
80%
60%
40%
20%
0%
Control
APAP
CisPt
tBHP
CdCl2
Passage number: 3 6 9 12 15

Figure 2


A
Dehydrogenase activity [% of control]
120%
100%
80%
60%
40%
20%
0%
Control
APAP
CisPt
tBHP
CdCl2
Passage number: 3 6 9 12 15
B
GSH [% of control]
120%
100%
80%
60%
40%
20%
0%
Control
APAP
CisPt
tBHP
CdCl2
Passage number: 3 6 9 12 15