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Department of Biological and Biochemical Sciences

Jan Čapek

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Author: **Mgr. Jan Čapek**

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

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References

ČAPEK, Jan. Fluorometric methods detecting cell damage. Pardubice, 2020. 132 pages. Doctoral thesis. University of Pardubice, Faculty of Chemical Technology, Department of Biological and Biochemical Sciences. Supervisor doc. RNDr. Tomáš Roušar, Ph.D.

Abstract

Fluorescent methods are specific, sensitive and selective. These properties are frequently used in scientific studies to detect various cellular parameters. Using fluorescence probes, we are allowed to detect intracellular concentrations of analytes, membrane potentials in cells and organelles or to visualize cell organelles.

The aim of our work was to optimize spectrofluorimetric method for routine measurement of glutathione (GSH) concentration in cells. Other partial aims of the study were use this and other fluorometric methods to characterize the toxic effect of CdCl2.

We used the fluorescent probe monochlorobiman to detect intracellular GSH concentrations. Firstly, we optimized the evaluation of fluorescence intensity signal over time. Consequently, we compared the glutathione concentrations obtained using the optimized calculation method with both the standardly used method and with the GSH levels measured using the reference *o*-phthalaldehyde method. We found the similarity of the results obtained using optimized and reference methods when we obtained a very strong correlation of both methods in the analysis of biological samples ($r = 0.96$).

Subsequently, we detected changes in GSH concentration, DNA fragmentation, mitochondrial membrane potential and ROS production in cells incubated with CdCl2.

Abstrakt

Fluorescenční metody jsou specifické, citlivé a selektivní. Často jsou pro tyto vlastnosti využívány v přírodních vědách pro detekci různých buněčných parametrů. Pomocí fluorescenčních sond jsme schopni detekovat intracelulární koncentrace analytů, potenciály na membránách buněk i organel či vizualizovat buněčné organely.

Cílem naší práce bylo optimalizovat spektrofluorimetrickou metodu pro rutinní detekci koncentrace glutathionu (GSH) v buňkách. Dále pak využít tuto, i další fluorescenční metody pro charakterizaci toxického působení $CdCl_2$.

Při detekci intracelulárních koncentrací GSH jsme využili fluorescenční sondu monochlorobiman. Při vyhodnocení získaného fluorescenčního signálu v čase jsme optimalizovali analýzu detekovaného signálu. Koncentrace glutathionu vypočtené pomocí nově vypracované metodiky jsme následně porovnali jak se standardně používaným zpracováním naměřených dat, tak i s koncentracemi GSH naměřenou pomocí referenční metody využívající *o*-ftalaldehyd. Kromě shodných výsledků naměřených optimalizovanou i referenční metodou jsme prokázali také velmi silnou korelaci obou metod při analýze biologických vzorků ($r = 0,96$).

Následně jsme v poslední části práce detekovali změny koncentrace GSH, fragmentaci DNA, mitochondriální membránový potenciál a produkci ROS u buněk inkubovaných s CdCl2.

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1. Introduction

1.1 Glutathione

Glutathione, γ-L-glutamyl-L-cysteinylglycine, is a low molecular weight thiol occurring in two free forms – the reduced form (GSH) and glutathione disulfide. It is a crucial intracellular antioxidant due its role in detoxification of free radicals, regeneration of other antioxidants, and maintaining of SH-groups in intracellular proteins. Additional roles of glutathione involve detoxification of xenobiotics, regulation of redox environment in the cell, and transport of cysteine (Dringen, 2000; Forman *et al.*, 2009; Sies, 1999). All these functions point to this molecule's significance. Therefore, knowledge of glutathione levels in cells is of crucial importance, and especially so in cell toxicity studies.

A number of assays based on a variety of analytical procedures have been used for glutathione detection. The most used are liquid chromatography methods. Their advantages lie in their high specificity and their sensitivity, the latter depending on the type of detection used, be that UV (Amarnath *et al.*, 2003; Garcia *et al.*, 2008; Glowacki *et al.*, 2009), mass spectrometry (Dieckhaus *et al.*, 2005; Dirven *et al.*, 1994; Guan *et al.*, 2003; Paroni *et al.*, 1995), or electrochemical (Hiraku *et al.*, 2002; Kominkova *et al.*, 2015). Their main disadvantages are their costliness and long time required for their analytical processes. The other glutathione assay most often used is a spectrophotometric method. Here, the glutathione reacts with 5,5′-dithiobis-2-nitrobenzoic acid (Ellman, 1959; Eyer *et al.*, 1986). This method has been known also as the recycling assay (Tietze, 1969). The advantages of the recycling assay are its great sensitivity and short measurement time. Its disadvantage can be seen in possible interference of glutathione reductase inhibiting compounds (Griffith, 1980). Other methods for glutathione measurement are based on bioluminescence (Romero *et al.*, 1998), electrochemistry (Inoue *et al.*, 2000; Safavi *et al.*, 2009), gas chromatography (Kataoka *et al.*, 1995), and capillary electrophoresis (Oshea *et al.*, 1993; Shackman *et al.*, 2007).

The latter group of methods for glutathione analysis consists of fluorometric assays. These methods are often preferred for their high sensitivity and specificity. The probe most used, *ortho*-phthalaldehyde (OPA), can react with GSH to form a highly fluorescent product (Cohn *et al.*, 1966; Hissin *et al.*, 1976; Rousar *et al.*, 2012). The analogue of OPA, 2,3-naphthalenedicarboxaldehyde, has been used for glutathione detection, too (White *et al.*, 2003). The ratiometric probe ThiolQuant Green has been developed for intracellular GSH quantitation recently (Jiang *et al.*, 2015). Still other fluorescent probes, bimanes, have been used less frequently.

Presently, the bimanes monochlorobimane (MCB) and monobromobimane (MBB) can be used for detecting GSH and other thiols (Anderson *et al.*, 1999; Barhoumi *et al.*, 1995; Fernandezcheca *et al.*, 1990; Chatterjee *et al.*, 1999; Kamencic *et al.*, 2000; Kosower *et al.*, 1987; Kosower *et al.*, 1979). In comparison to OPA, their substantial advantage can be seen in their ability to penetrate into the cell through cell membrane and to react directly with cellular thiols. This ability has been utilized in fluorescence microscopy assessment (Bellomo *et al.*, 1992; Keelan *et al.*, 2001; Stevenson *et al.*, 2002) and flow cytometry (Han *et al.*, 2016; King *et al.*, 2004; Xiao *et al.*, 2016). On the other hand, quantitative spectrofluorometric assessment of glutathione levels in intact cells has not yet been used frequently. Because a method for GSH assessment

using bimanes could be rapid, sensitive, and specific, and therefore suitable for routine assessment of GSH in intact cells, the aim of the present study was to optimize the bimanes-derived glutathione assay for this purpose. In addition, there is no comparison in the literature of bimanes-derived GSH assay with any other glutathione assay. An additional aim of our study, therefore, was to compare glutathione results obtained using our optimized bimane-derived method with those from the standard *ortho*-phthalaldehyde-derived method.

1.2 Mitochondrial membrane potential

The potential of the mitochondrial membrane ($\Delta\Psi$) is the result of redox transformations associated with Krebs cycle activity, Beta oxidation of fatty acids, pyruvate decarboxylation, and many other events. It serves as an intermediate form of energy that is used by ATP synthase for ATP synthesis. Transformations result not only in $\Delta\Psi$ but also in proton gradients, which together create a protonmotive force. Normally, cells very sensitively maintain the intracellular concentration of ATP and $\Delta\Psi$, which is essential for their physiological functioning. It is therefore a factor determining the viability of mitochondria, which are involved in the elimination of damaged mitochondria and, last but not least, it plays a role in the transport of charged compounds important for mitochondria functioning (Zorova *et al.*, 2018a; Zorova *et al.*, 2018b).

1.3 Reactive oxygen species

Free oxygen radicals, which are a group belonging to reactive oxygen species (ROS), are a highly studied group of substances. Reactive oxygen species arise as a result of metabolic processes. There is a number of cellular parts where ROS are formed. Due to the very fast turnover of oxygen, they include mitochondria, peroxisomes and endoplasmic reticulum. Of course, ROS sources can also be exogenous. Usually, the electron pairs in atoms or molecules are antiparallel, which strongly limits the oxidation properties of O_2. Single-electron reductions of O_2, are the most common which give rise to superoxide (Apel *et al.*, 2004; Cadenas, 1989). Gradual reduction of triplet oxygen produces reactive forms, which play an important role in the pathology of many diseases. One-electron reduction of O_2 to form superoxide radical ($O_2^{\bullet -}$) is the first initiation reaction of other oxygen radicals. This reaction is thermodynamically very unfavorable and the interaction of O_2 with another paramagnetic center is important for overcoming spin restriction.

1.4 DNA fragmentation

Cell nucleus localization is very important for describing cell integrity. In various pathological conditions, defensive cellular mechanisms are activated. Regardless of mutations in DNA, we can say that in acute cellular damage, rapid defensive mechanisms (concentration of antioxidants) are activated. Consequently, a large number of additional mechanisms are added in an attempt to bring the cell out of the pathological state. In the last phase, nuclear damage is usually a factor which is a marker of late-stage cell damage. Nuclear structure damage can be observed after toxic impairment, especially in apoptosis (Prokhorova *et al.*, 2015), and, furthermore, in a number of diseases, including Alzheimer's and Parkinson's disease (Candy *et al.*, 1983).

In later stages of apoptosis, chromatin condensation and DNA fragmentation occur. Chromatin condensation is followed by caspase 6 activation. The substrates for caspase 6 are nuclear laminins responsible for the homogeneity of nuclear chromatin. After the laminins are released by caspase 6, cell chromatin condenses finally. Condensed chromatin is a useful marker in the detection of apoptotic cells using fluorescent techniques (Ruchaud *et al.*, 2002).

2. Experimental part

2.1 Aims of Ph.D. study

The aim of our work was to optimize spectrofluorimetric method for routine detection of glutathione concentration in cells. Furthermore, to optimize the spectrofluorimetric method for the detection of fragmented DNA and in the last part of the work to use fluorescence methods to characterize the toxic effect of $CdCl_2$.

2.2 Cell culture

Two cell lines (HK-2 and A549) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Human kidney (HK-2) cells are 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). The adenocarcinomic human alveolar basal epithelial cell line (A549) is derived from human lung adenocarcinoma cells (Foster *et al.*, 1998).

2.3 Measurement of glutathione levels – bimanes assay

The working solutions of MBB and MCB were prepared fresh at the time of analysis by dilution in Dulbecco's phosphate buffer (pH 7; 1 mM) and tempered at 37 °C. The cells were incubated in cell medium (100 μL) on 96-well plates with the tested compounds for appropriate time periods: diethylmalonate (DEM; 0.5 and 2 mM; 3 h), *tert*-butyl hydroperoxide (50 and 500 μM; 24 h), cisplatin (5, 30 and 50 μM; 24 h), and $HgCl_2$ (20 μM; μM; 24 h). After incubation, 20 μL of the bimane solution was added to cells and the measurement was started. The final concentrations of MCB and MBB in a well were 40 μM and 10 μM, respectively. The fluorescence (Ex/Em = 394/490 nm) was measured for 60 min using a Tecan Infinite M200 fluorescence reader (Tecan, Austria) while incubated at 37 °C. We compared two calculations of glutathione levels from the fluorescence signal: 1) "standard" – the fluorescence was calculated as the difference between the fluorescence signal at 60 min and 0 min; and 2) "optimized" – the fluorescence was expressed as the slope of change of time/fluorescence on a linear part of the curve after 10 min of measurement (Δ fluorescence$_{(0\text{-}10\ min)}$/10 min). The glutathione levels were expressed as the percentage relative to glutathione levels in control cells (control = 100%).

2.4 Measurement of glutathione levels – ortho-phthalaldehyde assay

The measurement of glutathione levels using OPA was performed according to the published protocol (Rousar *et al.*, 2012). The cells were lysed in cold 10% metaphosphoric acid for 10 min at 4 °C. The cells were then scraped and cell lysates were centrifuged (5 min, 4 °C, 20.000g). The supernatant was used for the fluorometric assay. Briefly, 50 µL of supernatant was mixed with 1000 µL of phosphate buffer (100 mM; ph 8; with 1 mM EDTA), then 60 µL of the mixture was again added to 900 µL of phosphate buffer and 60 µL of OPA (0.1%; w/v in methanol). After 15 min, 75 µL of hydrochloric acid (1 mM) was added and the samples and standards were incubated at 4 °C until analyzed. The fluorescence was measured at Ex/Em = 340/420 nm using an AmincoBowman 2 spectrofluorometer (Thermo, USA).

2.5 Comparison of bimanes and ortho-phthalaldehyde assays

To compare the use of MCB and MBB to detect glutathione levels in cells, we used the reference fluorometric method based on reaction of glutathione with OPA. All three methods were used for detecting glutathione levels in the two cells lines (HK-2, A549) treated with the glutathione-depleting agent DEM (0.5 and 2 mM). After 3 h incubation, the glutathione concentrations were measured and calculated as percentages of levels in untreated control cells (control = 100%).

An additional comparison was performed between the MCB and OPA assays. The two cell lines (HK-2, A549) were treated with selected toxic compounds. We used DEM (0.5 and 2 mM), *tert*-butyl hydroperoxide (50 and 500 µM), cisplatin (5, 30 and 50 µM), $HgCl_2$ (20 µM), and hydrogen peroxide (500 µM). The toxic compounds were diluted in cell culture medium and the cells were treated for 24 h. The results were used to calculate the correlation coefficient for the MCB-derived method versus the reference method using OPA (n = 20).

2.6 $CdCl_2$ toxicity – glutathione

For cadmium toxicity, HK-2 cells were incubated in cell medium (100 µl) in a 96-well plate and then the cells were treated with $CdCl_2$ (0, 5, 25 and 200 µM) for 2, 6, 10 and 24 hours. Concentration of GSH was detected as described in chapter 2.3.

2.7 $CdCl_2$ toxicity – 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 (0, 5,25 and 200 µM Cd in various time), 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.

2.8 $CdCl_2$ toxicity – reactive oxygen species

We detected ROS production using CM-H_2DCFDA probe (Thermo, USA). The CM-H_2DCFDA probe working solution was prepared fresh in DPBS buffer (pH 7) before each analysis. After incubation, CM-H_2DCFDA was pipetted into the cells. Cells were incubated with CM-H_2DCFDA for 90 min. The final concentration was 1 μM CM-H_2DCFDA. After the incubation period, cells were washed with DPBS buffer (pH 7; 37 ° C). Fluorescence was measured for 60 min using a Tecan Infinite M200 spectrofluorometer at 37°C ($\lambda_{EX/EM}$ = 485/535 nm). A Nikon Eclipse 80i microscope was used for microscopic detection using a FITC fluorescence filter. The resulting intracellular ROS concentration was related to control cells (= 100%).

2.9 $CdCl_2$ toxicity – fragmentation of DNA

The Hoechst 33258 probe was used to stain fragmented DNA in cells. Hoechst 33258 working solution was always prepared fresh in DPBS buffer (pH 7) prior to each analysis. After incubation intervals, cells were pipetted with Hoechst 33258 probe. The cells were incubated with Hoechst 33258 probe for 20 min. Fluorescence was measured using a Tecan Infinite M200 spectrofluorimeter at 37 °C ($\lambda_{Ex/EM}$ = 352/461 nm). The fluorescence intensity in $CdCl_2$ treated cells was expressed relatively to control cells (= 100%).

2.8 Statistical analysis

All experiments were repeated at least three times independently. All values were measured at least in duplicate. The data were expressed as glutathione levels as a percentage of control values (control = 100%). The results were expressed as mean ± SD. The differences between groups of samples measured by different assay were evaluated using one-way analysis of variance (ANOVA), $p = 0.05$ (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). The correlation coefficient between OPA and MCB assays was calculated after analysis of similar samples.

3. Results

3.1 Optimized calculation of GSH levels detected using MCB

Monochlorobimane has been used for detection of cellular glutathione mostly in flow cytometry and fluorescence microscopy (Barhoumi *et al.*, 1995; Bellomo *et al.*, 1992; Cook *et al.*, 1991; Chan *et al.*, 2000; Keelan *et al.*, 2001; Stevenson *et al.*, 2002). According to the literature, the increase of fluorescence caused by the reaction between MCB and glutathione has commonly been measured after 60–90 min of incubating cells with MCB. Then, the difference in fluorescence between the end and start of measurement has been calculated and the results expressed as the percentage of fluorescence intensity relative to controls (Bellomo *et al.*, 1992; Flampouri *et al.*, 2016; Keelan *et al.*, 2001; Sebastia *et al.*, 2003). We followed these conditions for introducing and optimizing the bimane-derived spectrofluorometric glutathione assay.

We used MCB as a probe and determined 40 μM MCB to be the optimal concentration for analyzing GSH levels in cells. The cells were treated with cisplatin (30 and 50 μM) for 24 h, which caused the characteristic glutathione depletion. After addition of MCB to cells, we measured the fluorescence at 394/490 nm for 60 min. As can be seen in Fig. 1, linearity of the fluorescence signal was found only in the initial part of the measurement.

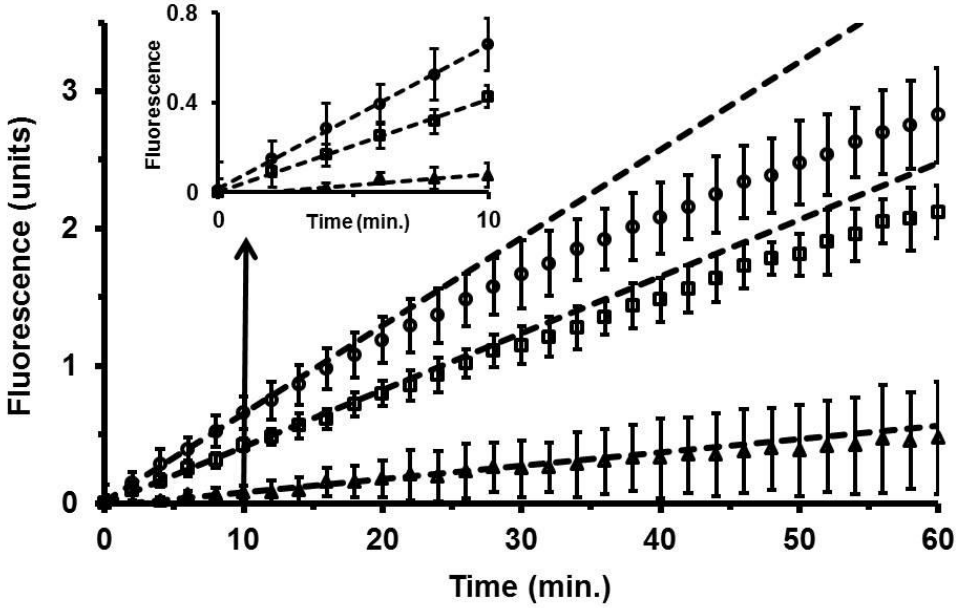

Figure 1.: Assessment of glutathione levels using monochlorobimane (MCB) in cells treated with cisplatin. Human kidney (HK-2) cells were treated with cisplatin for 24 h (○, control; □, 30 μM cisPt; ▲, 50 μM cisPt). Fluorescence ($\lambda_{Ex/EM}$ = 394/490 nm) was detected for 60 min. The fluorescence signal detected for 10 min was estimated by linear regression and the linear curve was fitted onto the graph (dashed line). The linearity of the fluorescence signal after detection for 10 min ($R^2 > 0.99$) is shown in the small graph above. Data are presented as mean ± SD (n=3).

The fluorescence signal at later times was not linear, especially in control cells with higher GSH levels. Inasmuch as the fluorescence signal is linear in the initial part of the measurement up to 10 min ($R^2 > 0.99$), we used the change of fluorescence at this time interval to calculate the slope per 1 min (Δ fluorescence$_{(0\text{-}10\ min)}$/10 min). Then the final slopes (Δ fluorescence/min) were related to the slope of the curve in control cells (control = 100%) and the GSH levels were expressed as percentages of control values. Using this calculation, we found that 30 μM and 50 μM cisplatin caused a decrease in glutathione levels of 64% and 15%, respectively, compared to controls.

In addition, we aimed to estimate whether the GSH levels assessed and calculated after 10 min of incubation with MCB were comparable with GSH levels obtained after 60 min of treatment. For this purpose, we calculated the glutathione levels in cisplatin-treated cells also at this time period. We found that GSH concentrations in cells treated with cisplatin were significantly higher after 60 min of fluorescence detection in comparison with results obtained from the linear part of the curve after 10 min. The glutathione levels were decreased by 75% and 17% in 30 μM and 50 μM cisplatin-treated cells, respectively, in comparison with controls.

3.2 Comparison of GSH levels measured by bimanes with OPA assay

Based on our results, we aimed to confirm if the GSH levels assessed and calculated after 10 min of incubation with MCB were comparable with GSH levels obtained after incubation for 60 min (Table 1). For this purpose, both the "optimized method" (i.e., the linear fluorescence signal was evaluated after 10 min of measurement and calculated as the change of fluorescence per 1 min) and the "standard method" (i.e., the fluorescence signal was evaluated after 60 min of measurement and calculated as the difference in fluorescence signal between 60 min and 0 min) were used. In addition to MCB, we used also another bimane, monobromobimane (MBB), as a probe. Thus, GSH levels were detected using MCB or MBB and evaluated by both calculation methods. In addition, we aimed to estimate the reliability of the two calculation methods, and that is why we compared the results to the reference fluorometric method using OPA.

We used two different cell lines (from kidney and lungs) treated with a typical glutathione-depleting agent, DEM (0.5 and 2 mM). The results in Table 1 show that dose-dependent glutathione depletion was detected using all methods in both cell lines after DEM treatment. The results also show that GSH levels calculated using the standard method were always higher than GSH levels calculated using the optimized method in the case of both MCB and MBB. Another comparison showed that all GSH levels detected using MCB in cells were lower in comparison with GSH levels assessed using MBB.

Finally, GSH concentrations detected using MCB or MBB and calculated by standard or optimized methods were compared to those detected using the reference OPA assay. Table 1 shows that GSH levels obtained by both standard and optimized calculations in MBB-treated cells were always significantly higher than GSH concentrations detected by the reference OPA assay. On the other hand, the use of MCB together with the optimized calculation method resulted in GSH levels similar to those from the reference OPA assay in both cell lines treated with both DEM concentrations. Based on these results, the latter aim of our study was to confirm this outcome.

Table 1.: Comparison of cellular glutathione levels measured by fluorometric methods using monochlorobimane (MCB), monobromobimane (MBB), and ortho-phthalaldehyde (OPA). Human kidney (HK-2) and human pulmonary (A549) cells were treated with diethylmalonate (DEM; 0.5 and 2 mM) for 3 h. Glutathione (GSH) levels were measured by MCB, MBB, or OPA fluorescent probes. GSH levels measured by MCB and MBB were evaluated both by a "standard" calculation method as the difference in fluorescence increase after 60 min and by the "optimized" calculation method from the linear part of the fluorescence signal after 10 min. GSH levels in cells were expressed as percentages of GSH levels in control cells (control = 100%); mean ± SEM (n=6–8). The results were compared with GSH levels measured by OPA assay using ANOVA (*, $p < 0.05$; ***, $p < 0.001$; vs. control of appropriate method).

Cell type	**Probe**	**Concentration of GSH (% of control)**			**Method for calculation of GSH levels (time)**
		Control	**DEM 0.5 mM**	**DEM 2 mM**	
HK-2	MBB	100 ± 4%	59 ± 2% *	31 ± 2% ***	*Optimized (10 min)*
		100 ± 4%	73 ± 2% ***	38 ± 1% ***	*Standard (60 min)*
	MCB	100 ± 7%	44 ± 4%	16 ± 3%	*Optimized (10 min)*
		100 ± 12%	67 ± 3% ***	18 ± 1%	*Standard (60 min)*
	OPA	100 ± 5%	42 ± 2%	17 ± 1%	*(Reference)*
A549	MBB	100 ± 4%	83 ± 2% ***	47 ± 4% ***	*Optimized (10 min)*
		100 ± 6%	100 ± 3% ***	76 ± 2% ***	*Standard (60 min)*
	MCB	100 ± 5%	53 ± 2%	21 ± 1%	*Optimized (10 min)*
		100 ± 10%	66 ± 2%	27 ± 1%	*Standard (60 min)*
	OPA	100 ± 3%	56 ± 6%	23 ± 3%	*(Reference)*

To compare optimized MCB and OPA assays, both HK-2 and A549 cells were treated with a variety of toxic agents (cisplatin, hydrogen peroxide, *tert*-butyl hydroperoxide, $HgCl_2$, and diethylmalonate). After incubation, GSH levels measured using the MCB assay and calculated using the optimized method were correlated with those obtained by OPA assay. As shown in Fig. 2, the correlation between MCB/OPA results was very strong, with correlation coefficient $r = 0.96$.

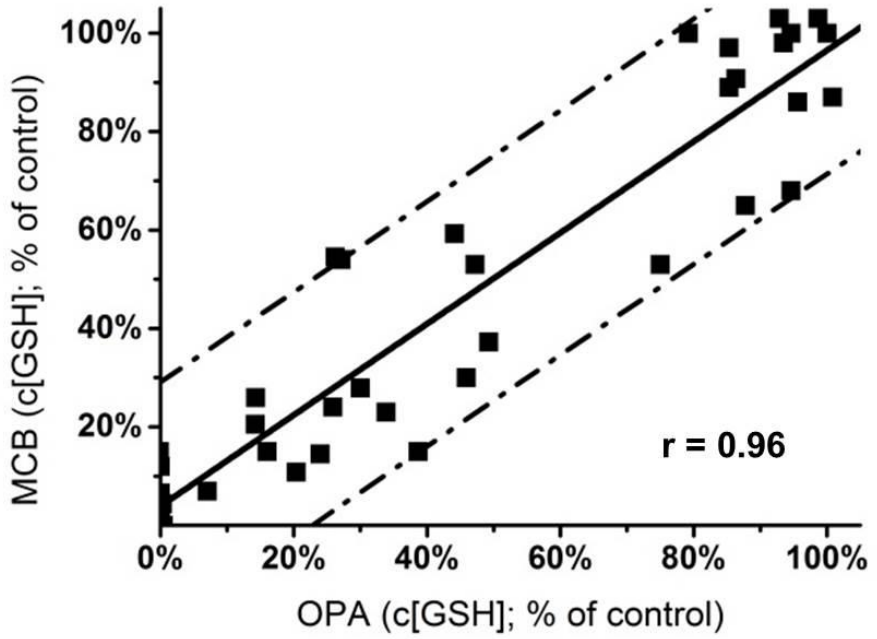

Figure 2.: Correlation of glutathione levels measured by optimized monochlorobimane (MCB) assay and ortho-phthalaldehyde (OPA) assay. The human kidney (HK-2) and human pulmonary (A549) cells were treated with selected toxic agents (cisplatin, hydrogen peroxide, tert-butyl hydroperoxide, HgCl2, and diethylmalonate) for 24 h (n = 20). Glutathione levels determined using the optimized MCB assay (i.e., after 10 min of treatment with MCB) and OPA assay were well correlated with one another (r = 0.96). The equation of the correlation curve was y = 0.926x + 3.871 (solid line). The curve is shown with the confidence interval (dashed and dotted lines).

3.3 $CdCl_2$ toxicity – glutathione

After incubation of cells with $CdCl_2$, the intracellular glutathione concentration was detected by MCB. As shown in Figure 3, no effect was observed at low $CdCl_2$ concentrations (5 and 25 μM) at any time interval. A significant decrease in GSH concentration was observed in cells exposed to 200 μM $CdCl_2$ at all time intervals. The most significant decrease in GSH concentration after exposure to 200 μM $CdCl_2$ was observed after 24 hours incubation by 95% compared to control cells.

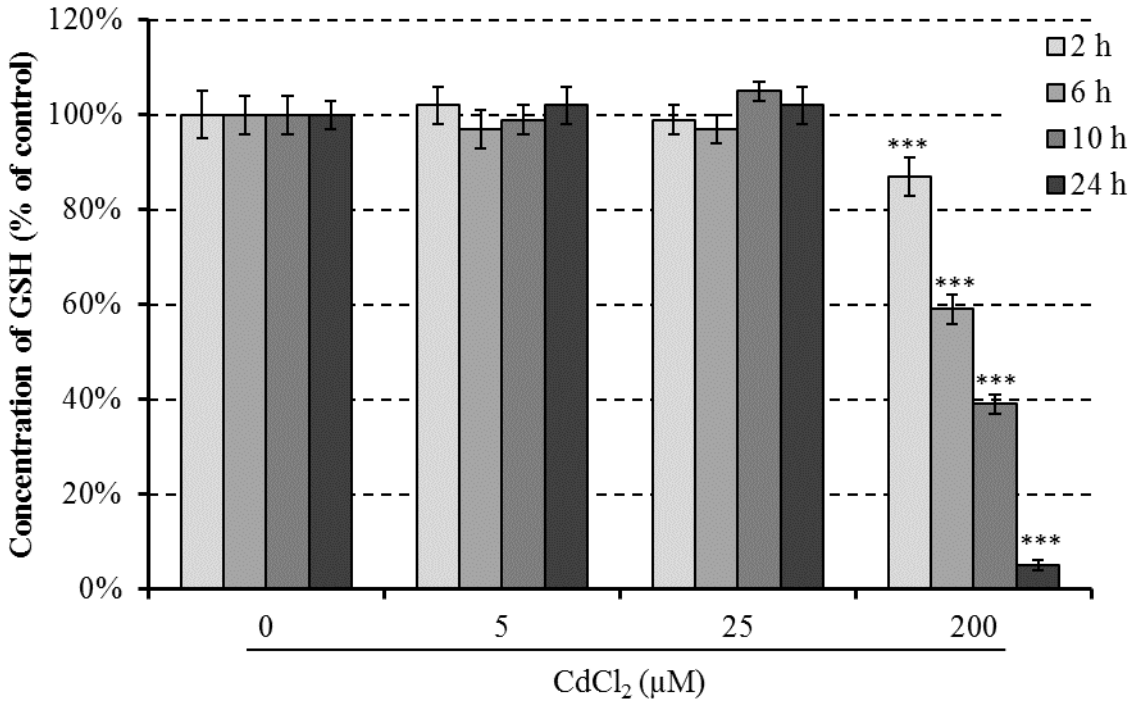

Figure 3.: Effect of $CdCl_2$ on glutathione concentration in HK-2 cells. The human kidney (HK-2) cells were treated with $CdCl_2$ (0–200 μM) for 2, 6, 10 and 24 h. (Control cells = 100 %); mean ± SD; ***; $p<0.001$; vs. control of appropriate time interval; n=6-10).

3.4 $CdCl_2$ toxicity – mitochondrial membrane potential

The effect of $CdCl_2$ on the membrane potential of mitochondria (ΔΨ) was tested in the HK-2 cell line. Detection was performed after incubation of cells with $CdCl_2$ using the JC-1 probe. ΔΨ values fluctuated depending on $CdCl_2$ concentration and time interval. As shown in Figure 4, a significant decrease of ΔΨ at 2 h was observed in 200 μM $CdCl_2$. Another decrease of ΔΨ occurred in 25 μM $CdCl_2$ treated cells at 10 h. The most significant decrease of ΔΨ was observed after treatement with 200 μM $CdCl_2$ at 24 h.

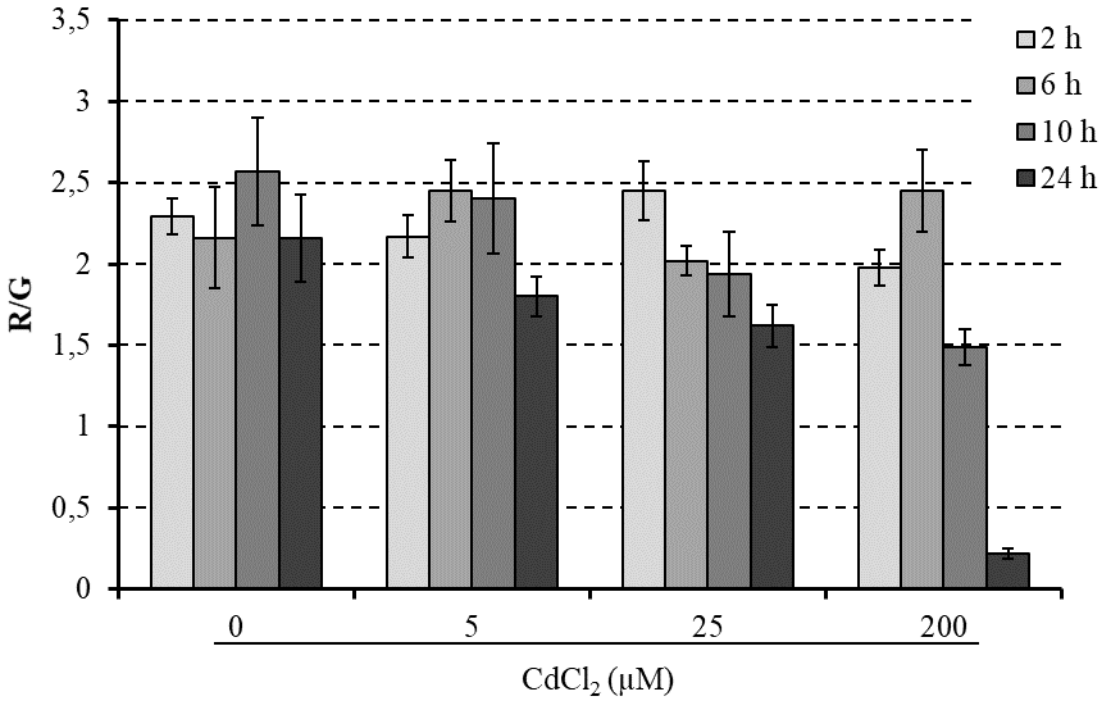

Figure 4.: Effect of $CdCl_2$ on the mitochondrial membrane potential detected by JC-1 in HK-2 cells; the mitochondrial membrane potential was expressed as the R/G ratio. The human kidney (HK-2) cells were treated with $CdCl_2$ (0–200 μM) for 2, 6, 10 and 24 h; mean ± SD; (***; $p<0.001$; vs. control of appropriate time interval; n=6-10).

3.5 $CdCl_2$ toxicity – reactive oxygen species

The production of reactive oxygen species was detected using the CM-H_2DCFDA probe. As shown in Figure 5, after two hours incubation of the cells with $CdCl_2$ (5 and 25 μM), there was a significant reduction in CM-H_2DCFDA probe fluorescence (40% reduction). In contrast, 200 μM $CdCl_2$ increased fluorescence by 48%. After 6 h with 200 μM $CdCl_2$, the fluorescence was significantly increased by 211% compared to the control cells. After 24 h with 25 μM $CdCl_2$, an increase in fluorescence by 104% compared to control cells was observed.

3.6 $CdCl_2$ toxicity – fragmentation of DNA

The Hoechst 33258 probe was used to detect the effect of cadmium on DNA fragmentation in HK-2 cells. As seen in Table 2, a significant increase in fluorescence, which can indicate the presence of fragmented DNA, is observed only in 200 μM $CdCl_2$ at both 10 h and 24 h.

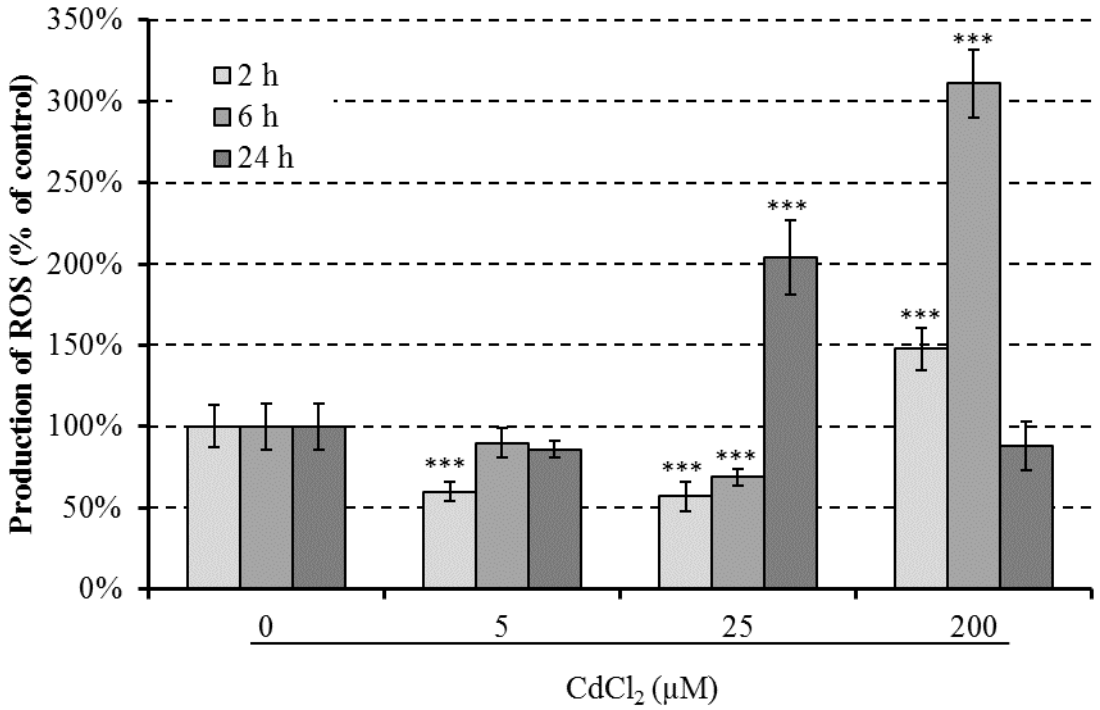

Figure 5.: Effect of cadmium on the production fo ROS detected by CM-H_2DCFDA in HK-2 cells; Concentration of ROS was expressed as % of control cells (control cells = 100%). The human kidney (HK-2) cells were treated with $CdCl_2$ (0–200µM) for 2, 6, 10 and 24 h; mean ± SD; (***; $p<0.001$; vs. control of appropriate time interval; n=6-10).

Table 2: Influence of $CdCl_2$ on DNA fragmentation in HK-2 cells (5, 25 and 200 µM) at 2, 6, 10 and 24 h using the Hoechst 33258 probe ($\lambda_{Ex/EM}$ = 352/461 nm). The DNA fragmentation was expressed as % of control cells (control cells = 100%); mean ± SD; **, (***; $p<0.01$; ***, $p<0.001$ (vs. control of appropriate time interval); n=6-10.

Time (h)	**$CdCl_2$ (µM)**			
	0	**5**	**25**	**200**
2	100 ± 11 %	94 ± 15 %	65 ± 13 %**	61 ± 17 %***
6	100 ± 50 %	107 ± 28 %	98 ± 5 %	87 ± 44 %
10	100 ± 30 %	69 ± 26 %	70 ± 21 %	563 ± 26 %***
24	100 ± 13%	74 ± 16 %	86 ± 8 %	561 ± 36 %***

4. Conclusion

A number of methods have been developed for assessing glutathione in cells. Fluorometric assays have been preferred for their high sensitivity and specificity, especially with OPA as a probe for glutathione detection. The bimanes are other probes usable for fluorometric detection of glutathione. They are non-fluorescent until reacted with a thiol. Their fluorescence after reaction with GSH and their ability to penetrate into the cell through cell membrane can be used for detecting glutathione within intact cells(Kamencic *et al.*, 2000; Kosower *et al.*, 1979)(Kamencic *et al.*, 2000; Kosower *et al.*, 1979)(Kamencic *et al.*, 2000; Kosower *et al.*, 1979). Although the both bimanes can be applied for detection of cellular thiols, MCB was proven to be more specific for detecting glutathione than MBB. That is because the reaction between GSH and MCB is more specific and is predominantly catalyzed by a cellular enzyme, glutathione-S-transferase. This is the reason why glutathione levels measured in different cell lines may be influenced by unlike glutathione-S-transferase activity. First, we used MCB as a probe for GSH assessment. The optimal concentration of MCB in cells was determined to be 40 μM. Also optimized were the incubation time of MCB in cells together with the duration of fluorometric measurement. We monitored the fluorescence for 60 min after MCB addition and found that fluorescence signal was linear only for 10 min. The results estimated from fluorescence signal in cells treated with cisplatin confirmed that GSH concentrations evaluated after 10 and 60 min of incubation with MCB differ considerably. Based on these results, we aimed to characterize the effect of incubation time in other experiments. Thus, we carried out additional tests in two types of cells treated with a typical glutathione-depleting agent, DEM. We used DEM because it causes fast glutathione depletion in comparison to other glutathione depleting agents. The extent of glutathione depletion was characterized using the two bimanes MCB and MBB after incubation for 10 and 60 min. We proved that the time of incubation of cells with MCB significantly influenced the GSH concentrations obtained, with GSH levels determined after 10 min of incubation with MCB always being significantly lower in comparison with GSH levels calculated from fluorescence after 60 min of treatment. This finding was obtained in both tested cell lines treated with MCB and also with MBB. In addition, we confirmed according to our expectations that GSH levels detected using MBB are considerably overestimated in comparison with those for MCB. Finally, we aimed to compare GSH results measured using the optimized MCB method with a reference assay of glutathione. We chose the optimized OPA-derived fluorometric glutathione assay for comparison with the MCB method. Our results showed that GSH levels obtained after 10 min of incubating MCB with cells are similar to GSH levels measured using OPA. This outcome was found in both cells lines treated with DEM. In addition, we confirmed that GSH levels detected using the optimized MCB method are strongly correlated with those estimated using OPA assay in cells treated with a number of various toxic agents.

In the next part of the study, we detected the effect of $CdCl_2$ on GSH concentration, $\Delta\Psi$, production of reactive oxygen species and DNA fragmentation. MCB was used to detect GSH concentration. A decrease in GSH concentration after 200 μM $CdCl_2$ treatment was observed at 2, 6, 10 and 24 h. This is due to the fact that GSH is an antioxidant that is present in cells at high concentrations and thus it is very effective in removing ROS. Furthermore, in our work we detected a significant decrease $\Delta\Psi$

especially in 200 μM $CdCl_2$, which corresponds to other results. Lastly, we detected fragmented DNA in cells after exposure to $CdCl_2$. The highest fluorescence signal of Hoechst 33258 was detected in cells affected by 200 μM $CdCl_2$ after 10 and 24 h. Each of our results confirmed the highest toxicity of 200 μM $CdCl_2$. It is important to consider selecting a fluorescent probe for the parameter we want to detect when characterizing cell damage. The cells are unique, complement and dynamic systems whose properties have to be taken into account in their characterization and understanding using biochemical methods, especially using intracellular fluorescent probes.

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6. List of Student´s Published Works

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