

Original Research

# Impact of Zinc Sulfate Exposition on Viability, Proliferation and Cell Cycle Distribution of Epithelial Kidney Cells

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## Abstract

Zinc is an essential trace element effective at very low concentrations, but it is also an important environmental pollutant dangerous after excessive intake or exposure. The aim of this study was to evaluate *in vitro* nephrotoxicity of zinc sulfate heptahydrate  $ZnSO_4 \times 7H_2O$  (1, 10, 50, 100, 200 mg/l) using rabbit epithelial kidney cells RK13 as the model cell line. The xCELLigence system (RTCA) for real-time monitoring of cell response and the end-point assays for determining metabolic activity (MTT test), cytotoxicity (LDH test), proliferation (BrdU test) and cell cycle analysis were compared. Exposure to zinc sulfate produced dose-dependent cytotoxicity. The inhibition concentration IC<sub>50</sub> value for xCELLigence monitoring was 101.8 mg/l, for MTT test 135.9 mg/l and 197.4 mg/l for BrdU test. There was a significant correlation between used assays ( $p \leq 0.05$ ) except for the LDH test. Based on mean of IC<sub>50</sub> values, the effect of zinc sulfate at 150 mg/l on cell cycle was evaluated. We observed the accumulation of cells in S phase accompanied with the reduction of cells in the G<sub>0</sub>/G<sub>1</sub> phase. In conclusion, the mean of our IC<sub>50</sub> values for kidney cells is relatively high in comparison to the recommended as well as therapeutic daily doses.

**Keywords:** xCELLigence, MTT test, BrdU assay, LDH, cell cycle

## Introduction

Zinc is an essential trace element with important biological functions that control many processes in the cell, such as DNA synthesis, normal growth, brain

development, behavioural response, fetal development and bone formation [1], regulation of response to insulin, reproduction, antioxidant cellular defense systems [2] and protein synthesis [3]. Zinc is effective at very low concentrations and therefore its excessive amount in body fluids could be harmful [4].

Zinc is a serious environmental pollutant and is present in the environment. Toxicity of zinc is a

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growing concern because of nanoparticles of zinc oxide (n-ZnO) being widely used in electronics and personal care products that accumulate in aquatic and terrestrial environments from many sources. The main route of exposure is inhalation, through the skin or by ingestion. After penetration to the body it causes irritation of the respiratory or digestive system, dental deterioration and ulceration of the skin. Zinc fumes are dangerous too, and may cause fever, chills, nausea, vomiting, muscular aches and weakness [1]. Zinc administration in experimental animals at relatively high amounts can initiate tissue/cellular damage, eventually leading to death [5]. Renal cells are often exposed to higher concentrations of xenobiotics, because kidneys play the most important role in eliminating various drugs and toxic substances. According to nature, the dose, route, and duration of exposition display diverse alterations in renal function ranging from mild tubular dysfunction to severe renal failure [6]. Zinc can induce damage to kidney cells by a mechanism dependent on zinc ions entering the cell, binding to the cell organelles and disrupting cellular processes [5]. Regarding renal excretion of zinc, it is known that about 2-3 mg of zinc are replenished daily in humans [7] and less than 5% is eliminated by kidneys [8]. According to the National Institutes of Health [9], recommended dietary reference zinc intakes for adults is 8-11 mg daily. However, higher therapeutic doses (150-660 mg daily) are indicated in some diseases, for example diabetes [10], urinary aminoaciduria [5], chronic kidney disease [11] or Wilson's disease [7]. Although many *in vitro* studies have indicated zinc toxicity [12-19], only a few studies have focused on its nephrotoxicity [5, 20]. Oral treatment with zinc can change energy metabolism and cause mitochondria and cell membrane impairment in rat kidneys via inducing nephrotoxicity [21]. Therefore, we evaluated the toxicity of zinc sulfate, the common chemical form of supplemental zinc, using rabbit kidney epithelial RK13 cell line. In this work, the xCELLigence system was used for continuous real-time monitoring of cell response after zinc sulfate treatment and the commonly used assays for determining metabolic activity, proliferation and cell cycle of treated cells were employed.

## Experimental

### Chemicals

Tested substance *Zinc sulfate heptahydrate* –  $\text{ZnSO}_4 \times 7\text{H}_2\text{O}$  of analytical grade purchased from Sigma Aldrich (St. Louis, USA) was diluted in 1% dimethyl sulphoxide (DMSO; Lachema, Brno, Czech Republic) and tested at final concentrations of 1, 10, 50, 100, 150 and 200 mg/l. In our experiment, DMSO at 1% concentration did not affect the behaviour of cells in comparison to control cells without treatment. Zinc

sulfate solutions were sterilised by filtration through a 0.22  $\mu\text{m}$  filter (Milipore, Watford, UK).

### Cell Cultivation

Epithelial RK13 cells (rabbit kidney cell line) were obtained from the American Type Culture Collection (ATCC CCL-37). Cells were cultured in Earl's Minimal Essential Medium (EMEM; Lonza, Valais, Switzerland) supplemented with 10% (v/v) foetal bovine serum (FBS; Lonza, Valais, Switzerland) and 50 mg/l gentamicin (Sigma Aldrich; St. Louis, USA) in a humidified atmosphere of 5%  $\text{CO}_2$  at 37°C. In experiments, RK13 cells were cultured in complete cultivation medium without antibiotics and were regularly checked for the absence of mycoplasma contamination [22].

### xCELLigence System (RTCA; Real-Time Cell Analyser)

The real-time monitoring of cell response to zinc sulfate was monitored using the xCELLigence system or real-time cell analyzer (RTCA; Roche, Applied Science, Mannheim, Germany), according to the manufacturer's instructions. This relatively new method was described in many studies [23-25]. Briefly, 100  $\mu\text{l}$  of antibiotic-free culture medium was added into 16-well E-plates (Roche, Applied Science, Mannheim, Germany) for the background measurement. Subsequently,  $1.5 \times 10^4$  cells/well in 50  $\mu\text{l}$  of RK13-cell medium were seeded in plates and inserted into plate station, kept inside a  $\text{CO}_2$  incubator at 37°C with 5%  $\text{CO}_2$  and humidified atmosphere. After 24 h when RK13-cells were within a log phase, the zinc sulfate in 50  $\mu\text{l}$  of culture medium was added to the cells. The cells treated with 1% DMSO only served as negative or solvent control. The cell response to zinc sulfate was monitored for 48 h. Cell index (CI) was measured automatically by the RTCA system once per hour until the end of the experiment. The parameter measured to specify the effect of the tested compounds was assessed as the normalized CI. The IC50 value at a given time point (24 h after zinc sulfate treatment) was calculated based on the concentration producing 50% reduction of normalized CI value relative to solvent control.

### MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) Test

Metabolic activity was evaluated using cellular thiazol blue (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide – MTT) uptake as an indicator of mitochondrial membrane integrity. Briefly, RK13 cells were seeded at a density of  $2.2 \times 10^4$  cells/well in 100  $\mu\text{l}$  of antibiotic-free culture medium in standard 96-well culture plate (Greiner-bio-one, Kremsmünster, Austria). After 24 h cells were treated with zinc sulfate at tested concentrations. After 24 h medium was refreshed

and cells were incubated with 0.5 g/l of MTT for 4 h in the dark. Formazan crystals were solubilised by the addition of concentrated DMSO. Sorensen's glycine buffer (0.1 mol/l glycine, 0.1 mol/l NaCl, pH 10.5) was added to stabilize the final product. The absorbance was recorded at 560 nm (Synergy HT, Biotek, Winooski, VT, USA). Cell viability (V, %) was expressed as fraction of the negative or solvent control.

#### Lactatedehydrogenase (LDH) Leakage Assay

RK13 cells were seeded in 100 µl of antibiotic-free culture medium in a 96-well culture plate (Greiner-bio-one, Kremsmünster, Austria) at a density  $2.2 \times 10^4$  cells/well and incubated for 24 h in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C. The growth medium was changed to a maintenance medium with 1% (v/v) FBS, and different concentrations of zinc sulfate were added for an additional 24 h. After the exposure period a non-radioactive colorimetric assay was used to quantify cytotoxicity/cytolysis by measuring LDH activity released from damaged cells (Cytotoxicity Detection KitPLUS, Roche Diagnostics, GmbH, Germany). The LDH test was carried out following manufacturer's instructions. Optical density (OD) was measured in an ELISA-multiwell reader Synergy HT (Biotek, Winooski, VT, USA) at 450 nm. To calculate percent cytotoxicity in each plate, a low-control (LC) and a high-control (HC) were set up and the percentage of cytotoxicity was calculated according to equation:

$$\% C = (OD_{\text{zinc}} - OD_{\text{LC}} / OD_{\text{HC}} - OD_{\text{LC}}) \times 100$$

...in which OD<sub>zinc</sub> is the mean value of OD of treated cells, OD<sub>LC</sub> (low control) is the mean value of OD-cells treated with the solvent control (DMSO) and OD<sub>HC</sub> (high control) is the mean value of OD cells treated with the lysis buffer (the maximum releasable LDH activity in the cells) measured at 450 nm.

#### Bromodeoxyuridine (BrdU) Proliferation Test

Cells were seeded the same way as in LDH assay, in 100 µl of antibiotic-free culture medium in a 96-well culture plate (Greiner-bio-one, Kremsmünster, Austria) at a density of  $2.2 \times 10^4$  cells/well and overnight incubated in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C. Subsequently, BrdU (10 µM) and different concentrations of zinc sulfate were added to the RK13 cells for 24 h. The colorimetric immunoassay was used to quantify cell proliferation (Cell Proliferation ELISA Kit, BrdU-colorimetric, Roche Diagnostics, GmbH, Germany). This test is based on the measurement of 5-bromo-2-deoxyuridine (BrdU) incorporation during DNA synthesis. Briefly, culture medium was removed after the exposure period, DNA of the cells were denaturated and cells on the bottom of the wells were fixed. Anti-BrdU-

peroxidase labelled conjugate (100 µl) was added to the cells for 90 min at 25°C. The immune complexes were detected by the subsequent substrate reaction (100 µl substrate solution) for 30 minutes at room temperature. The reaction was stopped by 25 µl 1 M H<sub>2</sub>SO<sub>4</sub> and the optical density (OD) was measured in an ELISA multiwell reader Synergy HT (Biotek, Winooski, VT, USA) at 450 nm. The mean optical densities were converted into a percentage of proliferative activity (% PA) according to the following equation:

$$\% PA = (OD_{\text{zinc}} / OD_{\text{control}}) \times 100$$

...in which OD<sub>zinc</sub> is the mean value of OD of cells treated with zinc sulfate and OD<sub>control</sub> is the mean value of OD of cells treated with the solvent only.

#### Cell Cycle Analysis

The effect of the zinc on RK13 cell cycle progression was assessed using flow cytometry. Cells were seeded at density  $1.72 \times 10^5$  cells per well in standard 24-well plates. Subsequently, overnight culture was treated with zinc sulfate at a final concentration of 150 mg/l. After 24 h incubation, both adherent and floating cells were harvested and washed in ice-cold phosphate buffered solution (PBS). Washed cells were fixed with ice-cold 60% ethanol and stored at 4°C overnight. Fixed cells were washed in PBS and resuspended in staining solution (25 mg/l propidium iodide and 100 mg/l ribonuclease A in PBS) and then incubated for 1 h at 37°C in the dark. Analysis was performed in a flow cytometer BD FACS Canto (BD Biosciences, San José, CA, USA) using BD FACS Diva software (BD Biosciences). The position of cells was gated on the dot plot FSC-A vs. SSC-A and subsequently doublets and aggregates were excluded from analysis on dot plot SSC-A vs. SSC-H. DNA fluorescence signal intensity on histograms FL-2 (575/25 nm) vs. counts was used for evaluating cell cycle phases. The results are expressed as a percentage of cells having subG1 DNA content and cells in the G0/G1, S and G2/M phases. A negative control consisted of cells exposed to medium with DMSO only.

#### Statistical Analysis

Results were expressed using the GraphPad Prism version 3.00 software (GraphPad Software, San Diego, CA, USA) as mean±standard deviation (SD; n = 3) by one-way analysis of variance (ANOVA), followed by Dunnett's multiply comparison test. The correlation coefficients (R<sup>2</sup>) were calculated by Pearson's test.

### Results and Discussion

Many *in vitro* studies have indicated zinc toxicity, for example to prostate cells [12], murine BAF-3 cells,

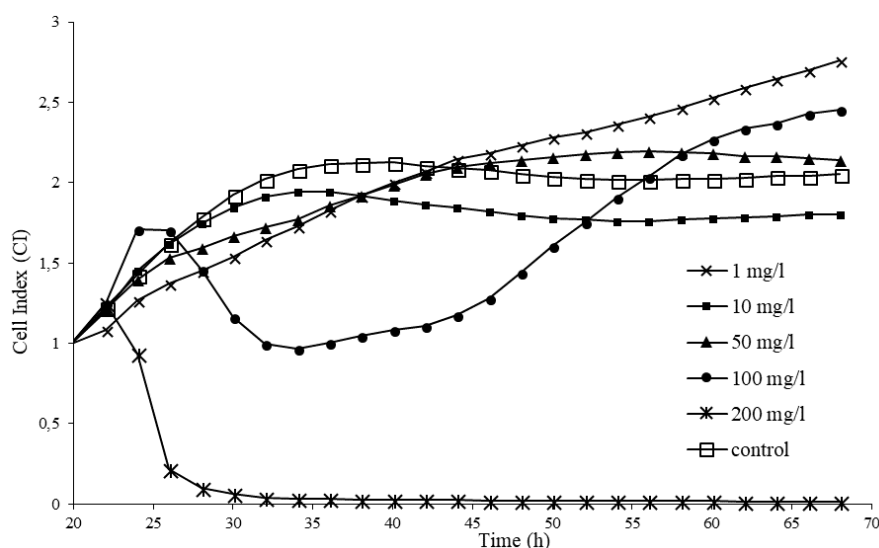


Fig. 1. The effect of zinc sulfate (1, 10, 50, 100, 200 mg/l) during 48 h exposition on the RK13 cell index (CI) measured by xCELLigence system (RTCA); results expressed as mean of normalized CI (n = 3).

human Jurkat cells [14], neuronal cells [13] as well as intestinal cells [2]. However, only a few studies have been focused on the nephrotoxicity of zinc ions [5, 20, 21]. Therefore, we evaluated the toxicity of zinc sulfate using the kidney RK13 cell line. The concentration range has been selected according to other publications about zinc cytotoxicity [19, 26] as well as our preliminary studies.

At first, the xCELLigence system (RTCA) was used for real-time monitoring of cell response after zinc sulfate treatment. The RTCA system utilizes a series of microwells whose bottoms are 80% covered with microelectrodes that measure impedance changes caused by cells interacting with the microelectrodes [23]. RTCA curves respond to treatment and clearly demonstrate cell-dependent toxic responses in

decreasing the measured CI. The effect of zinc sulfate during 48 h exposure of RK13 cells is recorded in Fig. 1. Cells treated with lower concentrations of zinc sulfate (1, 10, 50 mg/l) were grown similarly to control cells. Zinc sulfate at 100 mg/l concentration seems to decrease cell index in the first hours after treatment, but after a period of time the cells were able to continue growing with a slope similar to that of the control cells during their log phase at approximately 68 h into the experiment or about 48 h after treatment. A possible explanation for this observation is that the administered zinc sulfate dose did not kill all the cells in the well, or produce permanent damage to prevent them from growing. Thus, the surviving cells could continue growing after they adjusted to the conditions or the zinc sulfate concentration in the well decreased sufficiently

Table 1. The effect of zinc sulfate on adherence (RTCA), viability (MTT), cytotoxicity (LDH) and proliferation (BrdU) of the RK13 cells after 24 h; results are expressed as mean±SD (n = 3).

Tests		Concentration of zinc sulfate					
		1 mg/l	10 mg/l	50 mg/l	100 mg/l	200 mg/l	Control
RTCA	CI	2.15±0.16	1.99±0.09	2.08±0.01	1.17±0.10***	0.02±0.01***	2.09±0.01
	%	102.68±2.15	95.40±4.5	99.36±0.66	56.15±4.93	1.15±0.54	100
MTT	OD	1.37±0.44**	1.21±0.41	1.27±0.45	1.00±0.49***	0.23±0.01***	1.20±0.07
	% V	113.47±4.5	100.44±4.2	105.25±4.53	83.46±4.94	19.22±0.84	100
LDH	OD	1.09±0.00*	1.05±0.02	2.39±0.07***	2.58±0.00***	2.24±0.01***	1.00±0.03
	% C	3.69±0.18	1.91±0.76	55.50±2.77	62.76±0.02	49.38±0.48	100
BrdU	OD	0.75±0.08	0.64±0.07	0.56±0.06	0.68±0.06	0.18 ±0.01***	0.61±0.04
	% PA	123.76 ±11.03	104.84±9.37	96.10±7.65	107.82±10.78	29.24±2.03	100

CI – cell index, OD – optical density, V – viability, C – cytotoxicity, PA – proliferative activity  
Significant compared with control: \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$

Table 2. The IC50 values of zinc sulfate measured by different assays after 24 h exposure of RK13 cell line; results expressed as mean (n = 3).

RTCA	IC50	101.8 mg/l
	R <sup>2</sup>	0.9873
MTT	IC50	135.9 mg/l
	R <sup>2</sup>	0.9759
LDH	IC50	ND
	R <sup>2</sup>	-
BrdU	IC50	197.4 mg/l
	R <sup>2</sup>	0.8460

R<sup>2</sup> – Pearson's R square coefficient, *p* – *p* value; RTCA (Real-Time Cell Analyser); MTT – (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; LDH – lactate dehydrogenase; BrdU – 5-bromo-2-deoxyuridine

through metabolism or breakdown. With higher concentrations of zinc sulfate (200 mg/l), CI decreased in the first few hours to zero, meaning that no viable cells are attached to the microelectrodes, indicating the highest cytotoxicity.

Subsequently, based on the RTCA curves, we decided to assess zinc sulfate nephrotoxicity using end-point cytotoxic assays after 24 h treatment. These assays revealed that a 24 h exposure of RK13 cells to different concentrations of zinc sulfate produced a dose-dependent reduction of cell index (RTCA), cytotoxicity (LDH test), metabolic activity (MTT test) and proliferative activity of cells (BrdU test). The response of RK13 cells is shown in Table 1. For these different assays, IC50 values were calculated too (Table 2). IC50 values at 24 h of incubation were calculated as the concentration of zinc sulfate inhibiting CI, metabolic activity, proliferative activity or viability of cells by 50% in comparison to a solvent-treated control. The solvent used in our study, DMSO with a final concentration of 1%, did not affect the viability of RK13 cells. We found out that the IC50 values of RK13 cells for zinc sulfate was 101.8 mg/l (corresponding to 354.0 μM) for RTCA; 135.9 mg/l (corresponding to 472.6 μM) for MTT and 197.4 mg/l (corresponding to 686.2 μM) for BrdU (Table 3). These results are comparable with earlier research of Sanchez-Martin et al. [15], which showed that IC50 values of undifferentiated PC12 cells (from pheochromocytoma of the rat adrenal medulla) for zinc sulfate was up to 340 μM. The suitability of the usage of RTCA in combination with other assays for toxicity measurement was also confirmed by Xing et al. [23].

The toxic effect of zinc on kidney proximal tubular cells (LLC-PK1) has been studied recently and it has shown that zinc nephrotoxicity is in relation to zinc ions entering the cell, binding to the cell organelles and disrupting cellular processes rather than damage

initiated by free radical production [5]. On the contrary, other studies showed that zinc toxicity is accompanied by a massive ROS generation [15, 27].

It has been previously reported that diverse cytotoxicity assays can give different results due to the discrepancy of the assays employed as well as different sensitivity of cell lines to the test agent [28, 29]. We found that there is an excellent correlation between employed tests except the LDH cytotoxicity test (Table 3), for which the IC50 value was not detectable (Table 2). LDH leakage assay is based on release of the enzyme after disintegration of the cytoplasmic membrane [30]. Discrepancy between tests is most evident at the highest tested zinc sulfate concentration, where an increase in cytotoxicity is lower for LDH compared to other tests (Table 1). As mentioned above, diverse cytotoxicity/viability assays can give different results due to the discrepancy of the assays employed. The LDH test is based on release of the enzyme after disintegration of the cytoplasmic membrane and it is mainly used for determining cell necrosis [30] while other tests evaluate cells or monolayer attachment (RTCA), metabolic activity (MTT test) or proliferative activity (BrdU test) of cells – not their viability. Significantly increased LDH concentration (Table 1; *p*<0.001) indicate that the mode of cell death induced by zinc sulfate was necrosis. This is supported by the results of cell cycle analysis (Fig. 2). There was no increase in the number of cells having subG1 DNA content that is considered an apoptosis marker. On the contrary, Sharma et al. [27] found that zinc oxide nanoparticles induced in human liver HepG2 cells apoptosis mediated by mitochondria pathway, but independent of JNK and p38 pathways.

In this study, the effect of zinc sulfate on the distribution of RK13 cells in each phase of the cell cycle was also determined. The selected concentration of zinc sulfate was based on the mean of our IC50 values

Table 3. Correlation between the tests used for zinc sulfate nephrotoxicity evaluation.

		RTCA	MTT	LDH	BrdU
RTCA	P	-	0.0029	0.3543	0.0495
	R <sup>2</sup>		0.9644	0.2848	0.7727
MTT	P	0.0029	-	0.4405	0.0139
	R <sup>2</sup>	0.9644		0.2077	0.8996
LDH	P	0.3543	0.4405	-	0.4784
	R <sup>2</sup>	0.2848	0.2077		0.1788
BrdU	P	0.0495	0.0139	0.4784	-
	R <sup>2</sup>	0.7727	0.8996	0.1788	

R<sup>2</sup> – Pearson's R square coefficient, *p* – *p* value; RTCA (Real-Time Cell Analyser); MTT – (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; LDH – lactate dehydrogenase; BrdU – 5-bromo-2-deoxyuridine

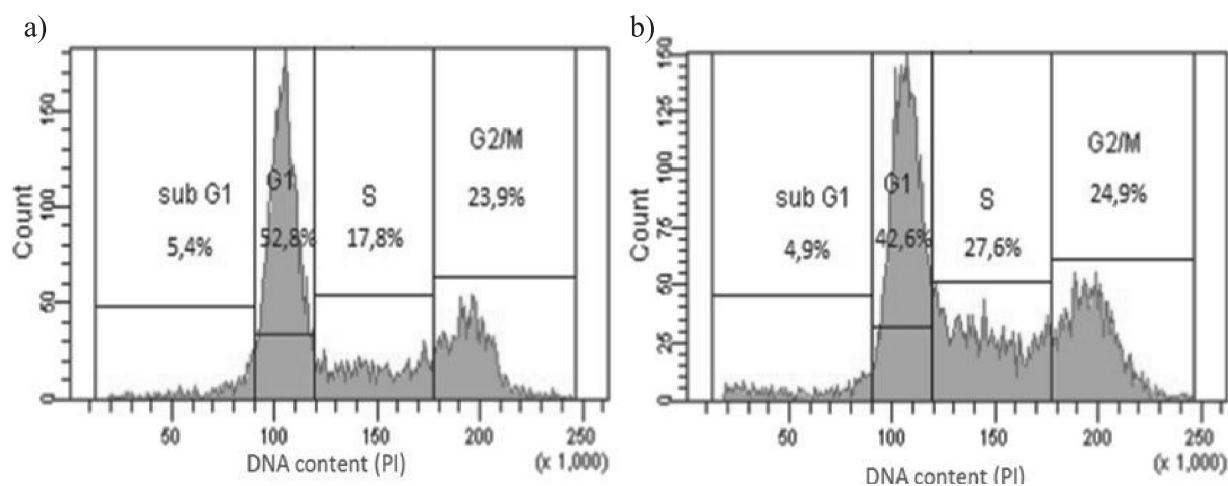


Fig. 2. Effect of zinc sulfate on the cell cycle of the RK13 cell line after 24 h incubation: A – negative control, B – cells were treated with zinc sulfate in 150 mg/l concentration; results are expressed as a mean ( $n = 3$ ); PI – propidium iodide.

(150 mg/L, 525  $\mu$ M). As depicted in Fig. 2, increased accumulation of cells in the S phase accompanied by the consequent reduction of cells in the G0/G1 phase was observed after 24 h treatment of RK13 cells. On the contrary, Wang et al. [19] showed that zinc sulfate (150–200  $\mu$ M) induced the arrest of human adenocarcinoma MDAMB231 cells in the G1 and G2/M phases. Moreover, the fraction of apoptotic MDAMB231 cells increased significantly in the presence of 200  $\mu$ M zinc sulfate. Similarly, zinc induced G2/M phase arrest of human prostate cancer cells in an earlier study of Liang et al. [12]. However, Yuan et al. [16] observed no alteration in the cell cycle of human lung adenocarcinoma (A549 cells) after treatment with lower concentrations of zinc sulfate (up to 100  $\mu$ M) for 6, 12 and 24 h. Mentioned studies that are not in correlation with our results used for zinc cytotoxicity assessment rapidly grew cancerous cell lines. However, some studies [17, 18] that used macrophages as an *in vitro* model are in accordance with our findings. They similarly observed zinc-induced S-G2/M cell cycle arrest accompanied with a decrease of cells in the G1 phase. The effect of zinc is clearly defined on different trophic levels after exposure to n-ZnO of freshwater mussels *Unio tumidus* oxidative injury, DNA fragmentation and caspase-3 mediated apoptosis were observed [31].

### Conclusions

In our work the combination of MTT, LDH, and BrdU tests with RTCA was employed. Based on the obtained results, we can conclude that the real-time monitoring of the cell response correlates significantly with commonly used biological methods for toxicity measurement except for the LDH test based on different principles. In the LDH test the higher toxicity caused an increased amount of released LDH, while in other tests the higher toxicity caused a decrease in the following

parameters. The IC<sub>50</sub> values were calculated for each of the used tests and the lowest inhibition concentration of zinc sulfate was recorded using xCELLigence system and the highest for the BrDU test. We found that the mean IC<sub>50</sub> value of zinc sulfate (150 mg/l) caused the accumulation of RK13 cells in S phase with a simultaneous decrease of cells in the G1 phase. The toxic effect of zinc sulfate on rabbit kidney increased at a concentration-dependent manner. In conclusion, the IC<sub>50</sub> values were relatively high in comparison to recommended as well as therapeutic daily doses.

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### Conflict of Interest

The authors declare no conflict of interest.

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