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Original Paper

Cytotoxic effect of aluminium ions on unicellular eukaryotic organism

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Aluminium is abundant in nature, food, or water and thus its exposition is part of everyday life. However, overexposure can result in cellular malfunctions. Therefore, the aim of this study was to investigate the effects of aluminium on eukaryotes, with the use of *Schizosaccharomyces pombe* as model organism. Spectrophotometry at OD_{600}, inductively-coupled plasma optical emission spectroscopy (ICP-OES) and microscopy techniques were used to analyse aluminium responses on the living system. Our results revealed that exposition of increasing aluminium concentrations lead to cell growth inhibition in a concentration dependent manner. Furthermore, aluminium incorporation by the cell from media markedly increased with rising Al concentration. Our results indicate that the yeast self-protection system in the presence of lower $Al(OH)_3$ concentration in the environment avoids to large extent dramatic uptake of aluminium by the cell while cells surrounded by higher aluminium concentrations lose this ability. Supplementation of the growth media with 100 µM $Al(OH)_3$ doubled the amount of Al in the cell compared to untreated control (232 mg/kg vs. 459 mg/kg), whereas addition of 1 mM $Al(OH)_3$ caused more than hundred fold increase of intracellular Al content (27,781 mg/kg). Here we also show that high concentrations of aluminium have an impact on cell morphology leading to cell integrity disruption. Findings presented in this study have the ambition to bring more light in an issue of how aluminium mediates impairments of the living organism.

Keywords: aluminium, *Schizosaccharomyces pombe*, toxicity, absorption, cell cycle

1 Introduction

Aluminium is widely used in daily life, and that is the reason of easy exposure to organisms. Uptake of aluminium in humans is possible from food, drinking water, cooking dishes or aluminium foils. Aluminium also occurs in agricultural soils, and through the crops can enter animal or human organism. In neutral pH, aluminium is bound in various minerals (most frequent is bauxite). In acidic environment (pH lower than 5) aluminium is released as aluminium ions Al^{3+} which are toxic to many organisms.

Chronic exposure of aluminium causes excessive accumulation in tissues thus having adverse effect on health. In the past, osteomalacia, microcytic anaemia and dialysis encephalopathy was observed in patients with kidney diseases due to presence of aluminium in dialysis fluid (Short et al., 1980; Li et al., 2012; Chen et al., 2018). Additionally, aluminium exposure can cause damages on cellular level as it increases the level of oxidative stress in cells. Elevated oxidative stress leads in mammals to apoptosis of brain cells contributing to development of neurodegenerative diseases (Maya et al., 2016; Colomina and Peris-Sampedro, 2017). Oxidative stress caused by aluminium enhances lipid peroxidation in brain and decreases activity of antioxidant enzymes superoxide dismutase (SOD) and catalase (Kaizer et al., 2005; Nehru and Anand, 2005). Moreover, formation of 8hydroxydeoxyguanosine, a common biomarker of oxidative DNA damage, is elevated in mitochondrial DNA after aluminium exposure. Expression of p53 protein has been increased as a result of oxidative DNA damage caused by Al^{3+} ions. Elevated expression of regulatory protein cyclin D1 due to Al^{3+} exposure can lead to cell cycle arrest in G1/S phase (Kumar

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et al., 2009). Investigations of effects of aluminium on the cell cycle revealed that its ability to interfere with cell components, disrupting the organization of microtubules and microfilaments results in alterations of cell cycle regulation. Defects after aluminium exposure were observed in microtubule cytoskeleton organization of mitotic cells of *Triticum turgidum* root tips. Stability of microtubules is affected by aluminium, tubulin forms atypical tubulin bundles, or ringlike aggregates causing incorrect spindle organization. Overdoses of aluminium impair kinetochore alignment during metaphase leading to abnormal arrangement of chromosome arms (Frantzios et al., 2000). Aluminium ions are responsible for slight depolymerization of cytoskeleton in alga *Spirogyra decimina* (Přibyl et al., 2008). Aluminium excess causes nucleolus disassembly which persists in the cell during mitosis (Qin et al., 2010; Zhang et al., 2014).

Studies analysing the possible mechanisms of aluminium toxicity for eukaryotic organism performed on yeasts *S. cerevisiae*, one of the most frequent model organisms (MacDiarmid and Gardner, 1996), showed that the yeast can undergo programmed cell death induced by aluminium exposure (Zheng et al., 2007). Analysis of the yeast genome has identified aluminium tolerant genes. Genes related to vesicle transport, genes responsible for signal transduction, and genes associated with protein mannosylation are aluminium tolerant, and can be responsible for aluminium metabolism and maintaining of the aluminium compromised cell integrity (Kakimoto et al., 2005). Intracellular aluminium ion accumulation can also generate abnormalities on protein level. In combination with diamide induces disulphide stress through generation of disulphide bonds between cysteines within, or between proteins leading to production of misfolded proteins that can be a cause of a disease (Wu et al., 2012; Tun et al., 2013). Another research has shown that even aluminium and acid tolerant yeast strain *Cryptococcus humicola* can be damaged by high concentration of aluminium. Incubation with concentration of aluminium above the tolerance level causes oxidative damage of membrane lipids resulting in size reduction and cell death (Nian et al., 2012).

It has been previously shown that aluminium ions are capable of interaction with many cellular components which has negative impact on homeostasis of microorganisms, plants and animals. Because of the toxicity of aluminium, for studies on its effect on living systems, the use of model organisms is required. *Schizosaccharomyces pombe*, a single-celled eukaryote, also known as fission yeast is a widely used model organism, as its genome comprises of many genes which are orthologous to genes reported to be responsible for certain diseases. In addition, this cell divides by mitosis similar to mitosis of multicellular organisms (Wood et al., 2002). Influence of aluminium exposition on cell cycle progression is to date only poorly examined. We used *S. pombe* to investigate the effect of aluminium on cell morphology, cell growth and proliferation, as this model organism exhibits similar responses as multicellular organisms and can thus help to elucidate toxic effects of the metal on cellular level.

2 Material and methods

2.1 Model organism and the growth conditions

The prototroph wild type (h^+) strain JG 15458 of the fission yeast *Schizosaccharomyces pombe* kindly provided from Doc. Dr. Juraj Gregan was used to analyse the sensitivity to aluminium ions. The standard rich YE+5S medium (YES), where indicated supplemented with defined $Al(OH)_3$ (Centralchem, Bratislava, Slovakia) concentrations was used for yeast growth. For media preparation the protocol from Sabatinos and Forsburg (2010) was used. Accordingly, for solid media, 20 g/L agar was added. Incubation temperature for the organism growth was 30 °C in both solid or liquid media. Moreover, aeration for yeast growth in liquid media was ensured by shaking at 130 rpm.

2.2 IC_{50} value determination

The half maximal inhibitory concentration (IC_{50}) represents the concentration of Al that causes the growth inhibition of the cells by 50%. Model organisms from the over-night culture were incubated for 3 hours at 30 °C with decreasing aluminium concentrations in a log 2 serial dilution from 1 mM to 1.5652 mM. The culture sample without aluminium addition was used as a control. Spectrophotometric analysis performed on Cary 60 UV-VIS spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) was used to determine the effect of tested metals on the cell growth. The differences in light absorbance at 600 nm before and after 3 hours of aluminium exposure denoted changes in the cell density revealing alterations in growth ability with the increasing metal concentrations. Cell growth ratio was calculated and IC_{50} value was determined using an IC_{50} calculator http://www.ic50.tk/.

2.3 Growth rate and cell morphology

To determine growth ability of the model organism under Al environment, cells from the over-night culture, diluted to OD_{600} 0.3, exposed to decreasing Al concentrations (1,000, 500, 250, 125, 60, 30 mM and 0 as a control) were incubated at 30 °C in water bath, and after 3 hours OD_{600} was measured on Cary 60 UV-VIS spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). Increase of

the cell density compared to time point 0 h defines the ability of cells to divide. The effect of the increasing Al concentrations to cell division is expressed as impairment of the cell density increase over time (Sabatinos and Forsburg, 2010). Cell morphology (length and width) was determined by the use of bright-field microscopy with 40× magnification on inverted microscope (Leica DMI 6000, Leica microsystems, Wetzlar, Germany). The length and width in [mm] was measured and compared in 100 cells exposed to 500, 150, 100 and 0 mM $Al(OH)_3$ by ImageJ software.

2.4 Spot test analyses

Spot test analysis was used in previous research (Pozgajova et al., 2013). Three aluminium concentrations 100, 150 and 500 µM were added to YES media containing agar, after sterilization by autoclaving. Cells from the over-night culture containing approximately 1×10^7 cells/mL were counted, serially diluted, and 10 µl of diluted cultures containing 1×10^4, 1×10^3, 1×10^2 and 1×10^1 cells/spot were plated on YES plates. Afterwards, plates were incubated at 30 °C for 2–3 days until growing cells formed colonies (spots). Individual spots formed on plates enriched by different Al concentrations were compared among each other and to the control. The number, size, and density of spots were evaluated.

2.5 Pre-analytical sample preparation of yeast

Cells were cultured overnight in 50 mL YES medium by shaking at 130 rpm and 30 °C. After culture growth, aluminium was added to sample in final concentrations: 100, 150, 500 and 1,000 µM. Control sample was left untreated. Cells were exposed to $Al(OH)_3$ for 3 hours followed by thorough washing at least three times with deionized water. Cells were pelleted by centrifugation, supernatant was removed, and samples were incubated at 55 °C for 12 hours. Dried yeast pellets were placed into PTFE digestion tubes and weighted. Five mL of ultra-pure TraceSELECT™ HNO_3 (Honeywell Fluka™, Seelze, Germany) is used for mineralization of the sample by pressure microwave digestion system ETHOS-One (Milestone, Srl., Italy). Mineralized samples were subsequently filtered with the use of quantitative Munktell filter paper No. 390 (Munktell & Filtrak, Bärenstein, Germany) into 50 mL volumetric flasks and filled with deionized H_2O (Kovacik et al., 2019). Three replicates of each sample were prepared to ensure statistical scoring.

2.6 Determination of aluminium content in yeast

Content of aluminium in mineralized yeast solutions was determined by optical emission spectroscopy with inductively coupled plasma ICP-OES (ICP-OES 720, Agilent Technologies Australia (M) Pty Ltd) (Kovacik et al., 2019).The detection limit for Al of the sample dry matter is 0,2 µg/kg.

2.7 Statistical analysis

Statistical significance of detected differences was evaluated by Student´s *T*-test using STATISTICA v.10 software (StatSoft Inc. Tulsa, OK, USA). To detect normality and homogeneity of variances the Cochran-Hartley-Barlett and Levene's tests were used. The limit of statistical significance was set up at P <0.05 *, 0.01 **, 0.001 *** for all statistically analysed samples.

3 Results and discussion

3.1 Effect of aluminium ions on the growth rate of cell culture in liquid medium

To investigate the influence of aluminium exposure on *S. pombe* growth, the spectrophotometry light absorbance measured at 600 nm was used (Figure 1). Optical density of the cell culture was determined after 3 hours of incubation with various concentrations of aluminium in liquid YES medium. As expected, increasing concentration of $Al(OH)_3$ led to cell culture growth inhibition. Similarly, growth rate inhibition of *S. pombe* was reported also after addition of the other toxic cations e.g. Cd (Clemens, 2003), As (Salgado, 2012) or Ni (Pozgajova et al. 2019). Significant drop in the cell growth was observed at concentration 250 µM. It demonstrates that exposure to high concentration of aluminium can reduce mitotic division, and thus disrupt normal growth of the cell culture compared to the control sample without an addition of aluminium. The half of the maximal inhibitory concentration IC_{50} was calculated and set on 234.53 µM $Al(OH)_3$.

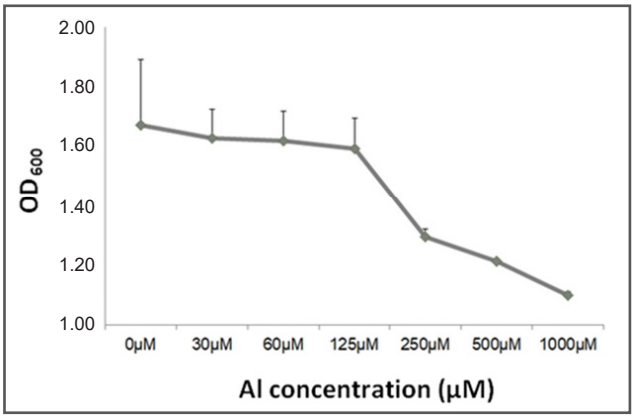

Figure 1 Dynamics of the growth of *S. pombe* cell culture after aluminium exposure in liquid YES medium. Change in optical density with increasing concentration of $Al(OH)_3$: 30, 60, 125, 250, 500 and 1,000 µM. Control sample was untreated with Al (0 µM). OD at 600 nm was measured immediately after $Al(OH)_3$ addition and after 3 hours of incubation

3.2 Spot test of cells on the solid medium with addition of aluminium

Spot test analysis confirmed the sensitivity of *S. pombe* to aluminium (Figure 2). In this test, the solid YES medium was enriched by 0, 100, 150 and 500 µM of aluminium. After serial dilution of the yeast culture 10, 100, 1,000, and 10,000 cells were spotted on plates, the growth ability was determined three days after incubation at 30 °C. Results of spot test corresponded to the results of the growth rate test, showing that lower $Al(OH)_3$ concentrations did not alter cell proliferation, whereas higher concentration, such as 500 µM $Al(OH)_3$ markedly reduced growth of cells.

Although small variations in growth rate of cell culture in these two methods could be observed due to different growth condition (liquid vs. solid medium, distribution of the cells on/in the medium, shaking, aeration), the trend of the cell culture growth under aluminium stress remained similar, and it was evident that growth rate decreased with rising $Al(OH)_3$ concentrations. Our results confirm that mitotic division of *S. pombe* cells was inhibited by high $Al(OH)_3$ concentrations, and thus we can conclude, that *S. pombe* yeast is sensitive to increasing aluminium exposure in the environment. Sensitivity of cells to aluminium exposure resulting in the growth inhibition was also confirmed on other model systems such as budding yeast or plants. Mitotic activity of root cells decreased with increasing concentration of aluminium ions (Huang et al., 2014; Jaskowiak et al., 2018). Wild type budding yeasts exposed to different aluminium concentrations showed growth rate inhibition in a dose dependent manner (Wu et al., 2012). However, in our hands, cell growth of *S. pombe* was inhibited by lower concentration of aluminium compared to experiments presented by Wu et al. (2012) with wild type *S. cerevisiae*.

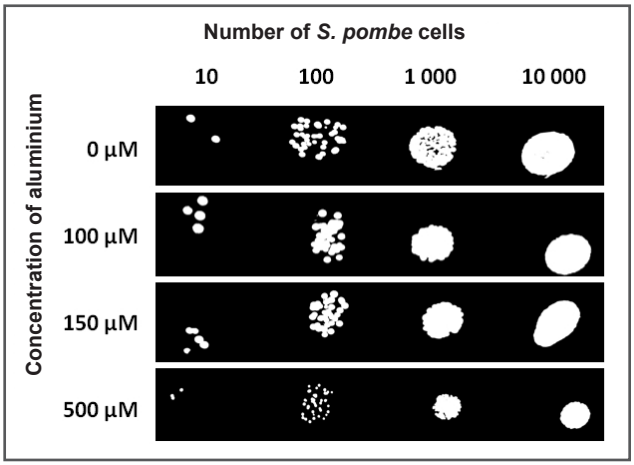

Figure 2 Spot test with 10, 100, 1,000 and 10,000 cells of *S. pombe* on agar plates in solid YES medium incubated for 3 days with concentrations of $Al(OH)_3$ 0, 100, 150 and 500 µM

We assume, that the resistance of *S. cerevisiae* cells to higher Al concentrations might be due to different experimental conditions and is caused by the use of $AlCl_3$, which shows lower toxicity to the cell compared to $Al(OH)_3$ (Zheng et al., 2007; Li et al., 2011).

Inhibition of the cell culture growth can be a result of aluminium interactions with intracellular structures responsible for proper mitosis. Experiments with plants revealed that accumulation of aluminium ions induces severe changes in the microtubular cytoskeleton and mitotic spindle, leading to significant destruction of mitotic cells. Furthermore, chromosome fragmentation, and chromosome stickiness was observed. This type of chromosome aberration is irreversible and leads to programmed cell death (Zhang et al., 2014, Vardar et al., 2016). Presence of aluminium in acid soil alters root growth by reducing the mitotic activity of the root tip cells as its exposure inhibits DNA replication resulting in the delay of the cell cycle (Jaskowiak et al., 2018). Thus, we suggest that the possible mechanism of the *S. pombe* cell culture growth inhibition is connected to aluminium interactions with intracellular components related to regulation of the cell cycle. Additionally, our results clearly demonstrate that aluminium effect on the cell division is largely concentration dependent.

3.3 ICP measurement of absorbed aluminium by the cells

In order to determine the amount of aluminium absorbed by the *S. pombe* cells, samples were analysed by ICP-OES. Concentration of incorporated $Al(OH)_3$ was measured after 3 hours of incubation. Obtained results have shown that concentration of absorbed aluminium rose with increasing concentration of aluminium present

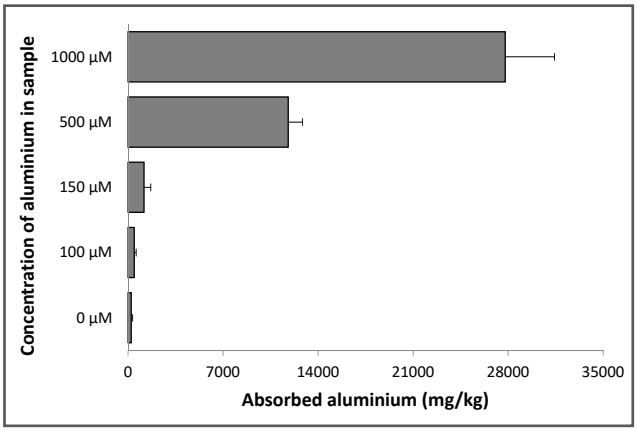

Figure 3 ICP-OES measurement of Al absorption. Atomic content of absorbed aluminium from $Al(OH)_3$ after 3 hours of incubation. Comparison of aluminium absorption into *S. pombe* cells in dependence on the increasing concentration of $Al(OH)_3$ in cell culture

Table 1 Added vs. absorbed Al^{3+}, measured by ICP-OES after 3 hours of incubation

Added $Al(OH)_3$ (µM)	Added Al^{3+} (µg)	Absorbed Al^{3+} (µg)
0	0	1.21 +/-0.67
100	54	2.63 +/-0.53
150	81	4.03 +/-0.69
500	270	48.10 +/-7.39
1,000	540	112.41 +/-18.83

in the *S. pombe* cell culture growth media. In the control sample, without aluminium addition, cells contained 232 mg/kg of aluminium. After addition of 100 µM of $Al(OH)_3$ to the growth medium content of aluminium in yeast cells doubled (459 mg/kg). Medium enrichment by 1 mM $Al(OH)_3$ caused more than hundredfold increase of aluminium content (27,781 mg/kg) in the cell (Figure 3). Uptake of aluminium by the cell exponentially rises with increasing aluminium concentration in the culture medium. Addition of 100 or 150 µM $Al(OH)_3$ led to incorporation of only one twentieth of Al^{3+} whereas under presence of 500 or 1,000 µM $Al(OH)_3$ in the medium cells were able to integrate one fifth of Al^{3+} ions (Table 1). We suggest that cells are capable to release aluminium up to certain concentration, while higher aluminium concentrations in the media cause aluminium persistence in the cell. Process of aluminium absorption and excretion by the cell is still not fully understood, however experiments with *S. cerevisiae* revealed some possible mechanisms through aluminium tolerant genes that are associated with secretory pathways (Kakimoto et al., 2005).

For instance, level of aluminium tolerance in plants is associated with citrate, oxalate and malate chelation of Al^{3+} to form a non-toxic complex. Release of aluminium from the cell is afterwards triggered by elution of aluminium complexes formed in the presence of elevated concentration of Al^{3+} in the environment (Jones and Ryan, 2003; Brunner and Sperisen, 2013). Accordingly, increased production of citrate was observed in aluminium tolerant yeast *Rhodotorula taiwanensis* RS1 after addition of aluminium above the tolerance level, which could be connected with possible detoxifying mechanism (Wang et al., 2013). Similarly, another research shows that accumulation of citrate in cellular and extracellular pools can mediate aluminium tolerance in S. cerevisiae strains (Anoop et al., 2003). Citrate and malate are present in all organisms, thus this mechanism of aluminium detoxification could be possibly used also by fission yeast. However, as these molecules are important for basic biochemical cycles (e.g. citric acid cycle), the capacity of binding Al^{3+} ions is probably limited, and therefore the mechanism of Al^{3+} detoxification cannot be conducted at high aluminium concentrations. Although, to date known molecular mechanism of toxic metal/metalloids, such as antimony, mercury, arsenic or cadmium, metabolisms in *S. cerevisiae* were described by Wysocki and Tamas (2010), and detoxification mechanism of cadmium and arsenic in *S. pombe* was suggested by Guo et al. (2016), the exact mechanism of how *S. pombe* deals with aluminium ions remains to be elucidated. Uptake of aluminium by *S. pombe* cells is still poorly understood and accordingly, many other aspects of metal biology remain largely elusive.

3.4 Cell morphology

Cells respond to nutritional stress e.g. starvation or presence of toxic elements, causing interruption of energy production, by immobilization of cellular compartments in a size-dependent manner (Heimlicher et al., 2019). Different types of cells exhibit different pattern of cell destruction and apoptosis caused by aluminium exposure (Zheng et al., 2007; Wu et al., 2012; Huang et al., 2014). Deformation of cell morphology was observed on S. cerevisiae cells upon aluminium exposure, size of the yeast cells was modified with typical apoptotic signs as cell shrinkage, nuclear fragmentation, vacuolization, and chromatin marginalization (Zheng et al., 2007). Cells of normal newly generated *S. pombe* divided by mitosis should be oval, 8 µm long, and 3.5 µm wide (Hoffman et al., 2015). Differences of the cell shape caused by aluminium ions analysed under microscope using 40× magnification, were detected after 3 hours of incubation (Figure 4A). Although, some cells were longer as they started a new cell cycle, it was obvious that increasing concentration of $Al(OH)_3$ caused conformational deformation of the cell. At high concentration of 500 µM, the morphology of cells was altered and irregular, and cells were markedly destructed. Attributes of 100 cells from each treated and untreated sample were measured and compared (Figure 4B). The results suggest that addition of aluminium causes modifications of *S. pombe* cells morphology in a dose dependent manner. Average length of the cells was higher, though average width was lower (although high concentration of aluminium led to complete destruction of some cells). When cells are in mitosis, the

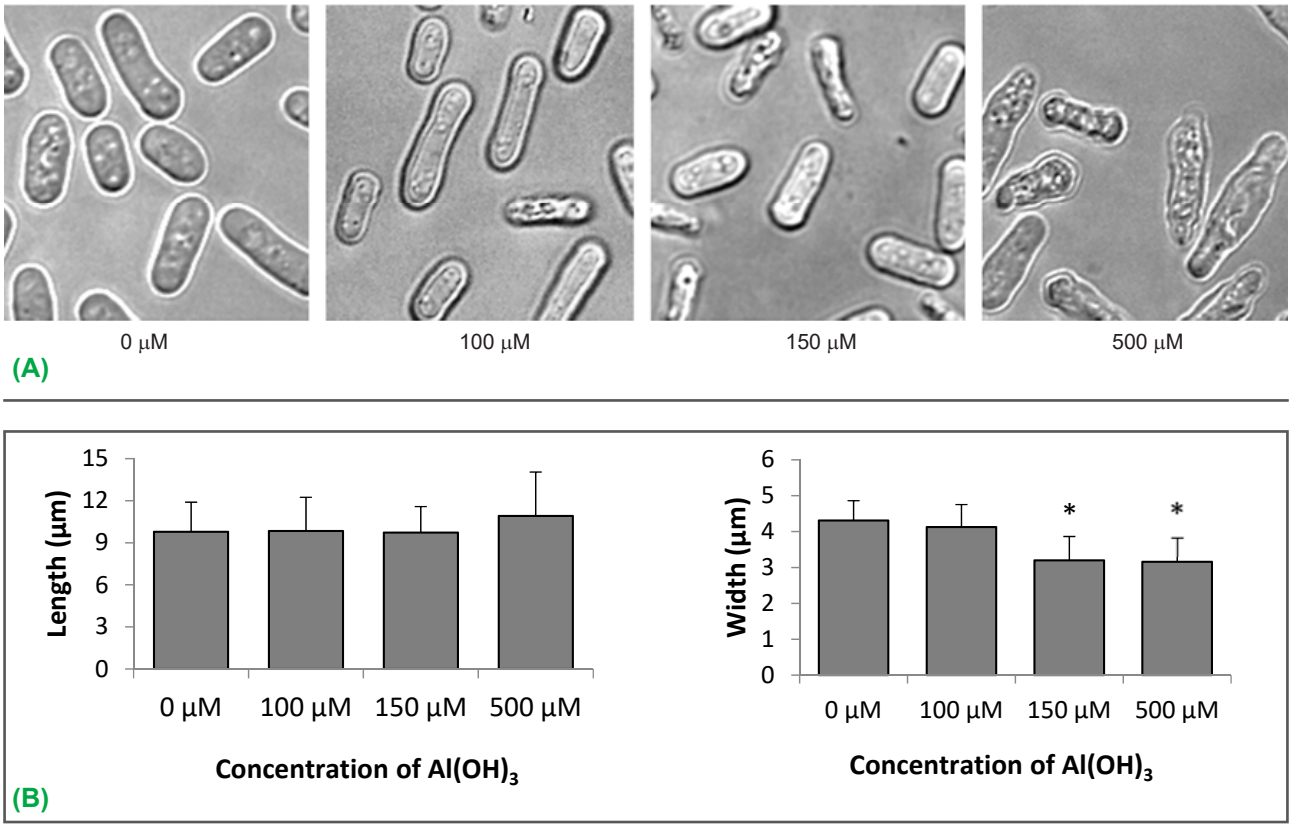

Figure 4 Morphology of the yeast cell after 3 hours of incubation with various concentrations of $Al(OH)_3$. (A) Representative pictures taken under the light microscope, magnification 40×. (B) Average length and width of the *S. pombe* in μm. Graph was created on the base of the pictures, length and width of 50 cells was measured by programme ImageJ. Statistical significance of obtained differences was determined by Student´s *T*-test, limit for significance was set up at $P < 0.05$*, 0.01**, 0.001 ***

length increases, but the width should stay the same. Our results show that according to the measurement, addition of high aluminium doses cause that cells seem to be "stretched" in their length and "shrinked" in their width. Accordingly, similar cell destructions after aluminium exposure were visible on *S. cerevisiae* under the electron microscope (Zheng, 2007).

4 Conclusions

Exposition of aluminium to the yeast *S. pombe* inhibits cell culture growth as it negatively affects the mitotic activity, together with cytoskeletal components responsible for cell division in a dose dependent manner. Additionally, higher aluminium concentrations cause irregular shape formation of the cells resulting in the overall cellular structure deformation. Intriguingly, our results show that uptake of aluminium by the cells is concentration dependent, as the amount of intracellularly absorbed aluminium is much greater in cells cultured under high aluminium concentration compared to conditions with lower Al content. This suggests that cells are able to eliminate certain proportion of aluminium from growth medium when they grow under conditions enriched by low although, compared to standard, still elevated aluminium concentrations. Mechanisms of aluminium excretion are not fully understood so far, thus further investigation is required. The effect of aluminium exposure on the cell, cell cycle and aluminium metabolism in model organism could broaden the understanding of fate of the aluminium compounds after exposure in multicellular organisms.

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