Curr Microbiol (2016) 72:545–550
DOI 10.1007/s00284-016-0987-y

Toxicity Induced by a Metal Mixture (Cd, Pb and Zn) in the Yeast *Pichia kudriavzevii:* The Role of Oxidative Stress

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Received: 2 October 2015 / Accepted: 11 December 2015 / Published online: 19 January 2016

Abstract The present work aims to contribute for the elucidation of the role of oxidative stress in the toxicity associated with the exposure of *Pichia kudriavzevii* to multi-metals (Cd, Pb and Zn). Cells of the non-conventional yeast *P. kudriavzevii* exposed for 6 h to the action of multi-metals accumulated intracellular reactive oxygen species (ROS), evaluated through the oxidation of the probe 2′,7′-dichlorodihydrofluorescein diacetate. A progressive loss of membrane integrity (monitored using propidium iodide) was observed in multi-metal-treated cells. The triggering of intracellular ROS accumulation preceded the loss of membrane integrity. These results suggest that the disruption of membrane integrity can be attributed to the oxidative stress. The exposure of yeast cells to single metal showed that, under the concentrations tested, Pb was the metal responsible for the induction of the oxidative stress. Yeast cells coexposed to an antioxidant (ascorbic acid) and multi-metals did not accumulate intracellular ROS, but loss proliferation capacity. Together, the data obtained indicated that intracellular ROS accumulation contributed to metal toxicity, namely for the disruption of membrane integrity of the yeast *P. kudriavzevii*. It was proposed that Pb toxicity (the metal responsible for the toxic symptoms under the conditions tested) result from the combination of an ionic mechanism and the intracellular ROS accumulation.

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Introduction

Heavy metals are one of the most important environmental contaminants. The chronic exposure of humans to heavy metals leads to a variety of diseases, which includes neurodegenerative disorders and dysfunction of vital organs [14]. Some metals are considered human carcinogens (for instance, As, Cd, Cr and Ni) [13] or probable carcinogens (Pb) by the International Agency for Research of Cancer [12].

Metals toxicity has been attributed mainly to their capacity to induce oxidative stress, as a consequence of the toxic levels of reactive oxygen species (ROS). The diatomic molecule of oxygen is relatively not reactive since the two unpaired electrons, present in the other shell, have the same spin. However, if one of these electrons is excited and changes its spin, then it originates singlet oxygen (O•), which is a powerful oxidant. Molecular oxygen can accept one electron being reduced to superoxide anion (O_2^-) that is the main ROS produced in vivo. The dismutation of O_2^- (which can occur spontaneously or catalysed by the action of the superoxide dismutase enzyme) originates hydrogen peroxide (H_2O_2), which is an oxidant with the ability to attack thiols. Hydrogen peroxide can be reduced to water by peroxidases. In the presence of transition metals, H_2O_2 originates the hydroxyl radical ($OH^{\cdot}$) one of the strongest oxidants. Hydroxyl radical reacts, indiscriminately with bio-molecules of all classes [4, 10].

Intracellular accumulation of ROS, metal-induced, can occur by several direct or indirect mechanisms. Metals like

Fe, Co, Cr and Cu can undergo redox-cycling reactions. In the case of redox-inactive metals, like Cd, Hg and Pb, they may induce oxidative stress by different indirect mechanisms, such as displacement of redox-active metals from cellular binding sites, inhibition of specific enzymatic antioxidant defences and depletion of pools of antioxidant molecules, such as reduced glutathione [2, 23, 26]. Metal-induced ROS can produce deterioration of biological molecules, such as lipid peroxidation [11], depletion of protein sulphydryl [6] and DNA damage [21]. Metal-induced oxidative stress damage may cause yeast cell death [5, 16, 20, 22].

The presence of heavy metals in soil, surface and ground water constitutes an important environmental problem. Most frequently, metal pollution is multi-element. This is the case of areas in the vicinity of mining and smelting sites [15] or electroplating industries [17]. The works found in the literature generally describe the impact of a single metal on the cells. Taking into account that metal pollution usually occurs due to the presence of multi-metals, the study of the simultaneous impact of various metals on the cell should be a more realistic approach.

The present work aims to contribute for the elucidation of the toxicity mechanisms associated with the exposure of *Pichia kudriavzevii*, a yeast with potential bioremediation capacity, to multi-metals (Cd, Pb and Zn). Specifically, the impact of multi-metals on the intracellular ROS level was addressed. The influence of single metal on ROS production was also investigated. Additionally, the effect of ascorbic acid on the mitigation of oxidative stress and toxicity was evaluated. Using the information previously achieved [18] and the data obtained in this work, a sequence of events occurring during the exposure of the non-conventional yeast *P. kudriavzevii* to metal mixture is proposed.

Materials and Methods

Yeast, Media and Growth Conditions

In this work, a strain of *P. kudriavzevii* CCMA 0136 was used. The strain belongs to the collection of cultures of agricultural microbiology (CCMA), from Biology Department (DBI), Federal University of Lavras (UFLA), Brazil. The gene sequence of the yeast has been deposited in the GenBank under the accession number KJ468031.1 (http://www.ncbi.nlm.nih.gov/nuccore/KJ468031).

The strain was maintained at 4 °C on YPD agar slants [10 g/l yeast extract (Difco-BD), 20 g/l peptone (Difco-BD), 20 g/l dextrose (Merck) and 20 g/l agar (Merck)]. Yeast pre-cultures were prepared in YPD broth. Cells were incubated at 25 °C on an orbital shaker at 150 rpm for 8–10 h. Cultures in the exponential growth phase were obtained by inoculating YPD broth with pre-cultures and then grown overnight ($OD_{600} \sim 0.5$) under the same conditions as the pre-cultures.

Exposure of Yeast Cells to Metal Stress

Exponential-phase cells were harvested by centrifugation (2500×*g*, 5 min), washed twice and re-suspended in deionised water at $\sim 1 \times 10^8$ cells/ml. Subsequently, cells were suspended in 10 mmol/l 2-(*N*-morpholino) ethanesulfonic acid (MES) pH buffer (Sigma-Aldrich), pH 6.8, at 1×10^7 cells/ml.

In the assessment of the influence of ascorbic acid (AA) on the intracellular ROS level and the cell viability, cells were suspended in 100 mmol/l MES pH buffer, pH 6.8, with 10 mmol/l AA (Merck) in the dark. Although AA in solution can undergo oxidation, it was described that at pH 7.0 (close to the pH value used), the percentage of non-degraded AA, after 6 h, was higher than 80 % [8]. The pH of the buffer solution did not change during the incubation with AA.

Metal mixture or individual metals were added at a final concentration of cadmium, 5 mg/l; lead, 10 mg/l; and zinc, 5 mg/l. The following stock standard solutions (Merck) were used: $CdCl_2$, 1000 mg/l; ($Pb(NO_3)_2$), 2000 mg/l; and Zn Cl_2, 2000 mg/l. The specific concentration of each metal was chosen according to previous screening experiments performed to select yeast strains tolerant to heavy metals, which were carried out in the Environmental and Industrial Microbiology Laboratory in UFLA/DBI, Brazil. Cell suspensions, at 1×10^7 cells/ml, were shaken in 100-ml Erlenmeyer flasks at 150 rpm at 25 °C, for 6 h; this incubation time corresponds to more than five times the doubling time of *P. kudriavzevii* in YPD broth.

Detection of Intracellular ROS Accumulation

ROS production was monitored with 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA; Sigma). Before metal treatment, cells were suspended at 1×10^7 cells/ml in MES buffer (10 mmol/l or 100 mmol/l in the case of the assay with AA, pH 6.8) and incubated at 25 °C for 20 min with 20 µmol/l H_2DCFDA (final concentration). Subsequently, yeast cells were exposed to metal mixture (in the absence or presence of 10 mmol/l AA) or individual metals and placed in quadruplicate in a 96-well flat microplate, 200 µl per well. The fluorescence intensity was measured (relative fluorescence units, RFU) in a PerkinElmer (Victor3) microplate reader at a fluorescence excitation of 485/14 nm and an emission of 535/25 nm. Fluorescence was corrected (subtracting cell, buffer and dye autofluorescence) and normalized considering the cell concentration.

Cells were also observed using a Leica DLMB epifluorescence microscope equipped with an HBO 100 mercury lamp and a GFP filter set from Leica. The images were acquired with a Leica DC 300 F camera using a 100× oil immersion N plan objective and processed using Leica IM 50-Image manager software.

Assessment of Membrane Integrity

Plasma membrane integrity was assessed by staining the cells with propidium iodide (PI). Cell samples were washed twice with deionised water and suspended in 10 mmol/l MES buffer, pH 6.8, at 1×10^7 cells/ml. Yeast suspensions were placed at a final concentration of 3 µmol/l PI (Sigma) for 5 min at room temperature. Cells were examined using an epifluorescence microscope equipped with I3 filter set from Leica. For each sample, at least three replicates of 200 cells (>600 cells) were scored in randomly selected fields. The images were acquired and processed as described above.

Measurement of Cell Viability by CFU Count

At defined intervals of time, samples (two replicates) were taken, serially diluted with sterile deionised water and plated on YPD agar (two replicates of the convenient dilutions). The colonies were counted after 1–2 days of incubation at 25 °C. No further colonies appeared after this period of incubation time. The viability was calculated using the number of colony-forming units (CFU)/ml at zero time as reference (100 %).

Reproducibility of the Results

All experiments were repeated, independently, three to five times. The data reported are the mean values ± standard deviation (SD), presented with 95 % confidence.

Results and Discussion

Exposure to Multi-metals Induces Oxidative Stress

Intracellular ROS accumulation in yeast cells was assessed using the probe H_2DCFDA. The nonfluorescent compound H_2DCFDA easily penetrates the plasma membrane and, once inside the cell, is hydrolysed to H_2DCF by non-specific esterases. In the presence of hydrogen peroxide, H_2DCF is oxidized to the high fluorescent 2′,7′-dichlorofluorescein [24]. *P. kudriavzevii* CCMA 0136 cells not exposed to metal mixture (control) remained unstained (Fig. 1a). Conversely, yeast cells incubated for 3 h in the presence of a metal mixture comprised 5, 10 and 5 mg/l of Cd, Pb and Zn, respectively, exhibited a strong green fluorescence, which indicated that cells contained substantial amounts of ROS (Fig. 1a).

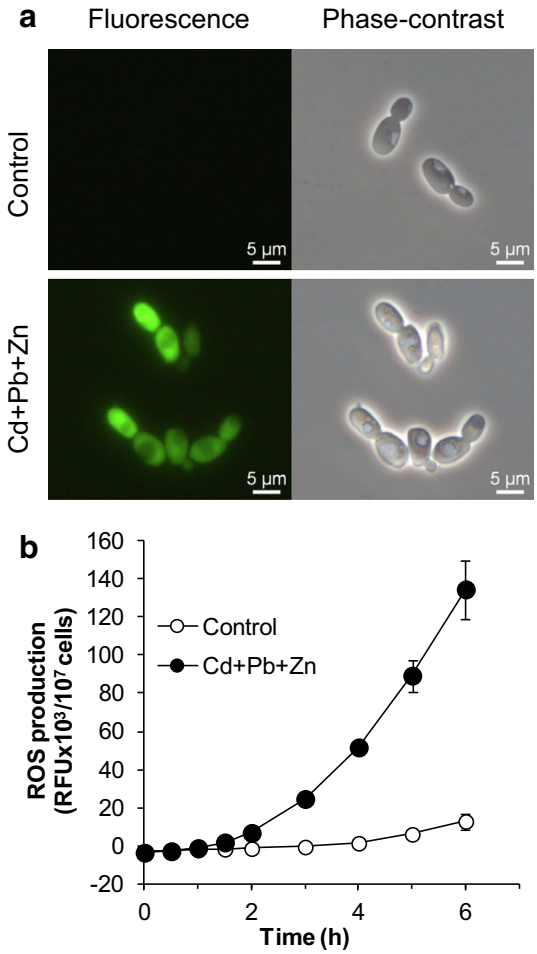

Fig. 1 ROS production in *P. kudriavzevii* CCMA 0136 exposed to multi-metals. **a** Visualization by fluorescence microscopy using 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA). Cells were pre-incubated with H_2DCFDA and subsequently exposed to a metal mixture comprised 5, 10 and 5 mg/l of Cd, Pb and Zn, respectively (Cd + Pb + Zn) for 3 h. Control: cells incubated in the absence of metals. **b** Kinetic of ROS production. Cells were pre-loaded with H_2DCFDA and subsequently incubated in the absence (control) or presence of the metal mixture (Cd + Pb + Zn). This is a typical example of an experiment performed three times. *Each point* represents the mean (±SD) of four fluorescent readings

The kinetic of the intracellular ROS accumulation in yeast cells not exposed (control) or exposed to the metal mixture was monitored. As it can be seen from Fig. 1b, the level of intracellular ROS, in yeast cells of *P. kudriavzevii* increased markedly after 2 h of exposure to metal mixture. After 6 h of metal exposure, yeast cells displayed high level of ROS. The level of intracellular ROS in non-treated cells remained low during the 6-h period (Fig. 1b).

Which Metal(s) is(are) Responsible for the Oxidative Stress?

In order to elucidate which metal(s) was(were) responsible for the oxidative stress, cells of *P. kudriavzevii* were exposed to a single metal, at the same concentration used in the mixture. As it can be seen from Fig. 2, Cd and Zn, at the concentrations used, did not induce ROS production. The intracellular accumulation of ROS was observed in the presence of Pb, in a similar pattern to the metal mixture (Figs. 1b, 2). These results allowed verifying that, with the concentrations used, Pb was the metal responsible for the oxidative stress observed in the metal mixture.

Loss of Membrane Integrity can be Due to Intracellular ROS Accumulation

Lipids of plasma membrane are one of the targets of the intracellular ROS. Extensive lipid peroxidation has been correlated with the loss of membrane integrity and cell death [3, 11]. Therefore, membrane integrity was assessed, during the exposure of yeast cells to metal mixture, through PI excluding assay. Cells that retained membrane integrity excluded PI (PI-negative cells), whereas cells with disrupted membrane were penetrated by PI and displayed an orange fluorescence (Fig. 3a). A slow loss of membrane integrity was observed during the exposure to metal mixture. After 3 and 6 h of metal exposure, ~6 and 18 %, respectively, of the cell population lost the membrane integrity (Fig. 3b).

Interestingly, the comparison of the kinetic of intracellular ROS accumulation (Fig. 1b) and the loss of membrane integrity (Fig. 3b) allowed verifying that the disruption of membrane integrity occurred after the enhancement of intracellular ROS accumulation.

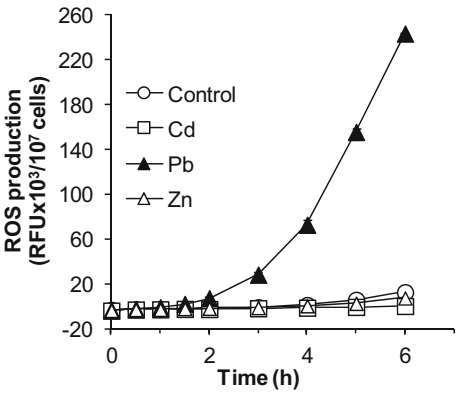

Fig. 2 Kinetic of ROS production in *P. kudriavzevii* CCMA 0136 exposed to a single metal. Cells were pre-loaded with H_2DCFDA and subsequently exposed to 5 mg/l Cd or 10 mg/l Pb or 5 mg/l Zn. Control: cells incubated in the absence of metal. This is a typical example of an experiment performed three times. Each point represents the mean (±SD) of four fluorescent readings

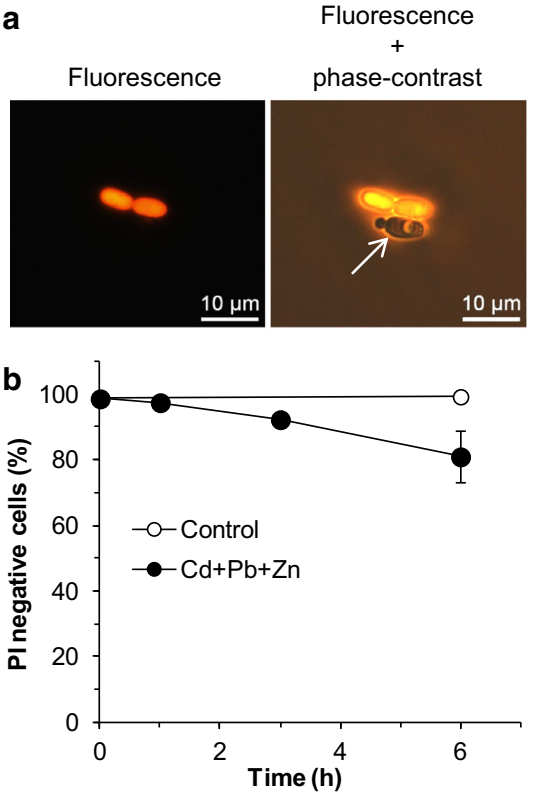

Fig. 3 Membrane integrity of the yeast *P. kudriavzevii* CCMA 0136 exposed to multi-metals. **a** Microphotographs illustrative of cell with intact membrane (PI-negative cell, *arrow*); cell with disrupted membrane presented an orange fluorescence (PI-positive cell). **b** Temporal assessment of the membrane integrity, evaluated by the microscopic determination of the PI-negative cells. Cells were exposed to the metal mixture (Cd + Pb + Zn) and subsequently stained with PI. Control: cells incubated in the absence of metals. The data represent the mean (±SD) of four independent experiments

Ascorbic Acid Impairs Oxidative Stress But Not the Metal-Induced Toxicity

If metal-induced toxicity is attributed to intracellular ROS accumulation, the decrease of ROS to physiological levels would mitigate metal mixture toxicity. To test this possibility, yeast cells were exposed to acid ascorbic (AA), a known ROS scavenger [1], in the presence or absence of metal mixture. As expected, the presence of AA was able to maintain yeast cells exposed to metal mixture with the same amount of intracellular ROS than the non-treated cells (control) (Fig. 4a). However, the presence of the antioxidant was unable to block the toxicity of metal mixture as it was revealed by the inability to sustain the loss of proliferation capacity (Fig. 4b). In fact, ~50 % of yeast cell population exposed for 6 h to metal mixture, in

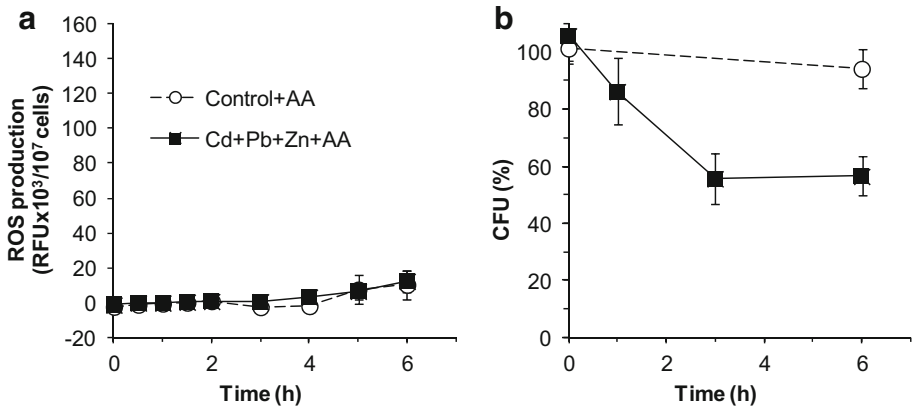

Fig. 4 Impact of ascorbic acid (AA) on the kinetic of ROS production and proliferation capacity of *P. kudriavzevii* CCMA 0136 exposed to the metal mixture. Cells were incubated with 10 mmol/l AA and the metal mixture (Cd + Pb + Zn + AA) or with 10 mmol/l AA without metals (control + AA). **a** Intracellular ROS assessed with H_2DCFDA. This is a typical example of an experiment performed three times. Each point represents the mean (±SD) of four fluorescent readings. **b** Yeast survival (colony-forming units, cfu, on YPD agar). The data represent the mean (±SD) of five independent experiments

the presence of AA, lost the proliferation capacity (Fig. 4b). The loss of proliferation capacity in the presence of AA followed a similar pattern to the one observed in the absence of AA [18].

Possible Toxicity Mechanisms of the Metal Mixture

The mechanisms of Cd and Zn toxicity, and particularly Pb toxicity, in the non-conventional yeast *P. kudriavzevii* are not yet known. However, most probably, Pb toxicity (the metal responsible for the toxic symptoms under the conditions tested) resulted from the combination of (i) an ionic mechanism and (ii) the ROS accumulation. It is known that in mammalian cells Pb is able to replace other bivalent cations like calcium and zinc, affecting several biological processes, such as intracellular signalling, metal transport, energy metabolism and enzyme regulation [9]. Consistent with this possibility, in a previous work, it was found that when *P. kudriavzevii* was exposed for 1 h to metal mixture, ~30 % of cell population lost the proliferation capacity and the metabolic activity [18].

The sequence of events which occurred during the exposure of yeast cells to metal mixture, allows observing that these toxic symptoms precede the enhancement of intracellular ROS accumulation (Fig. 5). Consistently, in the present work, it was observed that the ROS scavenger due to the presence of AA did not impair the loss of proliferation capacity (Fig. 4).

The continuous exposure to metal mixture induced the ROS accumulation (Fig. 1), most likely promoting membrane lipid peroxidation, which originated the disruption of plasma membrane (Fig. 3) and necrosis. In fact, oxidative stress is one of the most common mechanisms causing cell damage due to metal toxicity [2]. Cd and Pb, non-essential

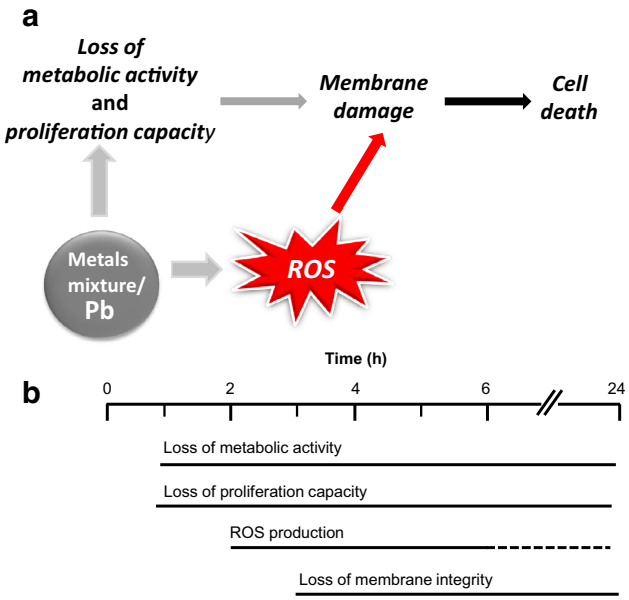

Fig. 5 Toxicity induced by the metal mixture in *P. kudriavzevii*. **a** Diagrammatic representation of possible modes of action of heavy metals in the yeast. **b** Sequence of events occurring during the exposure of yeast cells to the metal mixture. Proposal based on a previous work (loss of metabolic activity and proliferation capacity) [18] and from data obtained in this work. *Dashed line:* not evaluated

metals, are cellular toxicants that increase ROS in yeast cells of *S. cerevisiae* [19, 20, 22]. On the other hand, Zn, an essential metal for almost all organisms, is not redox active under physiological conditions [7]. However, when in excess, Zn is toxic to the cells [25].

In conclusion, *P. kudriavzevii* yeast cells exposed for 6 h to the simultaneous action of multi-metals (Cd, Pb and Zn) accumulated intracellularly ROS. The analyses of the action of single metals allowed determining that, under the

concentrations used, Pb was the metal responsible of the intracellular ROS accumulation. The oxidative stress seems to be responsible for the disruption of plasma membrane since the intracellular ROS accumulation preceded the loss of membrane integrity in P. *P. kudriavzevii.*

Acknowledgments The authors thank the FCT Strategic Project of UID/BIO/04469/2013 unit and the project RECI/BBB-EBI/0179/2012 (FCOMP-01-0124-FEDER-027462). Vanessa A. Mesquita gratefully acknowledges the grant from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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