Lead induces oxidative stress and phenotypic markers of apoptosis in *Saccharomyces cerevisiae*

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Abstract In the present work, the mode of cell death induced by Pb in *Saccharomyces cerevisiae* was studied. Yeast cells Pb-exposed, up to 6 h, loss progressively the capacity to proliferate and maintained the membrane integrity evaluated by the fluorescent probes bis(1,3-dibutylbarbituric acid trimethine oxonol) and propidium iodide. Pb-induced death is an active process, requiring the participation of cellular metabolism, since the simultaneous addition of cycloheximide attenuated the loss of cell proliferation capacity. Cells exposed to Pb accumulated intracelullarly reactive oxygen species (ROS), evaluated by 2′,7′-dichlorodihydrofluorescein diacetate. The addition of ascorbic acid (a ROS scavenger) strongly reduced the oxidative stress and impaired the loss of proliferation capacity in Pb-treated cells. Pb-exposed cells displayed nuclear morphological alterations, like chromatin fragmentation, as revealed by diaminophenylindole staining. Together, the data obtained indicate that yeast cells exposition to 1 mmol/l Pb results in severe oxidative stress which can be the trigger of programmed cell death by apoptosis.

Keywords Apoptosis · Ascorbic acid · Lead · Heavy metals toxicity · Oxidative stress · ROS production

Introduction

The release of heavy metals due to anthropogenic activities is a wild-world major concern due to its toxicity. Heavy metals represent an important environmental hazard as they are not degraded or destroyed, remaining for a long time in nature. As a consequence, heavy metals can be accumulated through the food chain, creating a threat to public health.

Several metals, like Ca, Cu, Fe, Mg, Ni and Zn, are incorporated by yeast cells since they are essential for its structure and metabolism; however, they become toxic when present at concentrations higher than those required for fulfilling its physiological functions. On the contrary, metals, such as Cd, Hg, or Pb, do not have any known cellular function; Cd and Hg are strong inhibitors of microbial metabolism, even at low concentrations (Gadd 1993). Pb can provoke a variety of adverse responses in humans, such as carcinogenesis, nephropathies and neuropathies (Silbergeld 2003). In yeast cells, Pb induces the loss of cell viability (Suh et al. 1999; Soares et al. 2002, 2003), inhibits cell growth (Chen and Wang 2007; Sakamoto et al. 2010) and metabolic activity (Van der Heggen et al. 2010), impairs ammonium assimilation, reduces DNA/RNA ratio (Chen and Wang 2007) and provokes DNA damage (Yuan and Tang 1999).

Reactive oxygen species (ROS), such as superoxide anion (O_2^{-}), hydroxyl radical ($OH^{\cdot}$) and hydrogen peroxide (H_2O_2), are usually produced during aerobic metabolism (Jamieson 1998). ROS can also be intracellularly accumulated by exposition of cells to ionic radiations (Jamieson 1998) to heavy metals such as Cu or Cd (Shanmuganathan et al. 2004; Liang and Zhou 2007; Nargund et al. 2008). ROS formation originates different harmful effects, which includes the lipids peroxidation (Howlett and Avery 1997) and proteins oxidation (Shanmuganathan et al. 2004; Sumner et al. 2005). DNA is highly susceptible to oxidative damage, while ROS attack to carbohydrates is comparatively slow (Avery 2001).

Intracellular ROS accumulation also induces and regulates yeast apoptosis (Madeo et al. 1999, 2009). Apoptosis is a form of programmed cell death (PCD), well-known in multicellular organisms, which plays important roles such as the removal of damage cells and maintenance of tissue homeostasis. *Saccharomyces cerevisiae* yeast cells can also undergo this mode of death which execution is carried out in a regulated manner. Yeast cell death by apoptosis is accompanied by diagnostic features resembling those observed in mammalian cells such as exposition of phosphatidylserine, DNA fragmentation and chromatin condensation in the absence of the loss of cell membrane integrity (Madeo et al. 1997, 2009). In yeast cells, several apoptosis inducers have been described such as hydrogen peroxide (Madeo et al. 1999), acetic acid (Ludovico et al. 2001), sugar (Granot et al. 2003), aspirin (Balzan et al. 2004), chronological and replicative ageing (Rockenfeller and Madeo 2008) and metals like Cd (Nargund et al. 2008), Cu and Mn (Liang and Zhou 2007).

In the present study, the generation of ROS and the occurrence of phenotypic apoptotic markers during the exposition of yeast cells to Pb were investigated. The mechanism of cell death induced by Pb in *S. cerevisiae* is also discussed.

Materials and methods

Strain, media and culture conditions

In this work, the brewing strain of *S. cerevisiae* National Collection of Yeast Culture (NCYC) 1214 was used. The strain was routinely maintained at 4°C on YEPD agar slants (10 g/l yeast extract (Difco-BD, USA), 20 g/l peptone (Difco-BD, USA), 20 g/l glucose (Merck, Darmstadt, Germany) and 20 g/l agar (Merck)).

Pre-cultures were prepared in 10 ml of YEPD broth in 100 ml Erlenmeyer flasks. Cells were incubated at 25°C on an orbital shaker Sanyo Gallenkamp IOC 400 (West Sussex, UK), at 150 rpm, during 24 h.

Cultures were obtained by inoculating 40 ml of YEPD broth, in 100 ml Erlenmeyer flasks, with 1 ml of pre-culture. The flasks containing the culture were incubated under the same conditions as the pre-culture. The time of incubation was 16 h, which corresponds to the end of the exponential respirofermentative growth phase/beginning of the diauxic lag phase (data not shown).

Preparation of cell suspensions

After growth, cells were harvested by centrifugation (2,000×*g*, 5 min) and washed twice with 30 mmol/l ethylenediaminetetraacetic acid (EDTA) solution (Merck). Subsequently, cells were washed twice with deionised water and resuspended in [2-(*N*-morpholino) ethanesulfonic acid] MES pH buffer (Sigma-Aldrich, St. Louis, MO, USA) 10 mmol/l, at pH 6.0, to approximately 1×10^7 cells/ml. MES is a suitable pH buffer for heavy metal toxicity studies because it does not complex lead (Soares et al. 1999), and yeast cells maintain viability when incubated in this buffer for 48 h (Soares et al. 2000).

Cell concentration was determined spectrophotometrically at 600 nm after appropriate dilution of the samples in EDTA solution (30 mmol/l) to prevent cell aggregation. Calibration curves (absorbance versus number of cells) were previously made.

Yeast cells exposure to Pb

Cell suspensions (40 ml) containing 1×10^7 cells/ml were placed in 10 mmol/l MES buffer (pH 6.0) with the appropriate volume of lead solution ($Pb(NO_3)_2$), from a stock standard solution of 1 g/l (Merck); Pb was added to a final concentration of 1 mmol/l. Cells suspensions, in 100-ml Erlenmeyer flasks, were shaken at 150 rpm, at 25°C, and exposed to Pb up to 6 h.

In the experiments, where the presence of a protein inhibitor or an anti-oxidant agent was tested, 25 μg/ml cycloheximide (Cyh; Sigma-Aldrich) or 10 mmol/l ascorbic acid (AA; Merck), final concentrations, was added to the cell suspensions just before the addition of Pb solution.

Evaluation of cell proliferation capacity

Before and after the addition of the metal, samples (100 μl; three replicates) were taken at defined intervals of time, serially diluted with sterile deionised water and plated on YEPD agar (two replicates of 100–200 μl of the convenient dilutions). The colonies were counted after 3 to 4 days of incubation at 25°C. The percent of survivors was calculated using the number of colony-forming units (c.f.u.) per millilitre at zero time as reference (100%).

Evaluation of cell membrane integrity

Cell membrane integrity was monitored with the dye bis (1,3-dibutylbarbituric acid trimethine oxonol) ($DiBAC_4(3)$) known as Oxonol (Molecular Probes, Invitrogen, CA, USA); 1×10^7 cells/ml, in MES buffer (10 mmol/l, pH 6.0), was incubated with Oxonol (in a final concentration of 1 μmol/l), during 20 min, at 25°C, in the dark. Subsequently, yeast cells were exposed to 1 mmol/l Pb. At defined intervals of time reported in the figures, fluorescence intensity (as relative fluorescent units) was measured, using a PerkinElmer ($Victor^3$) microplate reader, at fluorescence excitation of 485/14 nm and an emission of 535/25 nm. As a positive control (depolarized cells), yeasts were heated at 65°C, during 1 h (see below). The percent of depolarized cells was calculated using, as reference, the fluorescence of the positive control, where all cells are depolarized; in all cases (samples and positive control), fluorescence was normalized (considering cell concentration) and corrected by subtracting cell, buffer, lead (when appropriate) and dye autofluorescence.

In order to validate the Oxonol staining protocol, live, heated cells at 65°C and cells Pb-exposed were stained with Oxonol or propidium iodide (PI) and observed by epifluorescence microscopy; additionally, a double staining protocol with these two probes was also carried out. Oxonol and/or PI (Sigma, Steinheim, Germany) was added to yeast cells at 2 and 4.5 μmol/l final concentrations, respectively. The cell suspensions were incubated in the dark at room temperature for 20 min. Cells were examined using a Leica DLMB epifluorescence microscope (Leica Microsystems, Wetzlar GmbH, Germany) equipped with a HBO-100 mercury lamp. In the case of PI, a filter set I3 (excitation filter (band-pass filter, BP) BP 450–490, dichromatic mirror 510 and suppression filter (long-pass filter, LP) LP 515), from Leica, was used; for Oxonol, a GFP filter set (excitation filter BP 470/40, dichromatic mirror 500 and suppression filter BP 525/50) was used. The images were acquired with a Leica DC 300F camera (Leica Microsystems, Heerbrugg, Switzerland) using N plan objectives and processed using Leica IM 50-Image manager software.

All cells heated at 65°C, during 1 h, lost the membrane integrity since they were permeated to PI and were depolarised (evaluated with Oxonol) (Fig. 1S); the double staining protocol, carried out with these two probes, has shown similar results (Fig. 2S).

Evaluation of nuclear alterations

At defined intervals of time of contact between yeast cells and Pb, cells were taken and fixed with 3.5% (*w*/*v*) formaldehyde, during 2 h, at room temperature, with occasional mixing. Then, cells were harvested by centrifugation (2,000×*g*, 5 min), resuspended in phosphate-buffered saline (PBS) buffer (0.1 mol/l, pH 7.0) with 3.5% formaldehyde (*w*/*v*) and incubated during 24 h, at 4°C. Subsequently, cells were washed two times and resuspended in PBS buffer.

For nuclear staining, cells were incubated with diaminophenylindole (DAPI; Sigma-Aldrich), at a final concentration of 3 μmol/l, during 15 min, at room temperature, in the dark. Subsequently, cells were washed, resuspended in PBS buffer and placed on a microscope slide. A coverslip was mounted with a drop of ProLong Gold antifade reagent (Molecular Probes, Invitrogen); samples were cured overnight, at room temperature. Cells were examined under an epifluorescence microscope equipped with a filter set A (excitation filter BP 340–380, dichromatic mirror 400 and suppression filter LP 425), from Leica. Images were acquired and processed as described above.

Evaluation of ROS production

Intracellular ROS production was monitored with 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA; Sigma-Aldrich); 1×10^7 cells/ml was incubated with H_2DCFDA (in a final concentration of 20 μmol/l), during 20 min, at 25°C, in the dark; H_2DCFDA stock solution of 5 mmol/l was prepared in dimethyl sulfoxide (Sigma-Aldrich) and stored at −80°C. Yeast cells were subsequently exposed to 1 mmol/l Pb in the absence or in the presence of 10 mmol/l ascorbic acid. Fluorescence intensity was measured, at defined intervals of times reported in the figures, using a microplate reader at a fluorescence excitation of 485/14 nm and an emission of 535/25 nm.

For epifluorescence microscopy analysis, cells were treated in the same conditions described above and examined under an epifluorescence microscope equipped with a filter set I3, from Leica. Images were acquired and processed as described above.

Reproducibility of the results

All experiments were repeated, independently, at least two times. Although, in fluorescence measurements, absolute data were not comparable in the experiments performed on different days, the observed trends were fully consistent among the independent experiments and a typical example is shown. Fluorescence data were expressed as the mean ± standard deviation (SD) of quadruplicate measurements. The data reported for viability are the mean ± SD (presented with 95 % confidence) of three to five independent experiments performed in triplicate.

Results

The exposition of *S. cerevisiae* to 1 mmol/l Pb induced a progressive loss of cell proliferation capacity, assessed by the conventional spread technique. After 6 h of exposition to Pb, only 16% of cells retained the capacity to form colonies (Fig. 1a). Conversely, the integrity of cell membrane was maintained since no significant reduction of membrane potential occurred in cells exposed during 6 h to Pb, when evaluated using Oxonol [$DiBAC_4(3)$] dye (Fig. 1b). Viable cells are able to exclude the anionic $DiBAC_4(3)$ dye, due to the presence of a transmembrane potential; on the contrary, the fluorescent probe can freely enter in the cells with depolarized membrane, where it binds to intracellular proteins or membranes and enhances significantly the green fluorescence (Epps et al. 1994; Dinsdale et al. 1995). Similar results were observed, by epifluorescence microscopy, when PI was used (Fig. 2). This stain is membrane impermeable and is excluded from viable cells. On the other hand, cells with compromised plasma membrane incorporate PI; this stain binds to DNA (by intercalating between the bases) and exhibits an orange–red fluorescence (Haugland 2005). Pb-treated cells, during 6 h, retained their membrane integrity, as they were not able to incorporate PI. In the case of Fig. 2, a specific field containing one cell with compromised membrane was selected to evidence the staining. The double staining protocol, performed with Oxonol and PI, allowed to verify that cells treated with 1 mmol/l, during 6 h, retained the membrane integrity, when assessed by the ability to exclude PI and Oxonol (Fig. 2). The comparison of the results obtained by c.f.u. counting and by the fluorescent dyes indicates that after 6 h of contact with Pb, the majority of cell population (>90%) retain their membrane integrity but are not able to form colonies on solid YEPD medium (Fig. 1).

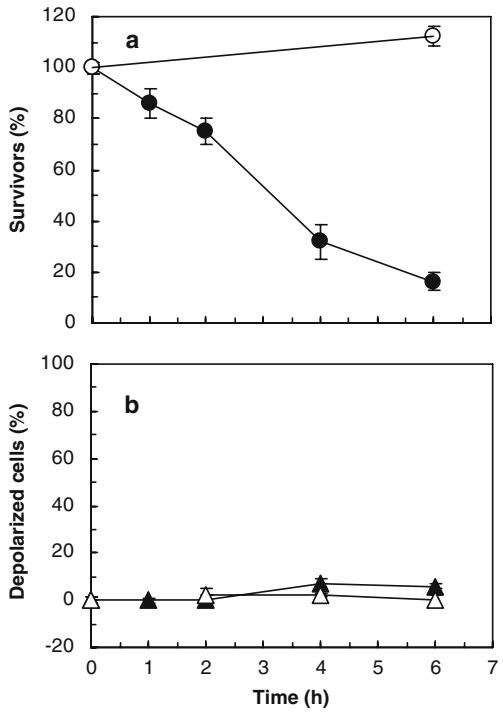

Fig. 1 Viability and cell membrane integrity of *S. cerevisiae* NCYC 1214 during the exposition to Pb; 1×10^7 cells/ml was suspended in 10 mmol/l MES pH buffer (pH 6.0) in the absence (*open symbols*) or presence (*closed symbols*) of 1 mmol/l $Pb(NO_3)_2$. Viability was estimated by c.f.u. counts (*circles*; **a**) and depolarised cells (evaluation of loss of plasma membrane integrity assessed with the fluorescent probe, $DiBAC_4(3)$ (Oxonol); *triangles*; **b**). For cell viability, each point represents the mean of five independent experiments. In the case of plasma membrane integrity, this is a typical example of an experiment preformed twice; each point represents the mean of four fluorescent readings. Standard deviations are presented; where no error bars are shown, they are within the points

Apoptosis is characterised by the participation of cell metabolism in the process of cell death (Madeo et al. 1999; Ludovico et al. 2001). In order to evaluate the requirement of protein synthesis on yeast cell survival during Pb-induced stress, yeast cells were simultaneously exposed to the action of 1 mmol/l Pb and 25 μg/ml of Cyh, a protein synthesis inhibitor at the ribosomal level in eukaryotes. Control experiments showed that Cyh, at the concentration used, was not toxic to yeast cells (data not shown). The presence of Cyh reduced the loss of cell viability (accessed by c.f.u. counting on YEPD plates) induced by Pb; the viability in the presence of Cyh is ~4 times higher than in its absence (Fig. 3).

It is well documented that the intracellular accumulation of ROS leads to cell damage (Avery 2001); in addition, ROS also act as cell death regulators and its presence has been linked with the phenomenon of apoptosis in yeast cells (Carmona-Gutierrez et al. 2010). With the help of H_2DCFDA, it was observed that Pb induced the intracellular accumulation of ROS (Fig. 4). H_2DCFDA is a cell-permeant indicator for ROS. Intracellular esterases remove the lipophilic blocking groups converting the probe into H_2DCF, a nonfluorescent and charged form of the dye that is much better retained by the cells than the parent compound; in the presence of hydrogen peroxide, H_2DCF is oxidized to the high fluorescent 2′,7′ dichlorofluorescein (Tarpey et al. 2004). The appearance of yeasts with a strong green fluorescence indicates that cells sustained considerable amounts of ROS (Fig. 4c); conversely, control cells, incubated in the absence of Pb, evidenced no fluorescent and appeared dark against the faint background fluorescence (Fig. 4a).

Using a kinetic approach, it was possible to observe a drastic increase of ROS production after 1 h of cells exposition to Pb (Fig. 5). After 2 h of Pb exposition, ROS were intracellular accumulated in the majority of the yeast cells, as it can be observed by fluorescent microscopy

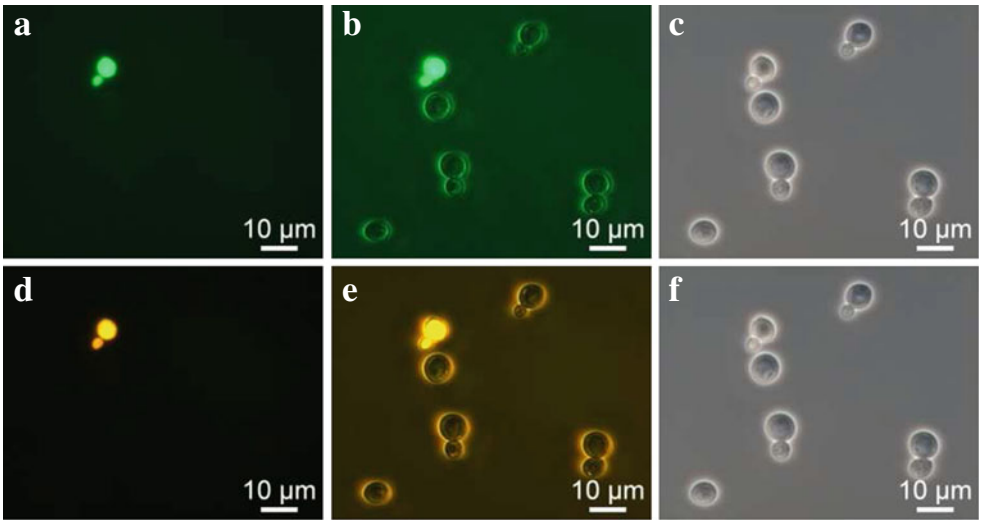

Fig. 2 Membrane integrity in cells of *S. cerevisiae* NCYC 1214 exposed to Pb. Cells were exposed to 1 mmol/l $Pb(NO_3)_2$, during 6 h, as described in Fig. 1. Subsequently, cells were double stained with Oxonol and propidium iodide. Cells with non-disrupted membrane are not able to incorporate Oxonol and/or propidium iodide. Fluorescent micrographs of the cells observed with filter set GFP (**a**) or I3 (**d**); fluorescence plus phase contrast (**b**, **e**) or phase contrast micrographs (**c**, **f**) of the same cells

(Fig. 4c). The analysis of ROS levels shows that Pb treatment indeed elicits a marked increase in ROS production in a similar way to that observed for loss of cell proliferation capacity (Figs. 1 and 5, respectively). Thus, it can be argued that the heightened production of ROS is responsible for the loss of proliferation capacity of yeast cells exposed to Pb.

In order to confirm the relationship between ROS production and the loss of viability, the effect of an antioxidant was tested. Cells were simultaneously exposed to 1 mmol/l Pb and 10 mmol/l of AA, an efficient scavenger of toxic free radicals and other ROS formed in the cells (Arrigoni and De Tullio 2002). The presence of ascorbic acid strongly impaired the rate of the intracellular accumulation of ROS and enhanced highly cell survival (evaluated by c.f.u.) in Pb-treated cells (Fig. 6). These results suggest that ROS is tightly associated with Pb-induced cell dead; the reduction of intracellular ROS accumulation confer increased resistance to Pb-induced toxicity (Fig. 6).

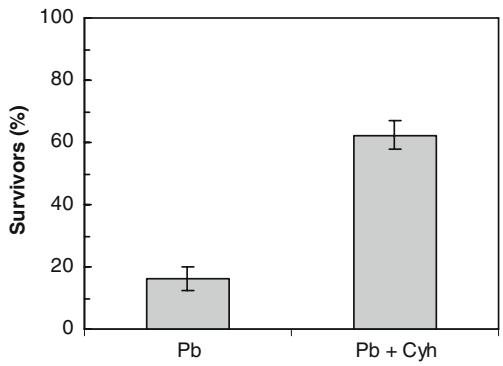

Fig. 3 Effect of cycloheximide in the lead-induced toxicity of *S. cerevisiae* NCYC 1214. Cells were incubated with 1 mmol/l Pb $(NO_3)_2$, as described in Fig. 1, in the absence or in the simultaneous presence of 25 μg/ml of cycloheximide (*Cyh*), during 6 h. Viability was estimated by c.f.u. counts. Each point represents the mean of three independent experiments; standard deviations are presented

The results obtained above prompted us to investigate if Pb-exposed cells displayed nuclear morphological alterations (a typical apoptotic marker); these nuclear morphological alterations were accessed with DAPI. This compound stain preferentially double-strand DNA (dsDNA); the binding of DAPI to dsDNA produces a ~20-fold fluorescence enhancement. DAPI stains nuclei and mitochondria specifically (blue fluorescence) with little or no cytoplasmic labelling (Haugland 2005). Cells not exposed to Pb exhibited nuclei with normal morphology (a single, bright and homogeneous spot; Fig. 7a); in these cells, mitochondria are predominantly located near the periphery of the cells as small dots with less blue intensity (Fig. 7a). Conversely, cells exposed to Pb presented nuclear alterations; after 4–6 h of exposition to Pb, cells showed chromatin fragments arranged in half rings (Fig. 7b) or nuclear fragments randomly distributed (Fig. 6c). After 4 or 6 h, cells not exposed to Pb or simultaneously exposed to 1 mmol/l Pb and 10 mmol/l of AA displayed normal morphology (data not shown).

Discussion

The action of a chemical or a physical agent to yeast cells can trigger two main cell death scenarios: cells necrosis or apoptosis. Necrosis has been traditionally seen as a form of cell death that results from an acute and intense cell injury. Nowadays, several evidences suggest that necrosis can also occur under non-extreme concentrations of cell death inducing agents. In both circumstances, necrosis characteristically leads to a plasma membrane rupture followed by the loss of intracellular content (Eisenberg et al. 2010).

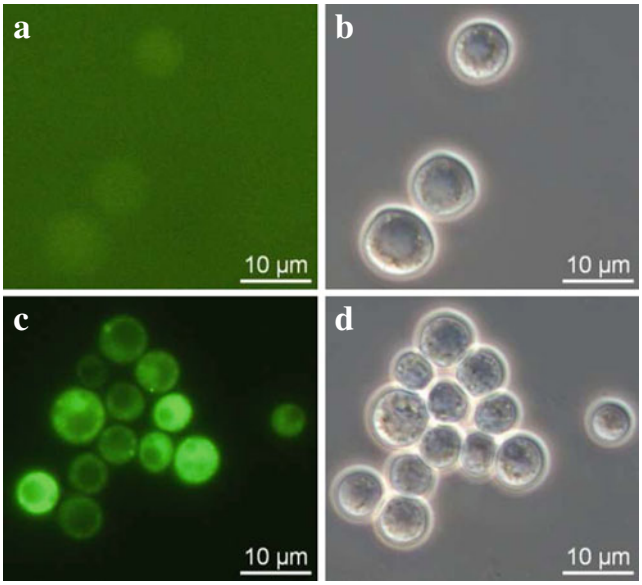

Fig. 4 Visualization of ROS production in *S. cerevisiae* NCYC 1214. Cells were pre-loaded with H_2DCFDA, during 20 min, and incubated subsequently in the absence (**a**) or in the presence of 1 mmol/l Pb $(NO_3)_2$, during 2 h (**c**). Fluorescence micrographs (**a**, **c**); phase contrast micrographs of the same cells (**b**, **d**). In the case of cells incubated in the absence of Pb, fluorescence images were shot with 4.25× shutter time used in the Pb-exposed cells

Conversely, apoptosis, as a PCD process, is mediated by an intracellular programme, energy dependent, in which the integrity of plasma membrane is maintained (Eisenberg et al. 2010).

The loss of survival in Pb-exposed cells was not accompanied by the loss of cell membrane integrity, which suggests that Pb induce in yeast cells an apoptotic scenario. The survival of the Pb-exposed cell population was

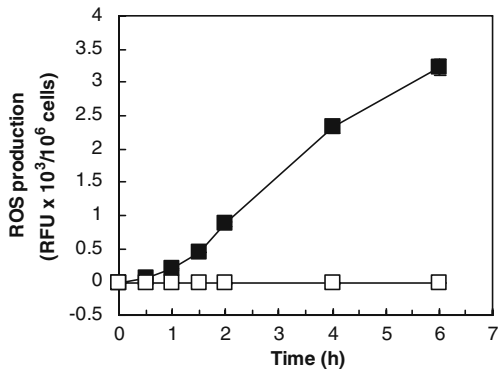

Fig. 5 Kinetic of ROS production in *S. cerevisiae* NCYC 1214 exposed to lead. Cells were pre-loaded with H_2DCFDA, during 20 min and incubated subsequently in the presence of 1 mmol/l Pb $(NO3)_2$ (*closed squares*) or in the absence of lead (*open squares*). This is a typical example of an experiment preformed twice. Each point represents the mean of four fluorescent readings; error bars are not shown because they are within the points

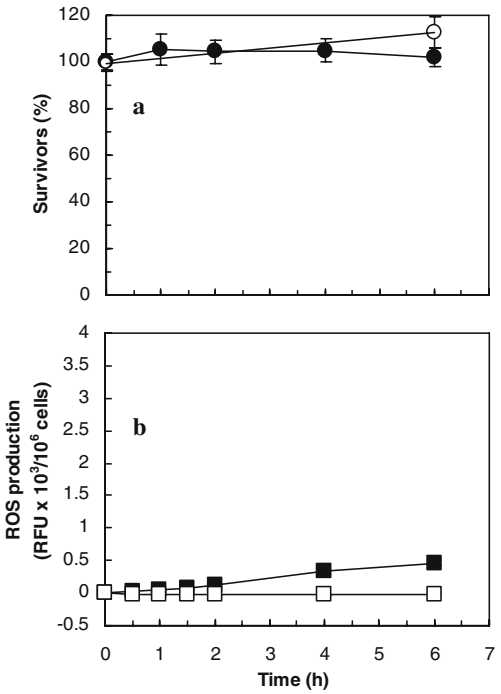

Fig. 6 Effect of the ascorbic acid in Pb-induced toxicity in *S. cerevisiae* NCYC 1214. Cells were incubated with 10 mmol/l ascorbic acid and 1 mmol/l $Pb(NO_3)_2$ (*closed symbols*) or with 10 mmol/l ascorbic acid without Pb (*open symbols*). Viability (*circles*) was estimated by the spread plate technique (**a**) and ROS production (*squares*) with H_2DCFDA (**b**), as described in material and methods. For viability, each point represents the mean of three independent experiments. For ROS production, this is a typical example of an experiment preformed twice; each point represents the mean of four fluorescent readings. Standard deviations are presented; where no error bars are shown, they are within the points

evaluated by a clonogenic assay. This assay allows, in a simple but a precise way, to determine the fraction of cell population able to form colonies (survival rate).

It is known that cell membrane of *S. cerevisiae* is a primary target of metals like Cu, Cd and Hg. Metal toxic effects increase the permeability of cell membrane with a consequent loss of cellular solutes, like K^+ or UV-260 absorbing compounds, and reduced the ability to maintain electrochemical gradients and consequently membrane potential (Gadd 1993; Soares et al. 2003). Conversely, cells exposed up to 1 mmol/l Pb, during 6 h, did not lose the cell membrane potential (Fig. 1), as evaluated using a lipophilic anionic dye [$DiBAC_4(3)$]; this dye is only accumulated in cells with reduced membrane potential. Additionally, Pb-treated cells retained membrane impermeability to PI (Fig. 2). These data are in agreement with our previous results, which have shown that plasma membrane seems not to be the immediate target of Pb toxicity, when yeast cells were exposed up to 1 mmol/l Pb, during 30 min (Van der Heggen et al. 2010).

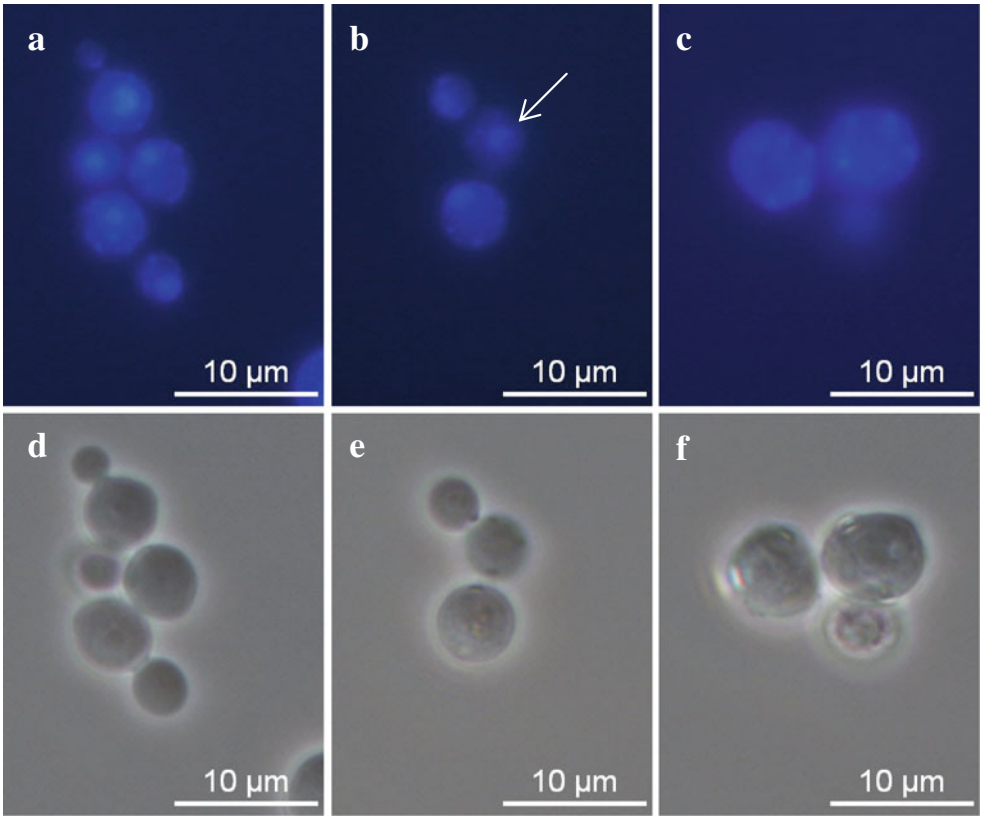

Fig. 7 Nuclear morphological modifications of cells of *S. cerevisiae* NCYC 1214 treated with Pb. Cells incubated in absence of Pb, during 6 h, display a normal nuclear morphology (**a**). Cells incubated with 1 mmol/l Pb $(NO_3)_2$ presented altered morphology of the nuclei as half-ring arrangements (*arrow*; **b**) or nuclear fragmentation (**c**); these cells were incubated with Pb during 4 (**b**) or 6 h (**c**). After incubation with or without Pb, cells were collected, fixed and stained with DAPI, as described in "Materials and methods". Fluorescence micrographs (**a**–**c**); phase contrast micrographs of the same cells (**d**–**f**)

It has been shown that metals like Cu and Mn are beneficial for yeast cells at subtoxic levels; however, both metals induced apoptosis in yeast cells at moderate toxic levels, while at even higher concentrations necrosis takes over (Liang and Zhou 2007). Similarly, Cd, at low concentrations, induces apoptosis in yeast cells (Nargund et al. 2008). These data, associated with the findings described above about the loss of cell proliferation capacity in the absence of the injury of cell membrane, prompted us to investigate other characteristic apoptotic phenotype markers in Pb-exposed cells.

PCD by apoptosis is a process of death which requires the participation of the cellular metabolism. In this context, it was described that Cyh prevented the apoptosis induced by H_2O_2 and acetic acid in *S. cerevisiae* (Madeo et al. 1999; Ludovico et al. 2001). In the present study, it is shown that the mechanism of Pb-induced toxicity seems to involve the protein synthesis. This is supported by the fact that the incubation of cells in the presence of Cyh attenuated the loss of cell proliferation capacity induced by 1 mmol/l Pb (Fig. 3). These data are in agreement with our previous results, which have shown that in yeast cells, when exposed to high Pb concentration (0.75–1 mmol/l), during 2 h, heavy metal toxicity was alleviated by the presence of Cyh (Van der Heggen et al. 2010). The enhancing of the survival percentage in the presence of Cyh indicates that Pb-induced cell death in *S. cerevisiae* is an active process. These data reinforce the possibility of PCD by apoptosis. Consistent with this possibility, recently, it was shown that Pb induced apoptosis in human leukaemia (Hl-60) cells (Yedjou et al. 2010). In addition, the prevention of the loss of proliferation capacity by inhibition of protein synthesis is a specific indicator that allows to distinguish apoptosis from necrosis (Madeo et al. 1999; Ludovico et al. 2001).

It has been proposed that ROS is a major cause of yeast apoptosis (Madeo et al. 2009). Pb-exposed cells accumulated appreciable amounts of ROS, which seems to be tightly associated with the loss of cell proliferation (Figs. 1 and 5); in fact, the kinetic of the ROS intracellular accumulation displayed the same pattern of the loss of cell proliferation capacity. The addition of an anti-oxidant (vitamin C) showed to be an effective yeast protector against the oxidative stress; the simultaneous addition of AA and Pb resulted in a small intracellular ROS accumulation and prevented cell death as ~100% of cells retained their proliferation capacity (Fig. 6). These results strongly suggest that ROS are the promoters of Pb toxicity. ROS formation metal-induced could occur by several mechanisms. In the case of redox-active metals like Fe, Cr or Cu, ROS are formed via Fenton or Haber–Weiss reactions; for redox-inactive metals like Cd, Hg or Pb, ROS can be produced by several indirect mechanisms as

displacement of redox-active metals from cellular bending sites or the inhibition of the anti-oxidant defence enzymes (Avery 2001).

DNA damage is a central aspect in the mechanism of heavy metals induced carcinogenesis; the maintenance of the genome integrity is critical to cell survival and normal cell growth (Silbergeld 2003). In the case of Pb, several mechanistic hypotheses have been considered for the DNA damage. Pb can bind to DNA and change its conformation or break nucleic acids (Silbergeld 2003). DNA is also very sensitive to the damage provoked by ROS (Avery 2001). Oxidative DNA damage heavy metal-induced constitutes another possibility. Metals such as Cd, Se and Cr(VI) promoted various types of DNA damage in *S. cerevisiae*. Several evidences supported that DNA damage ROS-mediated is an important mechanism of metals toxicity at least in the case of Cd and Cr(VI) (Avery 2001). Recently, it was shown that Pb increased mutation frequency in yeast cells, being the Pb-induced mutagenic effect largely oxidative stress dependent (Yu et al. 2009). Here, it was shown that yeast cells, under Pb-induced oxidative stress, displayed nuclear morphological alterations, like chromatin fragments arranged in half rings and nuclear fragments randomly distributed (Fig. 7). These nuclear morphological alterations are in agreement with another work, which described DNA damage in yeast cells exposed to Pb, evaluated by the single-cell gel electrophoresis assay (comet assay) (Yuan and Tang 1999). Conversely, in the simultaneously exposed to 1 mmol/l Pb and 10 mmol/l of AA, no nuclear morphological alterations were observed.

In conclusion, in the present study, it was shown that cells exposed to Pb displayed several phenotypic alterations characteristic of PCD by apoptosis, such as the loss of proliferation capacity in the absence of plasma membrane disruption and nuclear morphological alterations. The loss of proliferation capacity required the protein synthesis, which suggests the intervention of the cell machinery in the process of cell death. Pb induced the intracellular ROS accumulation which plays an essential role in the Pb-induced toxicity; abrogate of Pb-induced oxidative stress by addiction of ROS scavenger (vitamin C) impairs Pb-induced toxicity. These results suggest that ROS can be the trigger of PCD by apoptosis.

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