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Original Research

Nitric Oxide Modulates Lead Content during Lead-Induced Cell Killing in Yeast Cells

Ruigang Zhang, Huilan Yi*

School of Life Science, Shanxi University, Taiyuan 030006, Shanxi, P.R. China

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Abstract

In this study we investigated the role of NO in Pb-induced yeast cell death. We found that the rate of cell death increased with increasing concentrations of $Pb(NO_3)_2$ and prolonged exposure to $Pb(NO_3)_2$. NO production also increased significantly during Pb-induced yeast cell death. An exogenous NO donor increased Pb toxicity to cells, while NO synthesis inhibitors and NO-specific scavengers alleviated Pb toxicity. To further investigate the mechanism of NO in Pb toxicity, we measured the Pb content and mRNA expression of metal ion transport gene SMF1 in yeast cells. Our results showed that endogenous NO may enhance SMF1 expression, thereby increasing Pb content in yeast cells. Meanwhile, we found that the intracellular ROS levels increased with the increase of intracellular Pb content in yeast cells. Conclusion: Pb can promote the increase of intracellular nitric oxide levels. NO may promote intracellular Pb transport by enhancing the expression of SMF1 in Pb-induced yeast cell death. Intracellular accumulation of Pb further promotes the increase of intracellular ROS levels, and then lead to yeast cell death by oxidative damage.

Keywords: yeast, cell killing, lead, nitric oxide

Introduction

Lead (Pb) pollution is a global environmental problem [1-3]. Several studies have demonstrated that Pb^{2+} not only can affect physiological and biochemical reactions in plants, but can also induce numerous adverse responses in animals and humans [4-7]. The molecular mechanism of Pb toxicity has attracted a lot of attention in the field of abiotic stress research. Various mechanisms have been proposed involving oxidative stress, ionic processes, and apoptosis [8-10].

Nitric oxide (NO) is a common signaling molecule that plays an important role in physiological and biochemical processes in plants and animals [11-15]. NO responds to and has an important regulating effect on various forms of intracellular stress, including salt, drought, and heavy metal stress [16-18]. Recently, it has been shown that NO is an important signaling molecule associated with Pb resistance within plant and animal cells [19-21]. Some studies have shown that NO alleviates Pb toxicity by reducing the accumulation of intracellular reactive oxygen species (ROS) [22-23]. Other research has found that NO can increase Pb toxicity in cells. A dose-dependent relationship between Pb toxicity and NO production has been found in some cell lines, such as

*e-mail: yihuilani@yahoo.com

PC12 [24]. However, despite several published reports on the action mechanism of NO responding to Pb toxicity, it remains unclear how NO is involved in Pb-induced cell death.

Yeast cells are an ideal research tool for investigating the mechanism of cell death, due to their homogeneity and accessibility [25-28]. Investigating the mechanism of Pb-induced cell death in yeast should help further determine the mechanism of Pb-induced cell death across species. In addition, these findings will contribute toward a better understanding of the biological toxicity of Pb. In the present study, we investigated the toxic effects of Pb on yeast cells. We then explored the influence of Pb on NO content in yeast cells and the relationship between NO and yeast cell death. We also measured changes in the Pb content in yeast cells. Our results further the understanding of the regulating role of NO during Pb-induced cell death.

Materials and Methods

Cell Culture

The GS115 strain of Saccharomyces.*cerevisiae* was cultured in the YPD medium with 1% yeast extract, 2% peptone, and 2% glucose, and incubated at 30ºC on a rotary shaker at 200 rpm.

Measurement of Cell Viability

For each experiment, YPD medium was inoculated with a single yeast colony and cultured to logarithmic phase. Yeast cells that were not treated with Pb were used as a control group.

The treatment group was exposed to 2-100 $mg{\cdot}L^{-1}$ lead nitrate ($Pb(NO_3)_2$) solution, while the remission group was exposed to 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), NG-Nitro-L-arginine Methyl Ester (L-name), or sodium nitroprusside (SNP) to react with $Pb(NO_3)_2$. Cell viability was measured at 3, 6, and 24 h. Yeast cells were sampled to test cell viability using methylene blue staining, as described by Sami et al. [29].

Measuring NO

NO can move freely through cell membranes; therefore, NO released into the culture medium represents the amount of NO generated in yeast cells. Quantification of NO in the culture medium was previously reported by Hu et al. [30]. Briefly, a 1-mL filtrate of yeast cells was obtained via passage through a 0.22-μm micro-sieve. Next, 1 mL of Greiss reagent [1% sulfanilamide, 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride in 5% phosphate acid] was added for 30 min at room temperature, and the absorbance was quantified by a spectrophotometer at 550 nm. Different concentrations of $NaNO_2$ were used to prepare a standard curve.

Measurement of Intracellular Reactive Oxygen Species

The content of intracellular reactive oxygen species (ROS) in yeast cells was measured using the oidant-sensitive probe 2,7-dichloro-dihydrofluorescein diacetate (DCFH-DA) according to the methods by Wu et al. [31]. Reactive oxygen species was detected by Flow cytometry instrument (Becton Dickinson and Compangy, USA). Relative fluorescence intensity value was the average of the fluorescence intensity of 50,000 cells. Data collection and analysis were based on CellQuest 3.1f and ModFitLT3.0 software, respectively.

Determining Pb^{2+} Concentration

A modified version of the Chinese National standard of measurements of Pb in food (GB 5009. 12-2010) was used for Pb^{2+} measurements. Each sample was digested in a mixture of 5 mL HNO_3 (analytical grade) and 3 mL $HCLO_4$ (analytical grade) in a beaker overnight, and then heated over a graphite electric hot plate at approximately 220ºC for 20 min. Pb^{2+} concentrations were measured using an atomic absorption spectrometer (Skyray AAS6000, Jiangsu, China). Optimum conditions were applied: 283.3 nm wavelength, 6 mA hollow cathode lamp current, 0.8 L/min acetylene flow rate, 5.5 L/min air flow rate, and a 0.4 nm slit width. The correlation coefficient of the standard curve was 0.998, the recovery rate was 99.3%, and the limit of detection was below 0.006 μg/mL. A standard Pb solution obtained from the National Testing Center of Nonferrous Metals and Electronic Materials (GSB04-1742-2004, Beijing, China) was used as an analytical control.

Isolation of Total RNA and Quantitative Real-Time PCR

Total RNA was extracted from the yeast using TRIzol reagent according to the manufacturer's instructions. Next, RNA was reverse transcribed into cDNA using a Primer Script RT reagent kit with gDNA Eraser. PCR amplification was performed using a SYBR Premix Ex Taq II Kit, according to the manufacturer's instructions. The following primers were used: SMF1, forward 5'-GGTTTCGTTACGGGACTA-3' and reverse 5'-ATTCAAGGCAATCGCTGT-3'. Real-time PCR was performed using an ABI 7500 real-time PCR system (ABI, USA). PCR assays were performed three times with duplicate samples. The relative levels of gene expression were analyzed by using the $2^{-\Delta\Delta CT}$ method.

Statistical Analyses

All data were the average values of the three independent experiments. A data processing system (DPS) was used to carry out statistical analysis of all the data obtained. T-tests were used to test for differences

between the treatment and control groups, and $P<0.05$ was selected as significant.

Results and Discussion

Pb Induces Cell Death

Pb is a toxic metal widely distributed in the environment [32]. It can damage most organisms by affecting their biochemical processes and molecular structures [33-34]. However, the exact mechanism of Pb toxicity is still not clear. Therefore, further investigation of the signal transduction mechanism of Pb-induced cell death is warranted, and has the potential to reveal the toxic effects of Pb. In this study, yeast cells were used to study the mechanism of Pb toxicity.

As shown in Fig. 1a), after exposure to 2-100 mg·L^{-1} $Pb(NO_3)_2$ for 3-24 h, the yeast cell death rate increased with increasing lead concentration and exposure time. After short-term treatment with 2 mg·L^{-1} $Pb(NO_3)_2$, the increase of the yeast cell death rate was not significant. In contrast, the increase of the yeast cell death rate was significant after long-term treatment. However, short-term treatment with 100 mg·L^{-1} $Pb(NO_3)_2$ significantly induced cell death, with a death rate of 70% within 24 h. It turns out that $Pb(NO_3)_2$ can decrease yeast cell viability and increase cell death rate with the increase of Pb concentration and exposure time. This observation is consistent with a previous report in which *Vicia faba* root-tip cells were exposed to Pb [35]. Yeast cells were also treated in parallel with potassium nitrate solution that contained the same concentration of nitrate ions, in order to investigate whether Pb-induced yeast cell death was affected by nitrate ions or not. As shown in Fig. 1b), compared with lead nitrate treatment, potassium nitrate did not cause yeast cell death. This result suggests that it is the lead nitrate that caused the yeast cell death, presumably due to the Pb component.

The mechanism of Pb-induced cell death is complex and remains unclear. There are several possible mechanisms. Firstly, Pb regulates cell death by influencing levels of intracellular signaling molecules, including ROS, reactive nitrogen species (RNS), and Ca^{2+} [36-37]. For example, Pb can induce ROS generation and reduce the cellular antioxidant content at the same time, resulting in oxidative damage and apoptosis. Secondly, Pb directly influences the expression of apoptosis-related genes [38-39]. Thirdly, the mechanism of Pb toxicity is mainly due to Pb replacing other divalent ions, including Ca^{2+}, Mg^{2+}, and Fe^{2+}, thus affecting a variety of fundamental biological processes [40-41].

NO Contributes to Pb^{2+}-Induced Yeast Cell Death

NO is a diffusible signaling molecule with biological activity [42]. It has multiple roles associated with responses to biotic or abiotic stresses [43-45]. Many recent studies have shown that NO plays an important role in cellular responses to Pb stress [46].

As shown in Fig. 2a), treatment with 20 mg·L^{-1} $Pb(NO_3)_2$ significantly increased the NO level in yeast cells in comparison with the control group. This result is consistent with a previous report examining *Pogonatherum crinitum* root tip cells [47]. In that study and in ours, an increase in the NO level is closely correlated with the toxic effect of Pb on cells.

In yeast, NO is generated mainly via NO synthase-like activity [48]. An increased level of NO in response to Pb

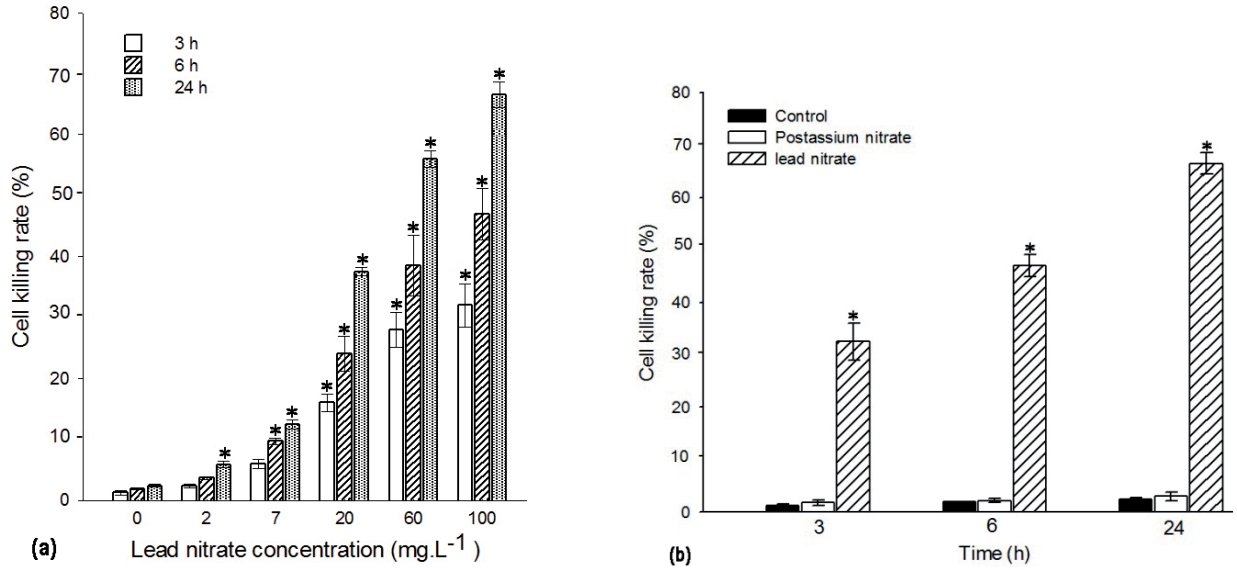

Fig.1: a) Viability assays of yeast cells exposed to lead nitrate and b) viability assays of yeast cells exposed to 100 mg/L lead nitrate and 61mg/L potassium nitrate. Each value represents the average of three experiments and bars indicated the standard error of the mean. "*" indicates the significant difference between the control and lead treatment groups.

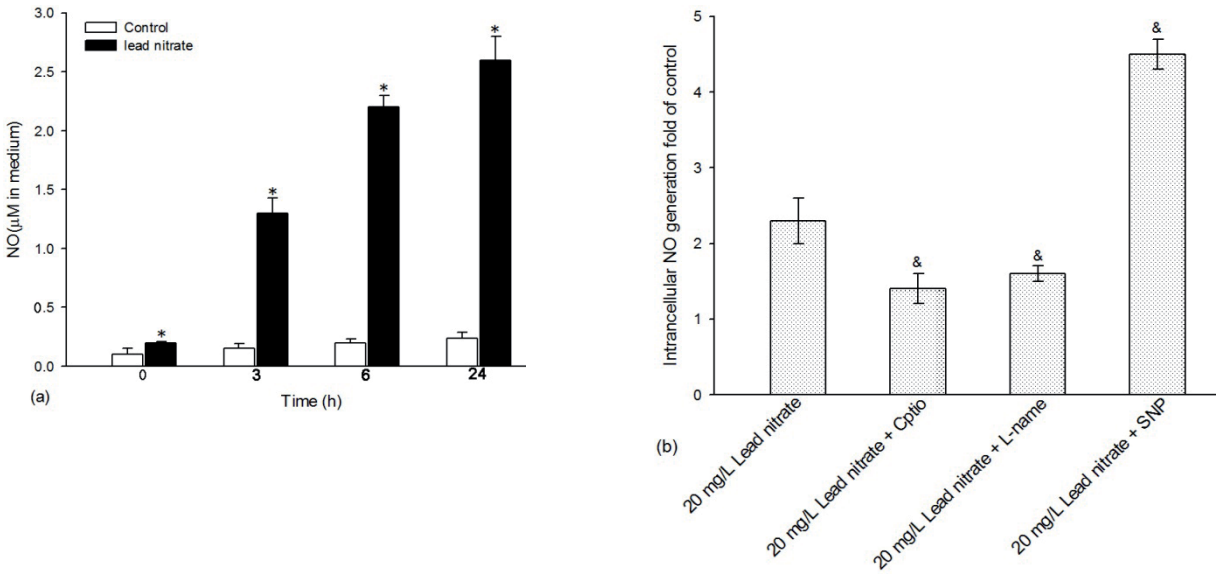

Fig. 2: a) The change of NO levels in yeast cells exposed to 20 mg/L lead nitrate exposure ("*" indicates the significant difference between the control and lead treatment groups) and b) effects of NO scavenger or NO donator on Pb-triggered NO generation in yeast cells ("&" indicates the significant difference between the lead treatment group and combination treatment groups).

exposure is mainly due to an increase in the expression of NO synthase-like genes [49-50]. NO belongs to the RNS family; therefore, NO can react with large molecules, including proteins, lipids, and nucleic acids [51]. Excessive amounts of NO can induce cell death by oxidative stress, interfere with energy metabolism, damage DNA directly, activate poly (ADP-ribose) polymerase, and disrupt calcium ion metabolism [52-53]. Therefore, the increased NO that arises in response to Pb exposure is likely related to the toxic effects of Pb.

To further investigate the influence of NO on Pb-induced yeast cell death, SNP, L-NAME, and cPTIO were used. Firstly, we tested the influence of SNP, L-name, and cPTIO on the NO content in cells. As shown in Fig. 2b), after treatment with SNP together with Pb, NO content in cells increased by 2.88 times, compared with Pb treatment alone. In contrast, L-name or cPTIO can significantly inhibit NO production in cells.

Secondly, after yeast cells were treated with cPTIO or L-name together with $Pb(NO_3)_2$ for 3 h, the yeast death rate was significantly inhibited compared to the Pb treatment group (Fig. 3a-b). Similar results are obtained after treatment for 6 h. In contrast, after treatment with SNP together with $Pb(NO_3)_2$, the cell death rate significantly increased compared to the Pb treatment group (Fig. 3c). Based on these results, we believe that Pb-induced NO can promote yeast cell death.

NO Influences the Pb Accumulation in Yeast Cells

There have been many studies showing that NO participates in heavy metal transport [54]. Ma et al. found that NO played a positive role in $CdCl_2$-induced programmed cell death by modulating Cd^{2+} uptake in BY-2 cells [55]. However, to date there have been no reports about the relationship between NO and Pb uptake in yeast cells.

To evaluate whether the NO content of yeast cells can affect their Pb content, we treated yeast cells with Pb for 6 h and then tested their Pb content. As shown in Fig. 4a, a positive correlation was observed between cell death rate and the intracellular content of Pb. Meanwhile, as shown in Fig. 4b), when SNP was applied together with $Pb(NO_3)_2$, the cell death rate and the Pb content in the cells simultaneously increased in comparison with the Pb treatment group. This result is mainly due to increased intracellular NO levels induced by SNP. Moreover, after treatment with L-name or cPTIO together with $Pb(NO_3)_2$, the Pb content in the cells and the cell death rate were simultaneously reduced in comparison with the Pb treatment group. This effect is mainly due to reduced intracellular NO levels by L-name or cPTIO. Together, these results suggest that NO participates in yeast cell death by altering Pb absorption by yeast cells. NO may modulate Pb uptake by regulating expression of ion transport-related genes.

Effects of NO on mRNA Levels of SMF1 in Yeast Cells

In the past few decades, several studies have reported that Pb enters cells primarily through transporters [56-57], but it is still unclear how NO regulates this uptake. SMF1 has been reported to be a metal transport-related gene in yeast. To investigate the effect of NO on Pb transport, we analyzed SMF1 expression. A positive correlation existed between SMF1 mRNA and the intracellular content of Pb (Fig. 5). After treatment with SNP together with Pb, SMF1 expression and the intracellular Pb content simultaneously

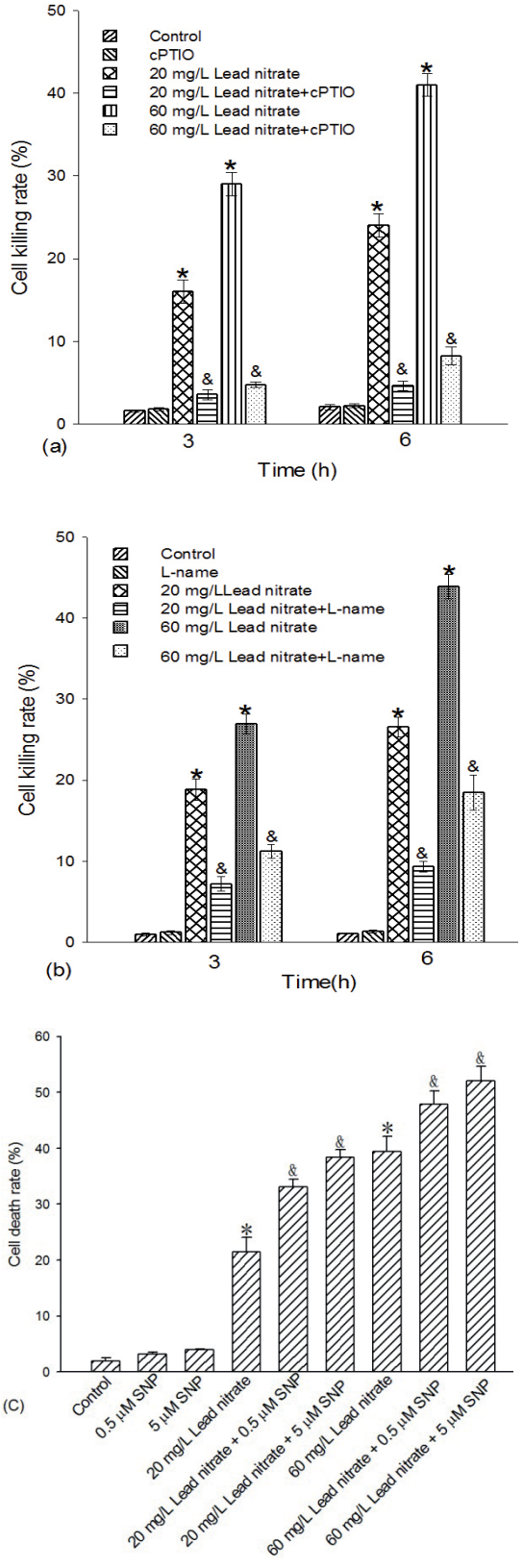

Fig 3. Effects of NO on Pb-induced yeast cell death: a) antagonistic effects of NO scavenger (cPTIO) on Pb-induced yeast cell death, b) antagonistic effects of nitrate reductase inhibitor (L-name) on Pb-induced yeast cell death, and c) stimulative effects of NO donator SNP on Pb-induced yeast cell death. "*" indicates the significant difference between the control and lead treatment groups, while "&" indicates the significant difference between the lead treatment group and combination treatment groups.

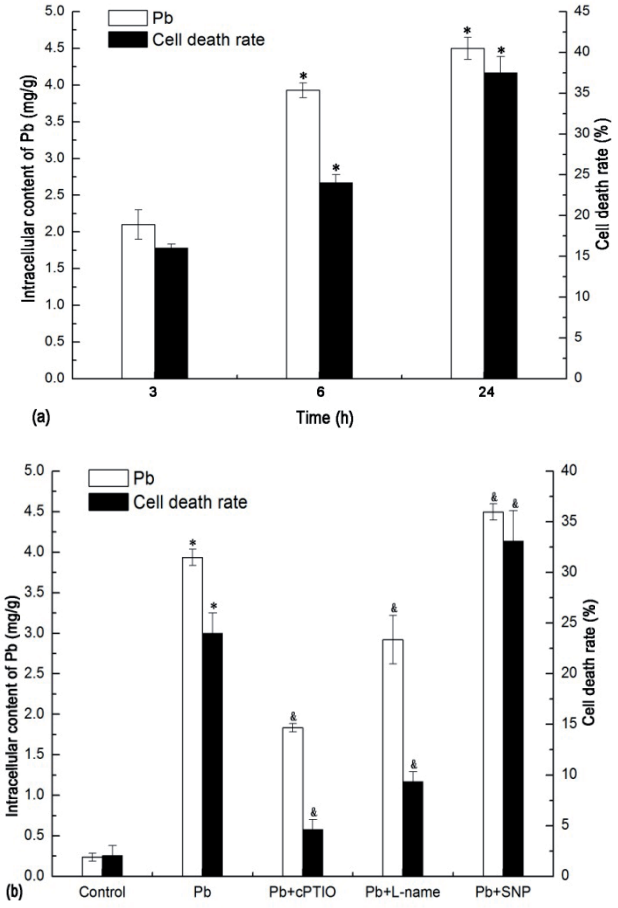

Fig. 4: a) Pb-triggered cell death and Pb accumulation in yeast cells and b) effects of NO scavenger or NO donator on Pb accumulation and cell death rate in yeast cells. Each value represents the average of three experiments and bars indicated the standard error of the mean. "&" indicates the significant difference between the lead treatment group and combination treatment groups.

increased in comparison with Pb treatment alone. This effect is mainly due to an increase in intracellular NO levels by SNP. In contrast, L-name or Cptio can be used to significantly inhibit SMF1 expression and intracellular Pb content, which is mainly due to decreased intracellular NO levels by L-name or Cptio. Taken together, these results suggest that NO may regulate the intracellular Pb content by regulating SMF1 expression.

Effects of Intracellular Pb Content on ROS Levels in Yeast Cells

Reactive oxygen species is an intracellular signaling molecule and can lead to cell death by oxidative damage [58]. To further study the effect of intracellular Pb accumulation on yeast cell death, we tested the changes of intracellular ROS levels. As shown in Fig. 6, the intracellular ROS levels increased with the increase of intracellular Pb content in yeast cells. It also suggested that intracellular accumulation of Pb further promotes the increase of intracellular ROS levels. Previous

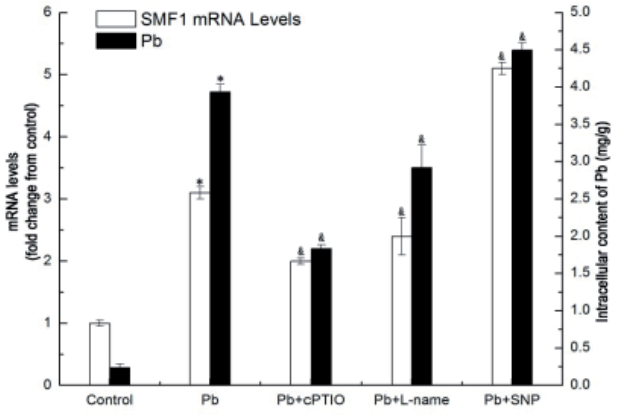

Fig 5. Effects of NO scavenger or NO donator on mRNA levels of SMF1 and Pb accumulation in yeast cells. Data are expressed as the mean. An asterisk indicates the significant difference between the control and lead treatment groups, and "&" indicates the significant difference between the lead treatment and combination treatment groups.

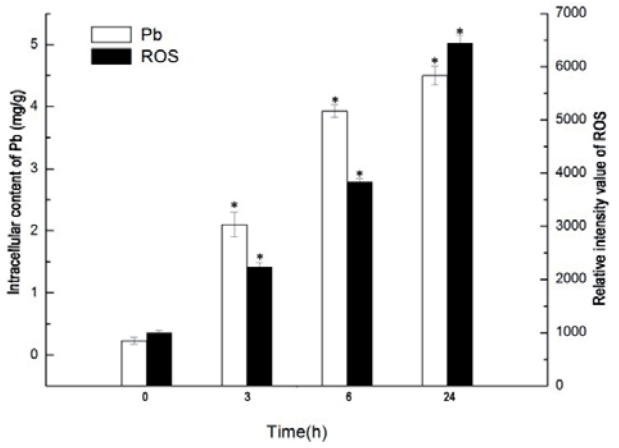

Fig. 6. The change of ROS levels in yeast cells exposed to lead nitrate exposure. An asterisk indicates the significant difference between the control and lead nitrate treatment groups.

research has shown that mitochondrial membranes were broken by high levels of ROS, leading to reduced membrane potential and causing cell death by activating relevant downstream signaling pathways [59]. Therefore, intracellular accumulation of Pb further promotes the increase of intracellular ROS levels, and then leads to yeast cell death by oxidative damage.

Conclusion

Our results show that endogenous NO plays an important role in Pb- induced cell death. Pb can promote the increase of intracellular nitric oxide levels. NO may promote intracellular Pb transport by enhancing the expression of SMF1 in Pb-induced yeast cell death. Intracellular accumulation of Pb further promotes the increase of intracellular ROS levels, and then leads to yeast cell death by oxidative damage. Further research on the mechanism of Pb-induced yeast cell death should help to further determine the mechanism of Pb-induced cell death across species. This will be important for understanding the toxic biological effects of Pb. A better understanding of Pb toxicity could lead to improved strategies for environmental Pb detoxification by bioengineering industrial yeast strains.

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