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Original Research

Toxicological Assessment of Aluminum Sulfate and Three Mitochondrial Respiratory Chain Inhibitors (Trimetazidine, Prednisolone, and Potassium cyanide) on Yeast *Saccharomyces cerevisiae*

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Abstract

Aluminum (Al) is a widely found metal with no known biological or clinical benefits, and thus causes considerable toxicological effects on biological organisms' health, including yeast. Mitochondria are the main cellular organelles involved in the production of oxygen and reactive oxygen species (ROS) in the cell. Yeast is the most scientifically used micro-ecologic, and the most sensitive microorganisms to various toxicants. As the toxicological effects of Al and mitochondrial respiratory chain inhibitors on *Saccharomyces cerevisiae* have not been elucidated; the present study was therefore, devoted to comparing the toxic effects of Al salts and three mitochondrial inhibitors, namely trimetazidine, Prednisolone, and Potassium Cyanide on *Saccharomyces cerevisiae*. Cells were exposed 2h increasing concentrations of Al (C1 = 0.017g/L, C2 = 0.034g/L, C3 = 0.342 g/L, and C4 = 8,5 g/L), and one concentration of mitochondrial inhibitor C1 = 0.0032g/L Potassium Cyanide (KCN), C1 = 0.013 g/L Trimetazidine (TMZ), and C1 = 0.018 g/L Prednisolone (PDN). Results showed an inhibition of cell growth in $Al_2(SO_4)_3$ and the used mitochondrial inhibitors, in particular, Cyanide and Prednisolone as evidenced by positive response percentages in exposed yeasts. Moreover, oxidative stress induction in Aluminum sulfate treatment was revealed by stimulation of catalase (CAT) and Glutathione S-transferase (GST) activity, along with increased levels of GSH and MDA compared with control.

Keywords: Aluminum sulfates, *Saccharomyces cerevisiae*, Oxidative Stress, Bioindicator, mitochondrial inhibitors

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Introduction

Aluminum (Al) is a non-trace element and environmentally omnipresent metal whose toxic exposure may result in undesirable effects on human and environmental health [1]. Due to its typical physicochemical properties, it is widely used, in either pure or alloy forms in large industrial applications, including cosmetics, drinking water, and food products [2], in addition to construction uses, the transport industry, kitchen utensils, containers and packaging, food preservation, and some paint manufactures [3]. In the human body, Al has no known biological role and is minimally metabolized by the liver, thus, human tissues and organs (especially the brain) are the main Al targets [4, 5]. Since the last few years, the undesirable effects of Aluminum have been the subject of numerous scientific researchers, claiming the necessity of the authorities to review and re-evaluate the benefit-risk ratio of Al and its derivatives during use. In this regard, several studies have reported serious human, animal, and plant diseases [6, 7], in association with induction of oxidative stress and lipid peroxidation through Al exposure [8, 9]. Nevertheless, its cytotoxicity and mechanism of action on microorganism cells are poorly documented [10].

In the cell, the mitochondria are the main cell organelles involved in producing energy in the form of ATP and regulating several biological processes such as apoptosis. In addition, a series of chemical reactions occur in ATP production, which consequently can be frequently altered by many toxicants through either inhibiting key steps in cellular respiration or altering the mitochondrial electron transport chain's membrane. Some other toxic chemicals may induce the formation of free radicals that damage organelle components, including mitochondrial DNA, resulting in the cell's ability to produce energy and induction of cell apoptosis [11].

Moreover, *Saccharomyces cerevisiae*, known as a eukaryotic cell with a perfect cellular organization comparable to superior organism cells [12], and a widely used biological model bio- indicators in cytotoxicity studies [13, 14]. Also, it has a fast life cycle and easy experimental use, enabling scientists to understand the mechanism of a xenobiotic action [15]. It is considered a privileged model for the studies of eukaryotic cells because it provides reliable results and basic knowledge of cellular and molecular deficiencies and disorders, such as mitochondrial dysfunction, cell division, apoptosis, diabetes...etc. [16]. In this context, the main purpose of this paper is to evaluate the cytotoxicity associated with reactive oxygen species (ROS) generation of Aluminum salts on *Saccharomyces cerevisiae* by estimating Catalase activity (CAT), the first line and a key enzyme of free radical defense [17]. At the same time, we quantified reduced Glutathione (GSH), essential for ROS neutralization [18]. We also monitored Glutathione S-Transferase (GST) activity, a phase II biotransformation enzyme [19]. And assessed the level of Malondialdehyde (MDA), the final product of lipid peroxidation [20]. On the other side, the present work aims to compare the Aluminum effect on growth with three pharmacological molecules known as mitochondrial respiratory chain inhibitors.

This is performed to understand the mechanism of action of Al sulfates, given the close relationship between cell death and mitochondrial dysfunction.

Material and Methods

Cell Culture

Baker's yeast, *S. cerevisiae* strain, was obtained from SAF-INSTANT YEAST Company. The yeasts were washed with physiological water (0.9% NaCl) using at least three centrifugations at 3000Tr/min every 5 min. The resulting pellet representing the crude cell fraction (F9) was collected into a respiration-promoting culture medium as previously described by [21]: 10 g yeast extract, 0.25 g glucose, 25ml glycerol, and 980 ml phosphate buffer, pH 6.2. Respecting the optimal conditions for cell growth (T: 25ºC±2) and oxygenation condition for 2 hours, which corresponds to the exponential phase.

Treatment and Monitoring of Growth Kinetics

After 2 hours of cell oxygenation, each 50 ml aliquot was exposed to increasing concentrations of Aluminum sulfates $Al_2(SO_4)_3$ obtained from SAIDAL Company: C1 = 0.017g/L, C2= 0.034g/L, C3 = 0.342 g/L, and C4 = 8,5 g/L. Each sample was incubated under the same growth conditions to maintain cellular proliferation. The growth kinetics were monitored spectrophotometrically (JENWAY, 660nm) after 1, 2, 3, 4, and 24 hours of exposure to the test chemical [22].

Thus, the choice of Al sulfate concentrations was based on a performed preliminary study, the selected ones were the most relevant to the exposure level.

Response Percentage

The response percentage was determined by using the following equation [23]:

$$\%R = 100 \times (Nt - Nc)/ Nt$$

Where "***Nt*** "is the optical density of control cells and "***Nc****"* is the optical density of the treated cells.

Negative and positive values indicate, respectively, the growth stimulation and the cell proliferation inhibition.

Determination of Oxidative Stress Markers

The enzymatic activity of catalase (CAT) was determined following the method outlined by [24].

After cellular centrifugation at 15000 T/10 min, a solution of 0.050 ml of supernatant, 0.075 ml of phosphate buffer and 0.2 ml of H_2O_2 were disposed of in a quartz cuvette. The spectrophotometer lecture was realized at 240 nm during 1 minute, and the catalase activity was measured in nmol/min/mg of proteins. The measurement of Glutathione S-transferase (GST) followed the procedure described by [25]. This method involves the reaction of 200 µl of enzymatic extract with 1,2 ml of CDNB (1-chloro 2, 4-dinitrobenzène). Absorbance readings were taken every minute for 5 minutes at 340 nm using JENWAY 3600 spectrophotometer. Moreover, levels of the glutathione (GSH) were determined according to [26]**.** A cellular suspension was added to 1ml of EDTA (0.02M), then 0.8ml was mixed with 0.2 ml of ASS solution (Sulfosalicylic Acid 0.25%). After centrifugation, 0.5 ml of the supernatant, 1ml of EDTA and 0.025 ml of DTNB (5,5' dithio- 2- nitrobenzoic acid) were placed in a plastic cuvette and measured the absorbance at 412 min after 5 min of rest. The GSH level is expressed in µM/mg of proteins. Thus, the rate of Malondialdehyde (MDA) was quantified using the method established by [27]. The protein quantification of each sample is realized using brilliant blue reagent according to Bradford method [28].

Comparative Study

In order to understand the Al sulfate mechanism of action at the subcellular level. Our focus centered on mitochondria as a cellular important target, playing a crucial role in signaling, respiration, and production of ROS in yeast. A comparative cytotoxic study of 0.05 mM of Al and the three mitochondrial inhibitors (0.0032 g/L Potassium Cyanide (KCN) (SIGMA-ALDRICH Company), 0.013 g/L mM Trimetazidine (TMZ), and 0.018 g/L Prednisolone (PDN) (SARL BIOGALENIC Company) on *S. cerevisiae* growth were investigated.

Statistical Analysis

Data were analyzed using GraphPad Prism 9 using one- and two-way ANOVA tests and were displayed as mean ± error deviation (SEM). Significant differences were noted when P≤0.05, (P≤0.01), and (P≤0.001).

Results

As shown in Fig. 1A), the growth kinetics revealed an obvious growth inhibition in cells exposed to toxicants after 1 and 2 hours (exponential phase of yeast). This depletion is more pronounced in yeast treated with low concentrations (especially C2) compared to that in cells exposed to higher concentrations. After 24 hours of treatment, the cellular growth increased markedly in cells that received a high concentration of toxicants. Further, the determined response percentages (Fig. 1B) were all positive in the highest concentration treated yeast; 33.4% for C1 and 47% for C2. Moreover, the catalase activity was very highly significantly stimulated (P≤0.001) in the cells treated with C4: (11 µmol/min/mg of protein) compared to that of controls, showing a value not exceeding 2 µmol/min/mg of protein (Fig. 2A). Similarly, GST activity showed a marked simulation, especially in the Al higher concentration exposed yeasts with a value of 0.00029 µmol/min/mg of protein compared to the controls, showing a value equal to 0.00002 µmol/min/mg of protein (Fig. 2D). Results showed also a significant (P≤0.05) increase in the level of MDA in the treated cells compared to the controls.

The MDA values were ranging from 0.001µM/mg of protein for C1 to 0.0014 µM/mg of protein for C4 compared to controls, showing not exceeding a value of 0.0006 µM/mg of protein (Fig. 2B). Similarly, the level of glutathione was markedly increased in cells treated with all Aluminum concentrations, and highly significant (P≤0.01) in C4-treated cells. This increase ranged from 0.5µmol/mg of protein for C1 to 0.8 µmol/mg of protein for C4 compared to the controls, showing a value of about 0.3 µmol/mg of protein (Fig. 2C).

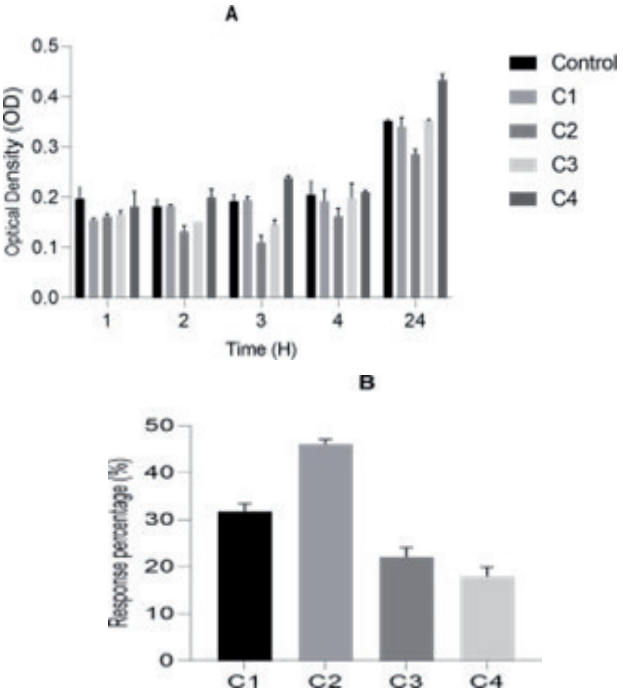

Fig. 1. A) Optical Density (OD) of the cell growth of control cells and those treated with Al for 24 hours. Data are displayed in mean±SEM, and analyzed by two way ANOVA test: Row Factor: *** (p = 0.003), column factor: * (p = 0.0196). B) Percentage of response of yeast exposed to Al. Data are displayed in mean±SEM, and analyzed by one way ANOVA test: **(p = 0.001).

The comparative study between the treated yeast with the same low concentration (0.05mM) of Al, TMZ, PDN, and KCN during 24h revealed an important cell growth inhibition following exposure to PDN ($p<0.0001$) and KCN ($p<0.0001$) compared to that seen in cells treated with TMZ ($p = 0.0022$) and Al salt, showing slight variation in the growth inhibition when compared with that of the control yeast (Fig. 3A). On top of that, the response percentage was significantly higher in yeast treated with PDN (26.3% $p = 0.0282$) and KCN (27, 4% $p = 0.217$) than that in cells treated with Al and TMZ (6.17% and 9.59%, respectively) (Fig. 3B).

Discussion

The present study is strongly related to that previously reported by [10] investigating the detrimental impact of Aluminum (Al^{+3}) on *S. cerevisiae* based on the evaluation of the major oxidative stress biomarkers, such as GSH levels, Catalase Activity, and TBARS levels. Our findings about the Aluminum sulfates support their hypothesis, indicating that this molecule has the potential to disrupt cellular integrity, induce oxidative damage, and influence the activities of antioxidant enzymes. By examining the same cells at different chemical concentrations, we were able to compare and validate the results obtained.

The bio-indicator microorganisms are a valuable tool for understanding the mechanism of action of toxicants resulting mainly in cell oxidative stress. As a result, the cell reacts to maintain its homeostasis or

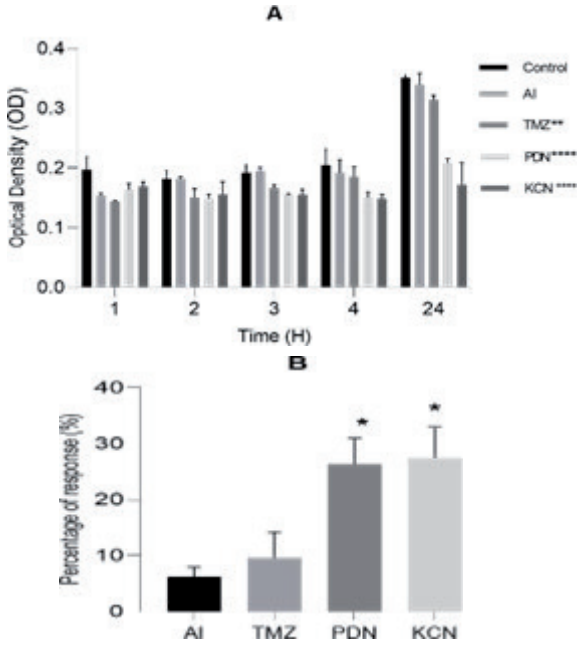

Fig. 3. A) Optical Density (OD) of the cell growth of yeast exposed to 0.017 g/L Al, 0.013 g/LTMZ, 0.018 g/LPDN, and 0.0032 g/L KCN and control cells. Results are expressed in mean±SEM and analyzed by TWO WAY ANOVA test: Row and column factor **** (<0.0001), and multiple comparisons are established in the case of TMZ: **($p = 0.0022$), PDN: ****($p<0.0001$), and KCN: **** ($p<0.0001$) comparing to control cells during 24hours of exposure**.** B) Data of the Percentage of response of the treated yeast expressed in means ±SEM, and analyzed by one WAY ANOVA test: PDN * ($p = 0.0282$) and KCN *($p = 0.217$).

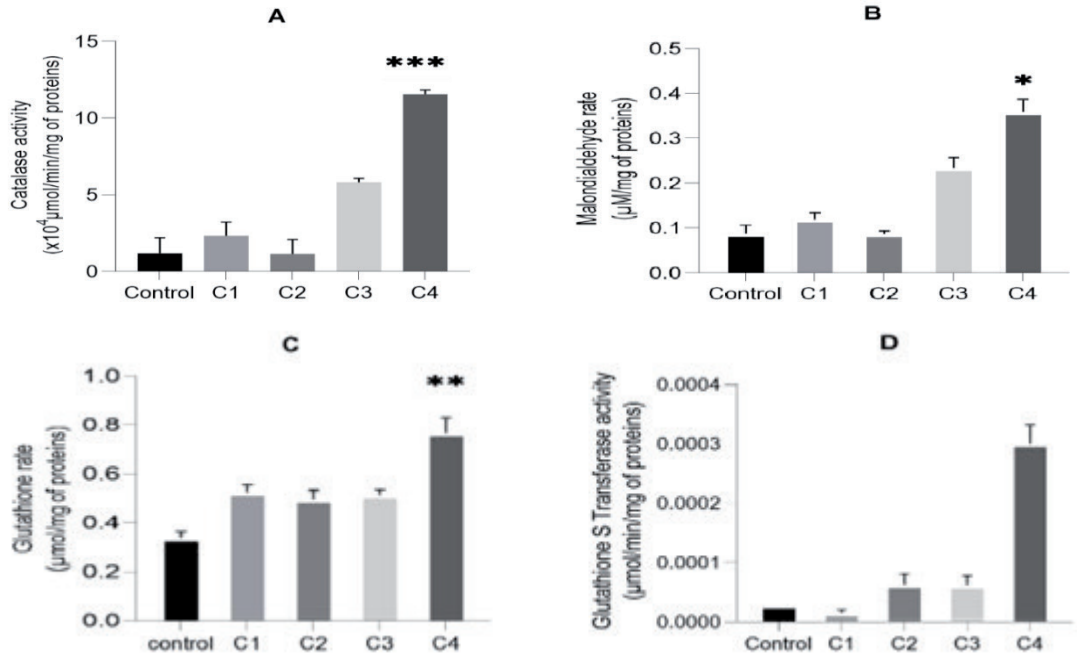

Fig. 2. Bio-indicator parameters of oxidative stress: A) (CAT), B) (MDA), C) (GSH), and D) (GST) in S. cerevisiae exposed to four increased concentrations of Aluminum salt: C1 50µM, C2 100 µM, C3 1mM, and C4 25mM. The data are expressed in mean ±SEM and analyzed by the One-WAY ANOVA test. (P≤0.05 significant, P≤0.01 highly significant, and P≤0.001 very highly significa.

to activate the defense and/or repair systems [29]. As reported [22, 30], the growth kinetics and cell metabolism are important responses of the bioindicator microorganisms via toxicity exposure. In this study, we have investigated Al-induced disturbance of cell growth, as evidenced by a significant decrease in cell proliferation, especially for the lowest concentrations. This disruption of cell growth is the first physiological response due to all Al concentration exposure. This result corroborates previous studies investigating Aluminum-induced stress in several yeast strains [10], the cytotoxicity of Al_2O_3, Mn_3O_4, SiO_2, and SnO_2 in yeast [15], and ZnO-induced or a 90% growth inhibition of *B. subitilis* (Gram-positive bacteria) [31], in addition to the work of [22], who studied the effect of Dihydropytidine on *Saccharomyces cerevisiae.*

Furthermore, the determination of the response percentage proves the inhibitory effect of the tested xenobiotic, and hence the obtained values were positive, similar to those of [23].

In this study, treatments caused a marked stimulation of catalase activity, and this is due to the reason that this enzyme is one of the first cellular defense lines against free radical attack [10]. The increased enzymatic activity of GST in treated yeasts compared with controls may explain its stimulation under the oxidative condition [32], and this concords with the work of [22]. Additionally, the Glutathione level was significantly increased in Al-exposed cells accordingly with the finding of [33], who evaluated the toxicity of some heavy metals on the behavior of the Coelomata group and that of [34], who found an increased level of GSH in Fe2O3- Helix aspersa. The interaction of toxic metals with GSH metabolism is an essential part of the metal response [35]. When the GSH level is depleted by any metal, GSH synthesis systems begin to produce more GSH from cysteine via the g-glutamyl cycle [35-37].

Consequently, its increase could be a sign of its direct reaction with metals, resulting in either the formation of complexes or the oxidation of GSH [38-40]. Alternatively, the increased GSH level could be explained by the synthesis of metallothioneins involved in the chelating metals through GSH-dependent mechanisms. On the other hand, the increased level of MDA, the end product of lipid peroxidation, may explain an early sign of the cell membrane lipid peroxidation process [41, 42] leading to the loss of membrane permeability and potential, and the inactivation of receptors and membrane enzymes [43], and subsequently to DNA damage and cell death [44].

This is in agreement with the results of [10], who determined the level of TBARS (another peroxidation product) and proved Al-induced oxidative stress by destabilizing cellular integrity, and those of [45] who reported an increase in MDA levels in yeast *S.cerevisiae*.

Moreover, the comparative study enabled us to support our results through the use of pharmacological molecules known as mitochondrial inhibitors. This organelle is the seat of ROS production, and the cell death signaling pathways [46]. Trimetazidine is a drug that inhibits B oxidation, more specifically, the enzyme 3-ketoacyl CoA thiolase, prednisolone which is a glucocorticoid acting indirectly by regulating the expression of the mitochondrial genome involved in glycolysis and/or ATP production, which can consequently disrupt mitochondrial function [47]. While cyanides inhibit cytochrome c oxidase (complex IV) of the mitochondrial respiratory chain, known as the relatively essential pathway of electron passage and ATP formation [48, 49]. A recent study conducted by [50] proved the beneficial therapeutic effect of TMZ in attenuating oxidative damage, reducing neuronal apoptosis, and preserving cellular integrity. However, the work of [51] confirms that the treatment of yeast with a non-lethal concentration of certain inhibitors, such as KCN, accompanied by restricted respiratory conditions resulted in mitochondrial damage responsible for ROS overproduction and cell death [52–54]. In a similar vein, [55] has demonstrated that the addition of KCN to wild-type yeast mitochondria resulted in a complete reduction of heam. The authors [56] reported that the very first studies revealed the presence of Glucocorticoid receptors in rat liver mitochondria, supporting the hypothesis of the direct interaction glucocorticoids in the mitochondrial genome. Conclusively, our study showed that yeast treated with PDN and KCN exhibit significant cell growth disruption compared to cells exposed to TMZ, since treatment with Al salts showed a slight growth inhibition compared to the other molecules. These data suggest that the mechanism of action of Al appears to be close to that of TMZ, and Al does not target the genome or the mitochondrial respiratory chain.

Conclusions

The study of Aluminum-treated yeast showed a noticeable decrease in cell proliferation, especially at the lowest concentrations, along with positive percentages of responses. Furthermore, the oxidative stress markers indicated the level of metal toxicity as well as its capacity to disturb mitochondrial functions by generating free radicals despite the membrane strength of *S. cerevisiae*. In addition, testing three mitochondrial inhibitors suggested that the Aluminum salts might have a similar effect to the Trimetazidine molecule.

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Data Availability Statement

The data supporting the findings of this study are available upon request.

Conflict of interest

The authors of the manuscript declare that there is no conflict of interest.

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