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Evaluation of oxidative stress induced by nanoparticles (ZnO) on a unicellular biological model (*Saccharomyces cerevisiae*)

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ABSTRACT

The aim of this study was to evaluate the ZnO nanoparticles toxic effect on yeast Saccharomyces cerevisiae. Oxygen consumption, reduced glutathione content and enzyme activities (glutathione S-Transferase, Guaïacol and Ascorbate Peroxidases) of Saccharomyces cerevisiae were evaluated after ZnO nanoparticles treatment at different concentrations. Obtained results showed that ZnO caused an inhibition of growth kinetic and oxygen consumption. Evaluation of oxidative stress biomarkers revealed that ZnO treatment resulted in a decrease of reduced glutathione concentration and an increase of all enzyme activities. These results suggested that ZnO nanoparticles caused an oxidative stress to yeast S. cerevisiae. Consequently, yeast could be a suitable model for assessment nanoparticles toxicity.

Key words: Nanoparticles, ZnO, Oxidative stress, Yeast.

INTRODUCTION

Nanoparticles (NPs) are important class of scientific materials that are being evaluated for various biotechnological, pharmacological, and pure technological applications. NPs are the particles with dimensions at the nanometer scale, less than 100 nm [1, 2]. At this length scale and depending on their form, nanoparticles possess unique properties that are different when compared to that of their respective bulk counterparts [3–6].

Metal oxide NPs are increasingly used in various consumer products such as cosmetics, sunscreens, dental fillings and textiles [7]. The occupational and public exposure to nanomaterials (NMs) is supposed to increase dramatically in forthcoming years and therefore there is an urgent need for information on toxicity and safety of manufactured NPs [8]. Zinc oxide nanoparticles (ZnO NPs) are important NMs that have been extensively used in industrial and consumer products, resulting in the increasing presence of NMs in the environment [9]. ZnO NPs have been shown to be toxic to algae [10–13], crustaceans [14, 15], bacteria [16], and fish [17, 18]. Studies of the biotoxicity of ZnO NPs suggest several mechanisms of action. Overproduction of reactive oxygen species (ROS) is believed to be a major mechanism of the toxicity of NPs [19, 20]. It has been reported that ZnO NPs disturb the balance between oxidation and anti-oxidation processes and cause oxygen stress responses in different organs of fish [18].

Yeast *Saccharomyces cerevisiae* is one of the most intensively studied unicellular eukaryotic model organisms in molecular and cell biology as its cellular structure and functional organization has much similarity with cells of higher-level organisms [21] and used for the study of the oxidative stress and aging [22, 23]. *S. cerevisiae* is also used in the toxicological evaluation of chemicals such as heavy metals [24-26], anticancer drugs [27], herbicides [28] or food preservatives such as monocarboxylic acids [29]. However, there are few studies about toxicity of NPs to *S. cerevisiae* [23].

The aim of this study was to evaluate the toxic effect of ZnO NPs on *S. cerevisiae* growth kinetics and rate of oxygen consumption, as well as on the oxidative stress by measurement of several biomarkers as reduced glutathione content (GSH) and enzyme activities of glutathione S-transferase (GST), ascorbate peroxidase (APx) and guaïacol peroxidase (GPx).

MATERIALS AND METHODS

Yeast strain:
Saccharomyces cerevisiae from industrial sources was conditioned from a culture conducted in rich middle substrates. It is therefore necessary to wash the yeast to remove any trace of substrate in the medium [30]. Yeast was washed in a 9 ‰ NaCl solution and then centrifuged twice at 3000 rpm for 5 min to remove the substrates contained in the supernatant.

Cultivation of yeast:
The cultivation was carried out in a nutrient medium based on phosphate buffer (pH 7.2) supplemented with 0.025 g of sucrose, 1 g of yeast extract, 2.5 ml of glycerol, 1g of fresh yeast in 100 ml of medium with gentle shaking and oxygenation for 2 hours.

Preparation of ZnO NPs:
The xenobiotic used is zinc oxide (ZnO) (25 nm diameter) from the Laboratory of Magnetism and Solid State Spectroscopy, University of Badji Mokhtar (Algeria). Elementary ZnO powder (purity 99.99%) was used as starting material. The mechanical alloying process was carried out in a crusher (Fritsch Pulverisette 7) at room temperature in an air atmosphere. The mixed powder was sealed in roller bottles with stainless steel balls. The total weight of the powder was 2 g, the ball weight ratio to powder was 20:1. The vial rotation speed was 500 rpm. The powder was ground for 3 h. In order to avoid excessive heating during grinding and to limit adhesion of the powder on the walls of vials, 30 min of grinding were followed by a pause of 10 min.

A concentrated stock solution was prepared in deionized water by sonication for 30 min (100W 40KHZ) and kept in the dark at 4°C. ZnO concentrations used were 0 (as control), 5, 10, 20, 50 and 100 µg/ml. Treatment was done after two hours of stirring and assays were performed after 1 hour of treatment.

Yeast growth kinetics:
The growth kinetics of the control and treated yeasts with different doses was carried out by measuring the optical density at 600 nm at several times during 2 hours (10, 15, 30, 60, 90, 120 and 180 min) [30].

Measurement of O_2 consumption:
O_2 consumption of control and treated yeasts was measured by assessing evolution of O_2 concentration in a medium using an oxygen electrode (Clark electrode) according to [31]. Briefly, the electrical system consisted in a cathode platinum (-) and in an anode silver (+) connected by a saturated solution of KCl as an electrolyte. They were separated by a membrane not permeable to water and ions but permeable to oxygen dissolved in the medium. Initial oxygen concentration in medium was estimated at 240 µM.

Preparation of enzymatic extract:
Saccharomyces cerevisiae culture was stopped in the exponential growth phase by a centrifugation at 1500 x g for 10 min. The pellet was stirred under ultrasonic in ice in potassium phosphate buffer (50 mM, pH 7.5) and centrifuged at 10 000 x g for 20 min at 4°C. Obtained supernatant containing enzymatic extract was immediately used for following assays.

Protein concentration:
Enzymatic extract total proteins concentration was quantified on control and treated ZnO NPs yeasts according to [32] at 595nm using Bovine Serum Albumin (Sigma) as standard. Results were expressed in µg/ml of extract.

Reduced glutathione (GSH) concentration:
GSH concentration of control and treated yeasts was quantified in enzymatic extract using the colorimetric method according to [33]. Oxidation of GSH by 5.5'-dithiobis sulfhydryl (2-nitrobenzoic acid) (DTNB) formed a yellow compound 5'-thio-2-nitrobenzoic acid (TNB), which was quantified at 412 nm. GSH concentration was expressed as µM of GSH per mg of protein.

Glutathione-transferase (GST) activity:
Control and treated yeast GST activities were estimated in enzymatic extract according to [34] by the conjugation of the thiol group of glutathione and the CDNB (1-chloro-2,4-dinitrobenzene) in presence of glutathione as co-factor. Absorbance was read at 340 nm and GST activity was expressed as µM per min per mg of protein.

Peroxidase activity (GPX and APX):
GPX (Guaïacol peroxidase) and APX (Ascorbate peroxidase) activities of control and treated yeasts were estimated in enzymatic extract according to [35] at 470 and 290 nm respectively. Enzymatic activities were expressed in µM/min/mg protein.

Statistical analysis:
Two-way analysis of variance (ANOVA) was performed to investigate possible time-effect relationships between studied parameters. Data were represented by mean ± standard deviation (m ± s). Differences were considered significant when $p<0.05$ (data analysis software R i386). Data normality was verified and differences between control and treated values were analyzed using the "t" test of Student.

RESULTS AND DISCUSSION

In this study, the oxidative stress induced by ZnO NPs on a single-cell model, the yeast *Saccharomyces cerevisiae* was evaluated. The growth kinetics and O_2 consumption were excellent bioindicators of toxicity. Obtained results (Fig.1) showed a concentration dependant toxic effect of ZnO NPs on the yeast growth. Compared to control, growth of treated yeast was inhibited by 5, 10 and 20 µg of ZnO/ml while it was stopped by the highest doses (50 and 100µg/ml). According to [23], nano ZnO as well as bulk ZnO both showed concentration dependent effects on yeast growth and about 80% inhibition of the growth was observed at 250 mg ZnO/l level for both types of ZnO formulations. Moreover, compared to control yeasts, estimation of O_2 consumption (Fig.2) by assaying the medium concentration of O_2 at different times showed that with low doses (5 and 10 µg/ml) this consumption was greater (66 and 80% respectively) than that of yeast controls (55% of O_2 consumed). However, an inhibition of O_2 utilization by treated yeasts with 20 and 50 µg of ZnO/ml (50 and 33% respectively) and a very low O_2 consumption (12%) with 100 µg of ZnO/ml was observed compared to control yeasts. So it seemed that with low ZnO concentrations, increase of O_2 consumption was probably used for activation of detoxifying activity whereas high ZnO concentrations (20, 50 and 100 µg/ml) involved a toxical effect. Comparable toxicity of nano and bulk ZnO has been demonstrated also for other organisms. For example, nano and bulk ZnO were of comparable toxicity also to unicellular algae *P. subcapitata* [36] and bacteria *V. fischeri* [11]. According to [37] ZnO nanoparticles inhibited growth of gram-positive bacteria *B. subtilis*.

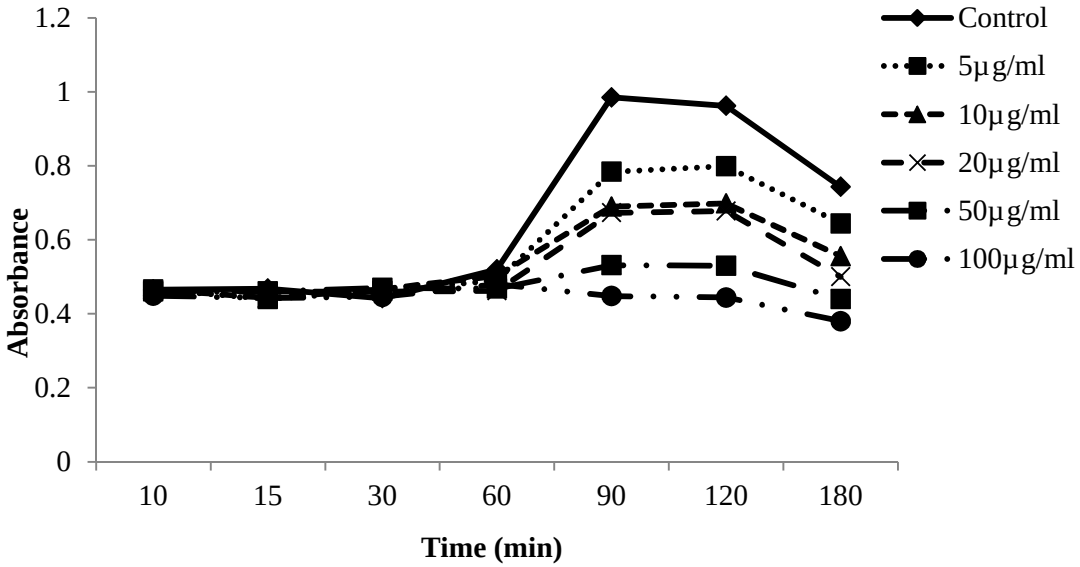

Figure 1. Growth kinetic of control and ZnO treated *Saccharomyces cerevisiae*

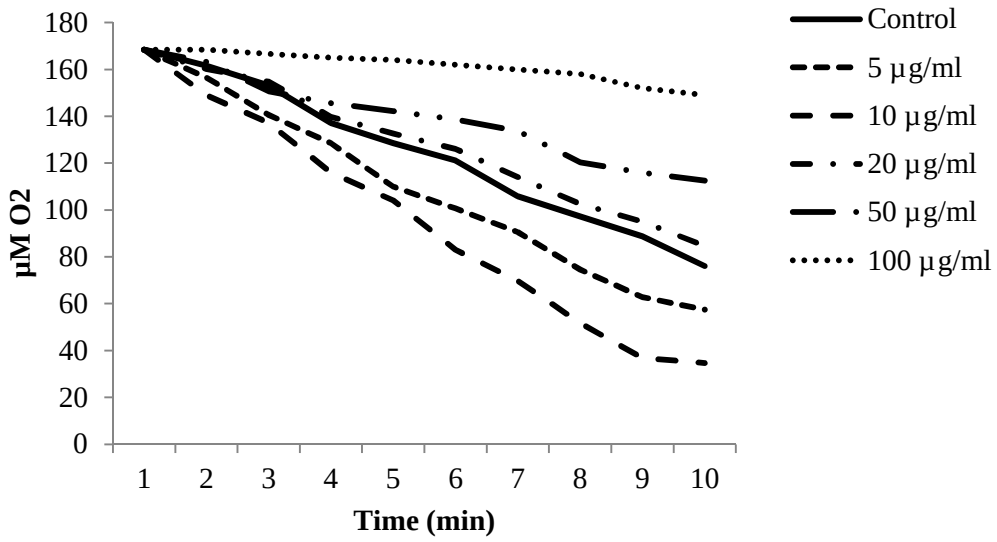

Figure 2. O_2 consumption (µM O_2 in medium) of control and ZnO treated *Saccharomyces cerevisiae*

Total protein and reduced glutathione concentrations were presented in table 1. Obtained results showed that protein content increased in the treated cells compared to the measured values in control cells. This increase was dose-dependent manner and could result in activation of detoxification system against oxidative stress. This activation could be verified by assessing the concentration of bioindicator of oxidative stress as reduced glutathione and activities of GST and peroxidases. In yeast treated ZnO, reduced glutathione concentrations decreased significantly (-50% with 10 µg ZnO/ml and -80% with 100 µg ZnO/ml) compared with control values (Tab1). This decrease reflected activation of "Glutathione" system. According to [20] who reported that the concentration of GSH significantly decreased in the treated ZnO Chlorella *Vulgaris alga*.

Table 1: Protein (µg/ml) and GSH (µM/ml) concentrations in control and ZnO treated yeast *Saccharomyces cerevisiae*

		ZnO concentrations (µg/ml)				
	Control	5	10	20	50	100
Protein (µg/ml)	0.45 ± 0.25 **a**	0,98 ± 0.21 **a**	1.55 ± 0.25 **b**	2.9 ± 0.49 **c**	4.21 ± 0.07 **d**	4.35 ± 0.09 **d**
GSH (µM/ml)	6.3 ± 0.25 **a**	5.82 ± 0.36 **a**	3.25 ± 0.2 **b**	2.11 ± 0.4 **c**	1.4 ± 0.25 **d**	0.94 ± 0.28 **d**

(Values followed by the same letter are not different at p<0.05).

It could be also confirmed by measuring GST activity (Fig.3) where the enzymatic activity increased (+450% with 100µg ZnO/ml) compared to GST activities of control yeasts.

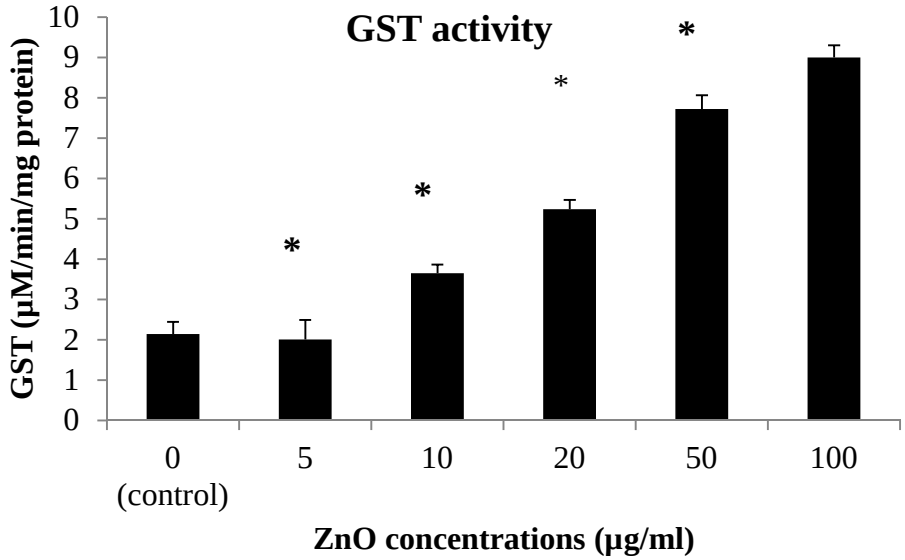

Figure 3: GST activity (µM/min/mg protein) in control and ZnO treated yeast *Saccharomyces cerevisiae*. (*: different from control p<0.05)

Peroxidase enzymes (APx and GPx) were also considered as system against oxidative stress [20, 23]. Evaluation of GPx (Fig.4) and APx (Fig.5) activities showed a dose-dependent increase (+330% and +350% respectively with the highest dose of ZnO) in treated yeasts compared to control. The antioxidant enzymes were also investigated in aquatic plant *Salvinia natans*, [20] where the activities of catalase, peroxidases and superoxide dismutase increased following treatment with ZnO.

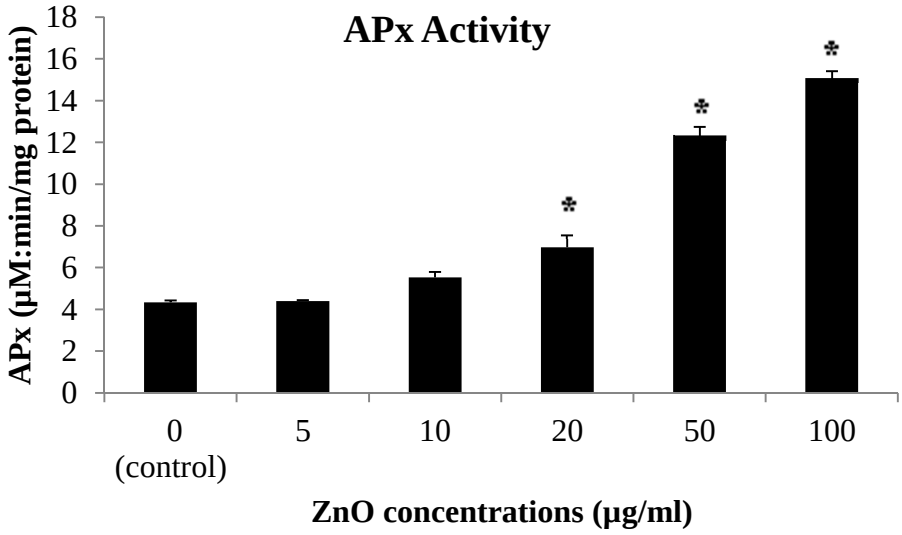

Figure 4: APx activity (µM/min/mg protein) in control and ZnO treated yeast *Saccharomyces cerevisiae*. (*: different from control p<0.05)

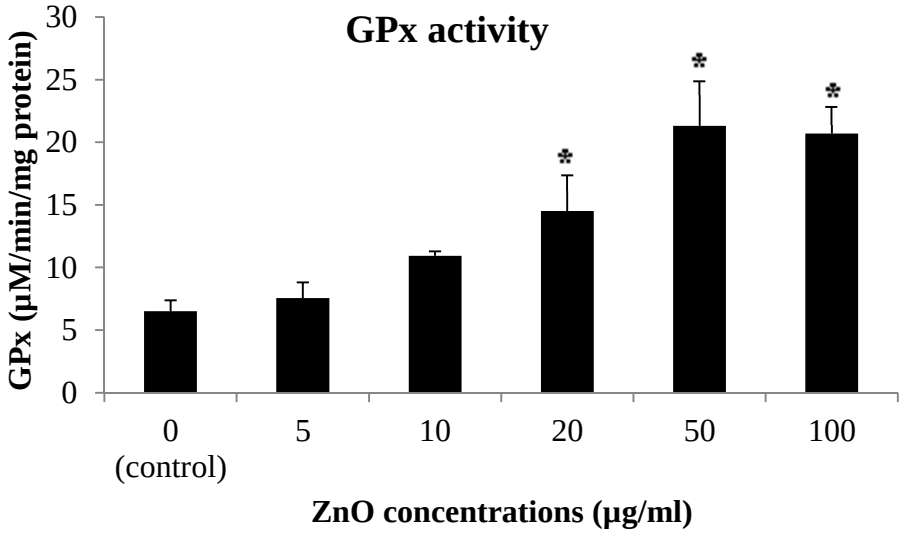

Figure 5: GPx activity (µM/min/mg protein) in control and ZnO treated yeast *Saccharomyces cerevisiae*. (*: different from control p<0.05)

CONCLUSION

Obtained results in this study showed that yeast treatment by ZnO NPs resulted in a strong inhibition of cell growth as well as a decreased reduced glutathione levels (GSH), increased enzyme activities GST, GPx and APx indicating generation of oxidative stress by the ZnO NPs. It therefore appears that the yeast *S. cerevisiae* can be a suitable model for the study of oxidative stress.

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