Zinc oxide nanoparticles impose metabolic toxicity by de-regulating proteome and metabolome in *Saccharomyces cerevisiae*

Piyoosh Kumar Babele

Department of Biological Sciences, Indian Institute of Science Education and Research Bhopal, Bhojpur, Bhopal, 462066, Madhya Pradesh, India

**Abstract**

As zinc oxide nanoparticles are being increasingly used in various applications, it is important to assess their potential toxic implications. Stress responses and adaptations are primarily controlled by modulation in cellular proteins (enzyme) and concentration of metabolites. To date proteomics or metabolomics applications in nanotoxicity assessment have been applied to a restricted extent. Here we utilized 2DE and $^1$H NMR based proteomics and metabolomics respectively to delineate the toxicity mechanism of zinc oxide nanoparticles (ZnO-NPs) in budding yeast *S. cerevisiae*. We found that the physiological and metabolic processes were altered in the *S. cerevisiae* upon ZnO-NPs exposure. Almost 40% proteins were down-regulated in ZnO-NPs (10 mg L$^{-1}$) exposed cell as compared to control. Metabolomics and systems biology based pathway analysis, revealed that ZnO-NPs repressed a wide range of key metabolites involved in central carbon metabolism, cofactors synthesis, amino acid and fatty acid biosynthesis, purines and pyrimidines, nucleoside and nucleotide biosynthetic pathways. These metabolic changes may be associated with the energy metabolism, antioxidation, DNA and protein damage and membrane stability. We concluded that untargeted proteomic and metabolic approaches provide more complete measurements and suggest probable molecular mechanisms of nanomaterials toxicity.

1. Introduction

Among engineered nanomaterials, zinc oxide nanoparticles (ZnO-NPs) the most common industrially produced nanomaterials. They gained much interest due to their applications in diverse fields ranging from sunscreens, to ceramics, superconductors and optoelectronic devices [1]. Owing to their extensive use and increased environmental and occupational exposure ZnO-NPs have become an important compound for public health issues therefore, an appropriate risk assessment for this compound is necessary. For these reasons, in the past few years several researches have been conducted [2–5]. The accumulating research evidences documented that exposure to ZnO-NPs can be toxic to biological system ranging from prokaryotes to higher eukaryotes including humans [2–5]. Among the eukaryotic microorganisms, the budding yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) represents a well-accepted and extensively used eukaryotic model system for basic and applied studies because its fundamental cellular processes and pathways are similar to those of higher eukaryotic organisms including humans [6]. Number of characteristics features and availability of yeast metabolome database (YMDB) makes it an excellent organism to carry out functional toxicological and metabolomic studies [7]. Several advantages are recognised by exploiting yeast cells, such as to dissect unknown mechanisms of action, to identify potential cellular targets and to characterize novel molecular biomarkers [8,9]. Although, few nanotoxicity studies have been done on this model organism [4,10], but the proteomic, metabolomic and systems biology based studies are scarce. In our previous research, we draw a conclusive mechanism(s) underlying the nanotoxicity of ZnO-NPs [11]. Although several toxicity pathways are reported but the studies explicitly dealing with the proteomic and metabolomic variations upon ZnO-NPs exposure to *S. cerevisiae* have gained relatively little attention.

In order to gain more information on the mechanism of nanotoxicity there is an urgent need to apply metabolomics in the field of nanotoxicology and in its integration with molecular profiling at protein level therefore, more studies are necessary in these directions [12,13]. We utilized proteomic and metabolomics to present a multi-omics approach for better understanding the nanotoxicity. Briefly, we investigated the variation in the ZnO-NPs exposed budding yeast *S. cerevisiae* at the proteome and the metabolome level. Qualitative data of de-regulated proteins obtained using two-dimensional gel electrophoresis (2DE) and quantitative data of de-regulated metabolites gained by utilizing $^1$H-NMR were interpreted using systems biology analysis. The aim of this study is to understand the biological processes that are modulated upon interaction of ZnO-NPs with living systems. This research contributing towards a detailed understanding of the biochemical mechanism(s) triggered in the yeast upon ZnO-NPs exposure. The
knowledge gained based on the information of networks, processes, and pathways modulated can be used to advance the design of nanoparticles and/or to discover definite biomarkers of nanotoxicity.

2. Material and Methods

2.1. Yeast Growth and ZnO-NPs Treatment

*S. cerevisiae*, BY4741 (MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0) cells were pre-cultured in liquid YPD medium. The overnight grown
culture inoculated in 300 ml (in duplicate) YPD medium (OD600nm 0.2), the cells were grown until mid-exponential growth phase (OD600nm 1.3–1.5). Cells were harvested by centrifugation and thoroughly washed thrice with sterile deionized water (DI) water. The cell pellets were resuspended and diluted using 300 ml sterile DI water. The cell pellets were used for ZnO-NPs treatment in duplicates. Cells were incubated for three hours (200 rpm, 30 °C) for toxicity evaluation in an incubator shaker. After exposure, the 100 ml control and treated cultures and rest 50 ml cultures were used for the metabolite and protein extraction respectively.

2.2. Protein extraction and Western blotting

Fifty ml culture was used for protein performed by a previously described method with desired modifications [11,14]. Briefly, control and ZnO-NPs treated cells were harvested and instantaneously frozen at −80 °C. Frozen cells were thereby washed three times with sterile DI water subsequently cell pellet was resuspended in 400 μl extraction buffer [50 mM Tris–HCl (pH 7.5); NaCl 200 mM; Triton X-100 0.1%; Glycerol 5%; EDTA 1 mM; DTT 5 mM; PMSF 0.5 mM]. Protease inhibitor cocktail (Sigma-Aldrich) and glass beads (acid washed; 0.5 mm; Sigma-Aldrich) were added to the cell suspension. Cells were disrupted by vigorous vortexing eight times for 40 s (samples were cooled on ice for 40 s in between the vortex steps). Remove all the glass beads from the cell extract and transferred cell extract to a fresh microcentrifuge tube and centrifuged at 12,000 for 10 min at 4 °C. The supernatant was recovered completely by transferring to a fresh microcentrifuge tube (fraction 1). The insoluble fractions were again suspended in 300 μl extraction buffer mix cell suspension by thorough vortexing and pipetting up and down with a 200 μl pipette tip. The sample was boiled for 5 min and immediately cooled on ice for 5 min. After centrifugation for 10 min (12,000 RPM, 4 °C), the supernatant (fraction 2) was then transferred to a fresh microcentrifuge tube and mixed with fraction 1. Consequently, add 100 μl of DNase and RNase solution [1% (w/v) DNase I, 0.25% (w/v) RNase A, 50 mM MgCl2, 0.5 M Tris–HCl, pH 7.0] and the combined fractions were incubated on ice for 20 min. The mixed protein extract was then purified by using a 2-D Clean-Up Kit (GE Healthcare, Uppsala, Sweden), and the purified protein sample was dissolved in rehydration solution [8 M urea; 4% (v/v) CHAPS; 5.4 mg/ml dithiothreitol; 0.002% (v/v) bromophenol blue supplemented with 2% (v/v) 3–10 IPG buffer (GE Healthcare). The total protein concentration was determined by Bradford method. Dissolved protein samples were stored at −80 °C before proteomic assays. The equal concentrations of protein extracts were separated by electrophoresis on SDS-polyacrylamide gel. Immunoblotting analysis of protein extracts was performed as described previously [11]. Blots were incubated (1 h) with primary antibodies anti-Tbp (polyclonal antibodies against recombinant yeast TBp) and anti-Rap1 (polyclonal antibodies against recombinant Rap1p). After that exposed to the IRDyeH 800CW anti-rabbit IgG secondary antibody for 1 h. Blots were scanned with an Odyssey Infrared Imager (LI–COR Biosciences).

2.3. 2-D gel electrophoresis (2DE)

2DE experiment was performed by a standard protocol described elsewhere [14]. For the first dimension (isoelectric focussing; IEF) 90 μg (make up the total volume with rehydration buffer to 125 μl) protein samples were loaded on a 7 cm Immobiline Dry-Strip pH 3–10 (GE Healthcare) avoiding air bubbles between protein samples and strips. The IPG strips were rehydrated overnight at room temperature. IEF was performed with Ettan IPGphore 3 system (GE Healthcare) at 20 °C with a 4 step gradient program: 100 V for 60 min (0.25 kV h), 500 V for
Table 2.

| Peak No. | Metabolites | Chemical Shifts (ppm) | Regulation | YMDB ID | KEGG ID | Cellular Functions |
|----------|-------------|----------------------|------------|---------|---------|------------------|
| 1        | NAD⁺        | 9.33 (s), 9.15 (d), 8.42 (d), 8.19 (m) | Down       | YMR00110 | C00020  | Maintain cellular energy homeostasis, as well as in signal transduction as cyclic adenosine monophosphate (CAP) binding molecular regulator. |}
| 2        | AMP         | 8.85 (s), 8.17 (d) | Down       | YMR00328 | C00020  | Works in the energy metabolism, serving as a precursor to ADP and ATP. It also acts as a second messenger in signal transduction pathways. |}
| 3        | UDP         | 7.86 (d), 7.05 (s), 3.96 (d), 3.22 (d), 3.12 (d), 3.01 (d) | No         | YMR00379 | C00035  | Important in nucleotide and lipid metabolism, serving as a precursor to UDP-glucose and UDP-N-acetylglucosamine. |}
| 4        | Histidine   | 7.43 (d), 7.36 (d), 7.32 (d), 7.29 (d), 7.27 (d) | Down       | YMR00379 | C00015  | Participates in the synthesis of purines and pyrimidines, and is also essential as a neurotransmitter. |}
| 5        | Phenylalanine| 7.18 (d), 7.05 (s), 7.04 (d), 7.03 (d), 7.02 (d) | Down       | YMR00379 | C00015  | Important in amino acid metabolism, serving as a precursor to tyrosine and tryptophan. |}
| 6        | Tyrosine    | 6.13 (d), 6.08 (d), 6.03 (d), 5.99 (d), 5.96 (d) | Down       | YMR00379 | C00015  | Important in amino acid metabolism, serving as a precursor to dopamine and norepinephrine. |}
| 7        | Trehalose   | 5.18 (d), 3.85 (m), 3.75 (dd), 3.64 (d), 3.44 (t) | Up         | YMR00379 | C00015  | Important in osmotic stress response and survival under adverse conditions. |}
| 8        | Lactate     | 4.11 (dd), 1.32 (d) | Up         | YMR00379 | C00015  | Produced during anaerobic glycolysis and serves as an energy source for many bacteria. |}
| 9        | Serine      | 3.94 (m), 3.83 (dd) | Down       | YMR00379 | C00015  | Important in amino acid metabolism, serving as a precursor to several amino acids including alanine, glycine, and proline. |}
| 10       | Glycerophospho-choline | 4.31 (m), 3.6 (d), 3.22 (d) | Down       | YMR00379 | C00015  | Important in lipid metabolism, serving as a precursor to cardiolipin and phosphatidylcholine. |}
| 11       | Pyruvate    | 2.36 (s) | Down     | YMR00379 | C00015  | Participates in the citric acid cycle and is a major energy source for aerobic respiration. |}
| 12       | Thiamine   | 5.46 (s) | Down     | YMR00379 | C00015  | Essential for energy metabolism, serving as a coenzyme in the metabolism of pyruvate, lactate, and other molecules. |}
| 13       | Ethanol     | 3.65 (q), 1.71 (d) | Down       | YMR00379 | C00015  | Produced during fermentation and is a product of the aerobic metabolism of pyruvate. |}
| 14       | Aspartate  | 3.88 (dd), 2.80 (dd) | Up         | YMR00379 | C00015  | Important in amino acid metabolism, serving as a precursor to several amino acids including methionine, threonine, isoleucine, and lysine. |}
| 15       | Leucine    | 3.71 (m), 1.69 (m), 0.95 (t) | Up         | YMR00379 | C00015  | Important in amino acid metabolism, serving as a precursor to several amino acids including valine, isoleucine, and threonine. |}

(continued on next page)
### Metabolite Extraction and 1H NMR sample preparation

Extraction of metabolites was done as described previously [15]. Briefly, the cells were collected by centrifugation, washed with 100 ml of ice-cold water and instantaneously frozen at −80 °C. Frozen cells were carefully washed thrice with 10 ml ice-cold water. Then cell pellets were resuspended in 4 ml 75% EtOH and an equal volume of glass beads; metabolites were extracted by vigorous vortexing 10 times for 35 s with 40 s breaks in ice. Subsequently, samples were centrifuged for 5 min at 2000 x g to remove cell debris and glass beads from the extract and equal volumes of the supernatant containing metabolites were evaporated under vacuum. Aqueous samples were dissolved in 600 μl of deuterated phosphate buffer (N₄P₂O₇ 100 mM, pD 7.5) in D₂O with 0.1 mM 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) as an internal standard for chemical shift and concentration.

### 2.6. 1H NMR experiments and spectra pre-processing

Spectra were recorded in an AVANCE III 700 MHz FT NMR spectrometer (Ascend™, Bruker), in a DQD mode at a frequency of 4323.96 Hz 5-mm QCI cryoprobe. Bruker pulse sequences were acquired with 128 free induction decays (FIDs) and 64 k data points over a spectral width of 20 ppm. 512 scans were used with a relaxation delay of 5 s. The FID values were multiplied by an exponential function with a 0.3 Hz line broadening factor. Spectra were automatically processed for the phase and base correction and after that referenced using the Topspin (AU program apk0.NOE) library. 1H NMR spectra were pre-processed with Topspin 3.5 pl 7 software (Bruker) and normalised to the sum of total spectrum intensity to minimise the effect of the differences in sample concentration. The regions corresponding to water and DSS were excluded during the normalisation and processing. For metabolites quantification, we exploited the algorithm called GSD (global spectrum deconvolution). Overlapping regions were deconvoluted and absolute quantification also performed for metabolites with resonances in crowded spectral areas [16]. For each compound, the mean value of the different assigned signals was determined.
2.7. Metabolite identification and quantification

Metabolite assignment was performed by a detailed, targeted metabolite profiling analysis of the 1H NMR signals using the YMDB [7]. Each spectral intensity data set was normalized to the assigned chemical compounds according to total sum of the spectral regions, converted to tab-delimited text (.txt) and comma-separated values (.csv) imported into Web-based MetaboAnalystv3.5 software to carry out normalization, scaling and multivariate analysis. Significance was determined with a p-value threshold (< 0.05). Metabolites levels were normalized using the log2 function and then, mean centering and pare to scaling was applied for all PCA and heatmap by MetaboAnalystv3.5 [17].

2.8. Isolation of total RNA and real-time quantitative PCR (qPCR)

RNA extraction, cDNA preparation and the qPCR experiments were performed by following the previously described methods with desired modifications [18]. Briefly, the exponentially growing cells were harvested (OD600 = 1.5) from each control and ZnO-NPs treated samples. Total RNA was extracted by a heat/freeze phenol method and treated with a TURBO DNA-free™ Kit (Invitrogen™), thereafter quantified in a NanoDrop 1000 Spectrophotometer (Thermo Scientific) for 260/280 ratio (i.e., 1.94) and its integrity was checked on 2% agarose gel. Genomic DNA contamination was checked by PCR on a total RNA template using primers targeting the ACT1 gene. For the synthesis of cDNA equal amount (1 μg) of purified RNA from each control and treated samples were used. cDNA was synthesized by a high capacity RNA-to-cDNA kit (Bio-Rad Laboratories Pvt. Ltd., Gurgaon, India) following the manufacturer’s instructions. Three technical replicates were performed for minimum three biological replicates using equal concentration of synthesized cDNA from every control and ZnO-NPs treated samples. Gene information and the primers used for real time PCR in this study were listed in Table 1. Real-time PCR experiments were performed using 2XSYBR green master mixes (ABI) in an ABI-7300 RT-PCR with Sequence Detection System v1.4 (Applied Biosystems, CA). Thermal cycler conditions included initial denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 20 s, annealing at 58 °C for 20 s, and elongation at 72 °C for 20 s. Melting curve analysis was performed for each primer pair, and relative changes in expression levels between control and treated samples were calculated by using the 2-ΔΔCT method. The relative expression fold change were calculated using the actin (ACT1) housekeeping gene as control, data represent the means ± standard errors of the results of three independent experiments.

3. Results and discussion

3.1. ZnO-NPs cause de-regulation in protein expression

To maintain the equilibrium between essential and non essential levels of certain toxic compounds, eukaryotic cells utilize complicated mechanisms to regulate its cellular uptake, sequestration to sub-cellular organelles and complexes, as well as detoxification [19]. Since metals nanoparticles cannot be degraded or modified like other toxic organic compounds they remains in cells and interfere with cellular
homeostatic pathways and can induce oxidative stress, alteration of enzyme and protein function, lipid peroxidation, and DNA damage [20]. In general metals and metalloids have the capacity to bind to proteins, often via thiol groups of cysteine residues, and inhibit enzyme function by inducing improper protein folding [21]. Most metals are weak mutagens and do not cause direct DNA damage however, they may trigger genotoxicity by interfering with DNA repair molecules and mechanisms [22]. Many metals including Zn have the capacity to influence membrane fluidity, which in turn could contribute to their toxicity [23]. Cellular response of *S. cerevisiae* at different “omics” levels to various environmental stress conditions has been comprehensively studied but this organism is poorly utilized to study the nanomaterials toxicity [8,9]. Earlier, it was established that despite, the rigid cell wall of yeast, ZnO-NPs induce mechanical damage by breaking the cell walls and get internalized inside the yeast cells with the help of endocytosis [4]. This may trigger several cellular and sub-cellular responses such as oxidative stress by generating radical oxygen species (ROS), inflammatory & immunological responses, genotoxicity and ultimately cell death [2–5]. It is very well established that metal nanoparticles impose oxidative stress thus induce defects in mitochondrial and ER enzymes [10,24–26]. The combined proteome and metabolome analysis and its integration with systems biology offers an opportunity to improve our knowledge about the toxic effects and mechanisms in nanoparticles and ER enzymes [10,24–26]. The combined proteome and metabolome analysis, it is essential that the protein concentration must be equal in both the control (untreated) and treated (ZnO-NPs) cells. Therefore, we performed western blotting using anti-TBP and -Rap1 as reference proteins. Western blots showed equal band intensity in both the control (untreated) and ZnO-NPs (10 mg L⁻¹) treated protein samples (Fig. 1A). Being assured, we analyzed the relative proteomic changes in the control and treated cell. The 2DE image gels analysis showed a total of 650 and 425 protein spots in the control and ZnO-NPs treated samples, respectively with an average percentage of matched spots across gels of 94% (Fig. 1B). Among the protein spots detected, 46 protein spots were found to be down-regulated and 20 spots were up-regulated by at least 2-fold in ZnO-NPs treated cells. A total number of 66 protein spots were differentially expressed and significant (p < 0.05) between the control and treated groups. Although we did not further characterize these proteins but qualitative 2DE gels showed a significant difference in the protein profile of control and ZnO-NPs exposed cells.

3.2. ZnO-NPs cause de-regulation in metabolites

Proteome analysis results motivated us to analyse the de-regulation in the metabolites. Untargeted ¹H NMR analysis approach has been utilized to evaluate the metabolic changes. Due to the complex ¹H NMR experimental set-up, we have chosen the most effective (EC₅₀) concentration of ZnO-NPs (i.e., 10 mg L⁻¹). To partly overcome the challenge of complex metabolomics data analysis, integrated metabolomics approaches were used to achieve insights into molecular pathways from identified metabolite annotations. The chemical shifts obtained after statistical analysis were submitted to the public database search for metabolite annotations and identification, we used the standard reporting system, described previously [16]. According to the chemical shifts data and coupling patterns, we identified 46 metabolites (including seven unknown metabolites) from the whole *S. cerevisiae* cell extract (Fig. 2). Chemical shifts of signals were assigned to the metabolites in the form of amino acids, organic acids, fatty acids, carbohydrates, and nucleotide derivatives. Results show that ZnO-NPs had a significant impact on the deregulation pattern (p < 0.05) of metabolites in the yeast cell. To our surprise, most of the annotated metabolites were significantly down-regulated and few of them were up-regulated. Metabolic pathway analysis revealed that ZnO-NPs repressed a wide range of key metabolites involved in central metabolism, including cofactors synthesis, amino acid & organic acid biosynthesis, purines &
pyrimidines and nucleoside & nucleotide biosynthetic pathways. These metabolites are classified by their chemical nature and functions associated with the metabolic pathways. Relative concentrations of the annotated metabolites and the parameters used for its calculation are presented in the supplementary Table S1. List of metabolites with their respective chemical shifts, de-regulation and YMDB, KEGG ID is presented in Table 2 and the main variations are summarized in the form of a bar graph (Fig. 3A, B). To visualize the differences between control and ZnO-NPs treated S. cerevisiae, the 46 identified metabolites were normalized and analyzed by Partial Least Squares Discriminant Analysis (PLS-DA) using metaboanalyst which is a supervised clustering method to maximize the separation between groups. The score plot shows that cells exposed to ZnO-NPs are clearly separated along the first principal axis (PC1), which explained 25.3% of the total variability (Fig. 3C) indicating that ZnO-NPs considerably altered the metabolic profiles of the yeast cell. The heat map obtained from the selected metabolites indicates that some metabolites are down-regulated while others are up-regulated upon ZnO-NPs exposure (Fig. 3D). To further validate these results we have checked the transcript level changes in few selected genes associated with the TCA cycle (ACO1 and KDG1) (Fig. 4A), oxidative pathway (SOD1, SOD2 and YAP1) (Fig. 4B), and lipid biosynthetic pathways (INO2, INO4, CHO1, PSD1 and CRD1) (Fig. 4C). We found that all the selected genes were down-regulated upon ZnO-NPs exposure. These observations are in favour of metabolic data and thus indicate that the ZnO-NPs impose toxicity by perturbing the mitochondria, ER and lipid homeostasis.

Mitochondria are essential bioenergetic organelles participate in several vital processes and fulfil the energy requirements of the cell. Metabolite profiling presented in this study showed improper mitochondrial functioning as evidenced by diminished biosynthesis of various metabolites of key mitochondrial processes i.e., glycolysis, the tricarboxylic acid (TCA) cycle, and the pentose phosphate pathway, indicating that ZnO-NPs affect the central carbon metabolism (CCM). The CCM network fulfils energy demand of the cell and is important for providing synthetic precursors for other primary and secondary metabolites. Thus, the regulation of CCM is important for the regulation and coordination of a wide range of metabolic reactions and pathways. Under stress conditions, the CCM and other metabolic pathways can be altered and these metabolic changes have been studied by using different approaches [31,32]. In aerobic metabolism, ROS are formed as a natural by-product, but in excess they can chemically modify various macromolecules and cause damage to vital organelles (mitochondria, ER, lysosomes etc.) [33,34]. Earlier it was suggested that zinc ions produced by dissolved ZnO-NPs affect the TCA cycle by repressing the enzymes and resulting in decreased levels of citrate, succinate and α-ketoglutarate [35]. Another study on rat lung confirmed that ZnO-NPs exposure...
down-regulate the key enzymes of glycolysis the tricarboxylic acid (TCA) cycle and the electron transport chain, resulting in diminished ATP generation and ultimately cell death [27]. Our results also showed decreased levels in the citrate and succinate this might affect the aerobic metabolism and the TCA cycle thus inhibits ATP production. Succinic acid is an important metabolite in the TCA cycle for energy generation, and it also participates in the GABA pathway involved in ROS stress alleviation [36]. Decreased levels of glucose and acetate while increased levels of lactate and alanine indicates that glucose can be changed into lactate or alanine in order to supply enough energy for survival. Result might entail the disturbance of aerobic metabolism and TCA cycle. Moreover, acetate is also viewed as an alternative energy source [37]. These alterations in metabolites provide evidence that there was induction of central elements of the oxidative stress response and the basic detoxification process against short-term ZnO-NPs exposure.

We did not observe any significant change in tyrosine, lysine and methionine while branched-chain amino acids (isoleucine, leucine and valine) and alanine were up-regulated. These essential amino acids are involved in the energetic pathways of eukaryotic organisms and their increased levels may be attributed to protein breakdown. Previous results also showed that branched-chain amino acids were increased in mouse model upon acrolein-induced lung injury this might be due do the result of the utilization of these amino acids as energy sources [38]. Another stress indicator found was the accumulation of trehalose. The biosynthesis of trehalose is considered as a cellular defence mechanism against ROS accumulation and other stressful conditions [12], the protective role of this carbohydrate against ROS has been confirmed in cell devoid of SOD [39]. Glycerol is a protective agent synthesized by yeast cells against environmental stress, which can function in maintaining the redox homeostasis [40]. The increased glycerol biosynthesis rate thus indicates the alteration in stress defence of the cells against ZnO-NPs toxicity. One of the most important metabolites found up-regulated is glycerophosphocholine, an important member of eukaryotic cellular membrane components. It is involved in the membrane choline phospholipid metabolism pathway, where phosphati-dylcholine can be converted to glycerophosphocholine, which can be reconverted to phosphatidylcholine. Cells consume glycerophosphocholine to generate phosphatidylcholine for maintaining cell membrane integrity [41,42]. Based on this we can propose a probable mechanism of membrane component maintenance upon ZnO-NPs exposure. Our finding is in parallel to the result of a research conducted in the rat lung upon acute inhalation of ZnO particles [27].

Protein synthesis is crucial for cellular stress response and adaptation. The basic amino acids are the building blocks of protein. During metabolic profiling several amino acids were de-regulated. Among them, threonine, histidine, phenylalanine, glutamate and glycine were down-regulated. The decrease in glycine and oxidised glutathione is reported that might have been a protective response to oxidative stress. Glycine is one of the precursor amino acids that can synthesise glutathione, which is an important antioxidant that provides redox defence against inflammation and oxidative stress induced by metal stress [43,44]. Nucleotides are crucial players in a huge number of diverse cellular processes. Nucleic acids are made up of purines and pyrimidines, and ATP is the central cellular energy supplier. The GMP/GTP and modified nucleotides for eg. cyclic AMP are signalling molecules. Finally, nucleotides are incorporated in cofactors (e.g., NAD and coenzyme A) and serve as precursors (e.g., UDP-glucose and GDP-mannose). In this study, all the above metabolites were down-regulated after ZnO-NPs exposure. Purine and pyrimidine synthesis occurs through distinct metabolic pathways, and these are highly conserved among both prokaryotic and eukaryotic species. The purine and pyrimidine pathways ensure net synthesis of nucleotides but also allow interconversion of nucleobases, nucleosides, and nucleotides, thus permitting a proper balance of the final products to be achieved. Formate is a possible alternative single-carbon source for the production of N5, N5-methylene-tetrahydrofolate, which is necessary for the bio-synthesis of nucleic acid. It might be a possibility that the under stressed condition cells utilize more amount of formate to produce purine nucleotides thus reflect low formate level and might indicate that ZnO-NPs cause DNA damage.

4. Conclusion

In summary, our results indicate that ZnO-NPs induced physiological and metabolic toxicity can be characterized by de-regulation of proteins and metabolites. An overview of the main interconnections for the de-regulated metabolites in S. cerevisiae is shown in Fig. 5. Taken together, it is clear that under stress conditions the enzymes of carbon, amino acids, lipid and nucleotide metabolism play important roles in maintaining optimal metabolite concentrations and eventually global metabolic homeostasis. On the basis of proteome and metabolome analysis we can hypothesise that ZnO-NPs affect physiology and metabolism of S. cerevisiae. In general the toxicity mechanisms showed decline in the growth-related processes such as carbohydrate metabolism and protein biosynthesis including the repression of genes of antioxidative and lipid metabolic pathways.

Conflicts of interest

The authors declare that they have no conflict of interest. Funding

This work was supported by a project grant by SERB- DST New Delhi India (File No. PDF/000200).

Transparency document

The Transparency document associated with this article can be found in the online version. Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jjogre.2018.11.004.

References

[1] A.B. Djurišić, X. Chen, Y.H. Leung, A.M.C. Ng, ZnO nanostructures: growth, properties and applications, J. Mater. Chem. 22 (2012) 6526–6535.
[2] A. Sirelkhatim, S. Mahmud, A. Seeni, N.H.M. Kau, L.C. Ann, S.K.M. Bakhori, H. Hasan, D. Mohamad, Review on zinc oxide nanoparticles: antibacterial activity and toxicity mechanism, Nano-Micro Lett. 7 (2015) 219–242.
[3] Y.H. Leung, X. Xu, A.P. Ma, F. Liu, A.M. Ng, Z. Shen, L.A. Gethings, M.Y. Guo, A.B. Djurišić, P.K. Lee, Toxicity of ZnO and TiO2 to Escherichia coli cells, Sci. Rep. 6 (2016) 35243.
[4] W. Zhang, S. Bao, T. Fang, The neglected nano-specific toxicity of ZnO nanoparticles in the yeast Saccharomyces cerevisiae, Sci. Rep. 6 (2016).
[5] M. Condello, B. De Berardis, M.G. Ammendola, F. Barone, G. Condello, P. Degani, S. Meschini, ZnO nanoparticle tracking from uptake to genotoxic damage in human colon carcinoma cells, Toxicol. Vitr. 35 (2016) 169–179.
[6] A.A. Duina, M.E. Miller, J.B. Keeney, Budding yeast for budding geneticists: a primer on the Saccharomyces cerevisiae model system, Genetics 197 (2014) 33–48.
[7] T. Jewison, C. Knox, V. Neveu, V. Djoumbou, A.C. Guo, J. Lee, P. Liu, R. Mandal, R. Krishnamurthy, I. Sinelnikov, VMD8: the yeast metabolome database, Nucleic Acids Res. 40 (2011) D815–D820.
[8] S.C. dos Santos, M.C. Teixeira, T.R. Cabrito, I. Sá-Correia, Yeast toxicogenomics: genome-wide responses to chemical stresses with impact in environmental health, pharmacology, and biotechnology, Front. Genet. 3 (2012) 63.
[9] D. Braconi, G. Bernardini, A. Santucci, Saccharomyces cerevisiae as a model in toxicological studies: a post-genomics perspective, J. Proteomics 137 (2016) 1–11.
[10] K. Kasemets, A. Ivank, H.-C. Dubourguier, A. Kahr, Toxicity of nanoparticles of ZnO, CuO and TiO2 to yeast Saccharomyces cerevisiae, Toxicol. Vitr. 23 (2009) 1116–1122.
[11] P.K. Babele, P.K. Thakre, R. Kumawat, R.S. Tomar, Zinc oxide nanoparticles induce toxicity by affecting cell wall integrity pathway, mitochondrial function and lipid
homeostasis in Saccharomyces cerevisiae, Chemosphere 213 (2018) 65–75.

S. Gómez, L. Lobo, P. Barbora, R. La Spina, G. Tomas, P. Urbán, A. Kinner-Ovaskainen, R. François, H. Chassagne, A combined proteomics and metabolomics approach to assess the effects of gold nanoparticles in vitro, Nanotoxicology 10 (2016) 736-748.

M. Farres, B. Pina, R. Tauler, LC-MS based metabolomics and chemometrics study of the toxic effects of copper on Saccharomyces cerevisiae, Metallomics 8 (2016) 790-798.

H. Jun, T. Kieselbach, L.J. Jonsson, Comparative proteome analysis of Saccharomyces cerevisiae: a global overview of in vivo targets of the yeast activator protein 1, BMC Genomics 13 (2012) 1.

C. Airoldi, F. Tripodi, C. Guzzi, R. Nicastro, P. Coccetti, NMR analysis of budding yeast metabolism: a rapid method for sample preparation, Mol. Biol. Cell. 11 (2005) 379-383.

F. Puig-Castellví, I. Alfonso, B. Piha, R. Tauler, 1H NMR metabolomic study of spontaneous starvation in yeast using multivariate curve resolution-alternating least squares for pathway analysis, Sci. Rep. 6 (2016) 30982.

J. Xia, I.V. Sinelnikov, B. Han, D.S. Wishart, MetaboAnalyst 3.0—making metabolomics more meaningful, Nucleic Acids Res. 43 (2015) W251–W257.

S.K. Sariki, P.K. Saho, U. Golla, V. Singh, G.K. Azad, R.S. Tomar, Sens, the homolog of human Senataxin, is critical for cell survival through regulation of redox homeostasis, mitochondrial function, and the TOR pathway in Saccharomyces cerevisiae, FEBS J. 283 (2016) 4056–4083.

R. Wysocki, M.J. Tamia, How Saccharomyces cerevisiae copes with toxic metals and metalloids, FEMS Microbiol. Rev. 34 (2010) 925–951.

D. Hosiner, S. Gerber, H. Lichtenberg-Frate, W. Glaser, C. Schüller, E. Klipp, Impact of acute metal stress in Saccharomyces cerevisiae, PLoS One 9 (2014) e81330.

S.K. Sharma, P. Goloubinoff, P. Christen, Heavy metal ions are potent inhibitors of protein folding, Biochem. Biophys. Res. Commun. 372 (2008) 341–345.

B. Beyersmann, A. Hartwig, Carcinogenic metal compounds: recent insight into molecular and cellular mechanisms, Arch. Toxicol. 82 (2008) 493.

J.J. García, E. Martinez-Ballarin, S. Millán-Plano, J. Allue, C. Albendea, L. Fuentes, J. Escanero, Effects of trace elements on membrane fluidity, J. Trace Elem. Med. Biol. 19 (2005) 19–22.

V. Sharma, D. Anderson, A. Dhawan, Zinc oxide nanoparticles induce oxidative DNA damage and ROS-triggered mitochondria mediated apoptosis in human liver cells (HepG2), Apoptosis 17 (2012) 852–870.

E. Huerta-García, J.A. Pérez-Arizti, S.G. Márquez-Ramírez, N.L. Delgado-Buenrostro, Y.I. Chirino, G.G. Iglesias, R. López-Marure, Titanium dioxide nanoparticles induce autophagy preceding apoptosis through mitochondrial damage and ER stress in RAW264.7 cells, Toxicol. Vit. 28 (2014) 1402–1412.

S.-H. Lee, T.-Y. Wang, J.-H. Hong, T.-J. Cheng, C.-Y. Lin, NMR-based metabolomics to determine acute inhalation effects of nano- and fine-sized ZnO particles in the rat lung, Nanotoxicology 10 (2016) 924–934.

K.A. Morano, C.M. Grant, W.S. Moye-Rowley, The response to heat shock and oxidative stress in Saccharomyces cerevisiae, Genetics 190 (2012) 1157–1195.

H. Taymaz-Nikerel, A. Cankorur-Getinkaya, B. Kirdar, Genome-wide transcriptional response of Saccharomyces cerevisiae to stress-induced perturbations, Front. Bioeng. Biotechnol. 4 (2016) 17.

Z. Minic, Proteomic studies of the effects of different stress conditions on central carbon metabolism in microorganisms, J. Proteomics Bioinform. 8 (2015) 80.

M.G. Wiebe, E. Rintala, A. Tamminen, H. Simolin, L. Salusjärvi, M. Toivari, J.T. Kokkonen, J. Kiuru, R.A. Ketola, P. Jouhten, Central carbon metabolism of Saccharomyces cerevisiae in anaerobic, oxygen-limited and fully aerobic steady-state conditions and following a shift to anaerobic conditions, FEMS Yeast Res. 8 (2007) 140–154.

W. Guo, Y. Chen, N. Wei, X. Peng, Investigate the metabolic reprogramming of Saccharomyces cerevisiae for enhanced resistance to mixed fermentation inhibitors via 13C metabolic flux analysis, PLoS One 11 (2016) e0161448.

J.P. Fabisiak, M. Mevedovic, D.C. Alexander, J.E. McQuinn, V.J. Concél, K. Bein, A.S. Jung, A. Berndt, L.I. Vuga, K.A. Brant, Integrative metabolome and transcriptome profiling reveals discordant energetic stress between mouse strains with differential sensitivity to acrolein-induced acute lung injury, Mol. Nutr. Food Res. 55 (2011) 1423–1434.

E. de Jesus Pereira, A.D. Panek, E.C.A. Eleutherio, Protection against oxidation during dehydration of yeast, Cell Stress Chaperones 8 (2003) 120–124.

C.M. Paget, J.M. Schwartz, D. Delneri, Environmental systems biology of cold-tolerant phenotype in Saccharomyces species adapted to grow at different temperatures, Mol. Ecol. 23 (2014) 5241–5257.

J.Z. Hu, D.N. Rommevereed, K.R. Minard, A. Woodstock, B.J. Harrer, R.A. Wind, R.P. Rhipps, P.J. Sime, Metabolomics in lung inflammation: a high-resolution 1H NMR study of mice exposed silica dust, Toxicol. Mech. Methods 18 (2008) 383–398.

G. Van Meer, D.R. Voelker, G.W. Feigenson, Membrane lipidic: where they are and how they behave, Nat. Rev. Mol. Cell Biol. 7 (2006) 112.

E. Hatem, V. Berthonaud, M. Dardalhon, G. Lagniel, P. Baudouin-Cornu, M.-P. Jeandel, Acrolein, a lung irritant, induces reactive oxygen species production and oxidative stress in primary human pulmonary artery smooth muscle cells, Biochim. Biophys. Acta (BBA)-Gen. Sub. 1780 (2012) 1217–1235.

S. Gioria, J. Lobo Vicente, P. Barboro, R. La Spina, G. Tomasi, P. Urbán, A. Kinsner-Pawełko, A. Kinsner, Toxicology Reports 6 (2019) 64–73.