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Saccharomyces cerevisiae yeast cells as a test system for assessing Zeocin toxicity

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Abstract

Having unique genetic machinery and a high degree of conservation with higher eukaryotes, the yeast *Saccharomyces cerevisiae* is recognised as a smart experimental system for studying the modes of chemical toxicity. The present study was undertaken to elucidate the changes in the intracellular redox homeostasis and key macromolecule structure following exposure to Zeocin. Cell populations of logarithmic, quiescent (Q) and non-quiescent (NQ) cells of *Saccharomyces cerevisiae* BY4741 were used as a model to examine the cytotoxic effect of this radiomimetic. The levels of endogenous ROS, oxidized lipids, carbonylated proteins, and glutathione were analysed after treatment with Zeocin (IC_{50}). An increase in ROS production and respectively increased oxidative stress was detected in all three types of cell populations, with the highest degree being observed in proliferating *S. cerevisiae* BY4741 cells. The stress response of both proliferating and stationary phase (Q and NQ) cells to Zeocin included an overexpression of glutathione. The quiescent cells also showed very low DNA susceptibility to high Zeocin concentration (100–300 μg/ml), presented as no induced double-strand breaks (DSBs) in the macromolecule. Based on our research it could be concluded that the cellular physiological state is a critical factor determining the resistance to environmental stress with Q cells being the most robust.

Keywords

Quiescence, stress response, yeast, Zeocin

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Introduction

The worldwide use of chemicals, drugs and other pharmaceuticals is significant. However, they represent toxic pollutants and their presence in the environment seriously endangers human health. Pharmaceutical residues can interact with biological targets and thus exert their different toxic effects at very low concentrations. These often-irreversible interactions triggered serious damages in lipids, proteins, and DNA molecules (Farrugia and Balzan 2012). Our ability to predict toxicological outcomes of exposure to drugs as well as understanding the mechanisms underlying toxicity in biological systems are of significant importance for human health and safety. *Saccharomyces cerevisiae* is one of the best-explored models of eukaryotic cells for studying cellular mechanisms that occur under stress conditions. The main advantage of using yeasts in drug testing is the possibility to identify sensitive biomarkers and mechanisms of drug-mediated cell toxicity in the cases when the toxic compounds are still unknown. In addition, *S. cerevisiae* is currently the only system that allows evaluation of all targets in the cell, simultaneously and *in vivo* (Smith et al. 2010). However, most of the yeast-based toxicological studies involve the use of proliferating *Saccharomyces cerevisiae* cells (Gasch et al. 2000; Ericson et al. 2008; Dos Santos et al. 2012; Braconi et al. 2016). Since quiescence is the most common cellular state found on Earth, using *Saccharomyces cerevisiae* in quiescent state will be the more valuable tool for prediction of cellular response to exposure of environmental toxic compounds. Correspondingly, this study compares the toxic response of proliferating and stationary phase (Q and NQ) *S. cerevisiae* BY4741 cells to Zeocin. With its radiomimetic properties, this compound is known to induce single (Miné-Hattab and Rothstein 2013) and double-strand breaks (Chankova et al. 2007; Kopaskova et al. 2012; Todorova et al. 2015a) as well as basic substitutions in the DNA molecule (Guénolé et al. 2013). Moreover, it has been shown that Zeocin has pro-oxidative, mutagenic, and carcinogenic effects in *S. cerevisiae* (Todorova et al. 2015b). Taking this into consideration, we investigated the effect of Zeocin (IC_{50}) on the level of intracellular reactive oxygen species (ROS), total intracellular glutathione, oxidized lipids, and proteins as sensor molecules for measuring induced oxidative stress during drug toxicity. The DNA damaging potential of high concentrations Zeocin on proliferating, Q and NQ cells was also estimated.

Materials and methods

Microorganism and growth conditions

Yeast strain *Saccharomyces cerevisiae* BY4741 (*MATa*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*) was used, obtained from the EUROSCARF Frankfurt collection (Germany). Yeast cells were grown on a liquid YPD medium at 30 °C on a reciprocal shaker (205 rpm) for 168 hours. Samples were withdrawn at exponential (24 hours) and late sta-

tionary phase (168 hours). The biomass was harvested by centrifugation at 5000 rpm for 10 min at 4 °C. The pellet was washed twice with distilled water and used for further analyses.

Isolation of quiescent ($G_{0/}$ Q) and non-quiescent (NQ) cells

Isolation of Q and NQ *S. cerevisiae* BY4741 stationary phase yeast cells (168 h) was performed in Percoll density gradient according to the protocol described by Allen et al. (2006).

Zeocin treatment

IC_{50} dose of Zeocin (Cayman Chemical Company, USA) was previously determined using a spot analysis (Daskalova et al. 2021). The toxic effect of IC_{50} concentration on the level of intracellular damages was studied after exposure to 50 μg/ml Zeocin. The yeast cells were incubated with the stress-inducing agent for 60 min at room temperature, and then washed twice with distilled water and subjected to disintegration. Different concentrations of Zeocin (100, 200 and 300 μg/ml) were used in the experiments for double-strand breaks (DSBs) detection.

Disintegration mode

Washed biomass of unexposed and exposed to Zeocin log, Q and NQ cells was mechanically disrupted according to the previously described procedure (Daskalova et al. 2021).

Biochemical analyses

Protein content was measured by the method of Lowry et al. (1951). The concentration of reactive oxygen species (ROS) was determined by the nitroblue tetrazolium test (NBT) method described by Kostova et al. (2008). The levels of oxidized proteins were assessed following the methodology of Mesquita et al. (2014). Quantitative evaluation of oxidized lipids was done using the method described by Hodges et al. (1999). The concentration of intracellular glutathione was determined using the method of Zhang (2000).

Constant field gel electrophoresis (CFGE) for detection of double-strand breaks

CFGE was performed as described in Todorova et al. (2015b) and Todorova et al. (2019). Logarithmic, Q, and NQ cell suspensions were treated with different concentrations of Zeocin for 1 min on ice. Cells were centrifuged and included into agarose plugs at concentration 1×10^6 cells/ml. The agarose plugs were then placed in 1 ml of lysis solution (pH = 8) containing proteinase K at final concentration 1 mg/ml. After cell lysis, plugs were washed in Tris- EDTA (pH = 7.5) and inserted into a series of wells

in an agarose gel. Electrophoresis conditions were: 40 h at a constant field of 0.6 V/cm (20 V). The levels of induced double-strand breaks (DSBs) represented as a fraction of DNA released (FDR) from the wells were quantified by detecting the ethidium bromide fluorescence using the Gene Tool Analyser G:Box Syngene. To evaluate the repair capacity of cell populations 30 and 60 min recovery time was given after Zeocin treatment.

Data analysis

Used data represent the mean values with Standard error of the mean (±SEM) of three independent experiments. The statistical analysis was performed using MICROSOFT OFFICE 365 EXCEL 2020 software. Differences in means were analysed using Student's t test with independent measures. Differences were considered statistically significant at the $p < 0.05$ level.

Results

ROS levels

A comparative study on the cytotoxic effect of Zeocin in logarithmic, quiescent, and non-quiescent *S. cerevisiae* BY4741 cells has been conducted. After their exposure to 50 μg/ml Zeocin, the induced intracellular changes were determined based on the level of generated reactive oxygen species and their harmful oxidative effect on key cellular macromolecules - proteins and lipids. Results presented in Fig. 1 show the level of accumulated ROS in untreated and Zeocin-treated logarithmic, Q and NQ cells. They clearly indicate that the action of Zeocin leads to an increase in the concentration of toxic radicals in the three types of cells. ROS values measured in treated logarithmic (400 μM/ml) and NQ (125 μM/ml) cells were almost four times higher than those in untreated cells. The lowest increase in the level of ROS (1.5 times) after Zeocin treatment was observed for the quiescence cells.

Levels of oxidized proteins

Next, the presence of carbonyl groups after exposure to the toxic agent has been studied (Fig.2). Q and NQ cells of *S. cerevisiae* BY4741 exhibited differential responses to the action of Zeocin. The highest increase in the level of carbonyl groups has been observed in G_0 cells – about 3-fold. Logarithmically growing Zeocin-treated cells as well as non-quiescent cells showed a slight increase in the amount of carbonyl groups compared to the control ones.

Levels of oxidized lipids

The data obtained after the measurement of malonaldehyde equivalents in the three cell types showed that Zeocin could also impair the prooxidant balance and activate

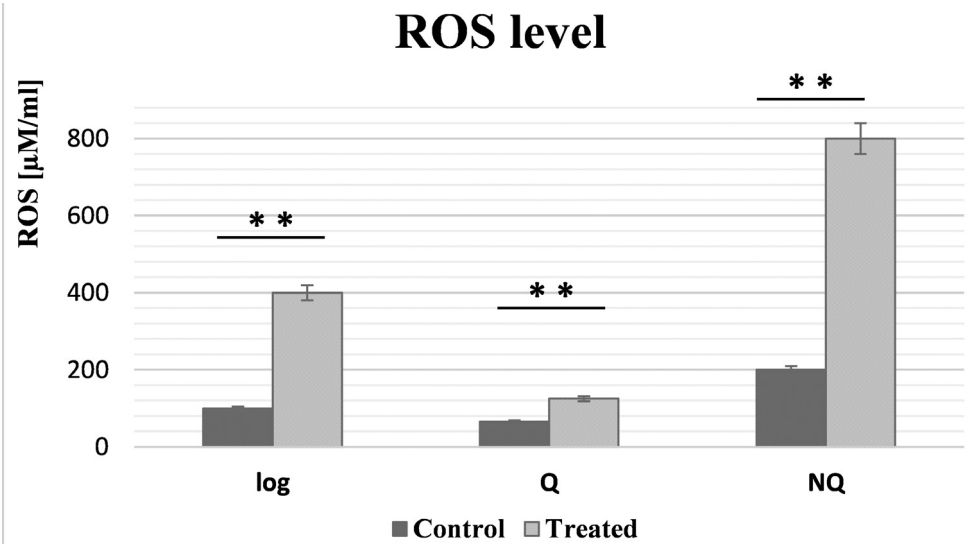

Figure 1. Comparative analysis of ROS levels in untreated (control) and Zeocin-treated proliferating, Q and NQ cells of *S. cerevisiae* BY4741. Each value represents the mean ± SEM (n = 3). Significant differences (* $p < 0.05$; ** $p < 0.001$) are presented.

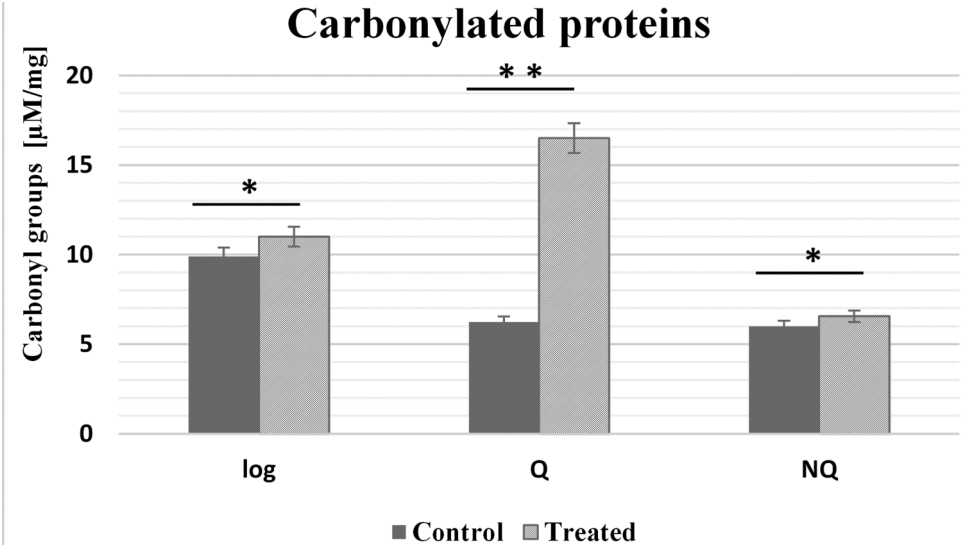

Figure 2. Comparative analysis of carbonylated proteins in untreated (contol) and Zeocin-treated logarithmic, Q and NQ cells of *S. cerevisiae* BY4741. Each value represents the mean ± SEM (n = 3). Significant differences (* $p < 0.05$; ** $p < 0.001$) are presented.

the process of lipid peroxidation (Fig. 3). The higher levels of oxidized lipids were observed in both Zeocin-treated proliferating and quiescent cells (1.88 and 1.4 nmol/mol, respectively). On the contrary, non-quiescent cells showed a very low level of lipid peroxidation whether or not they are treated with Zeocin.

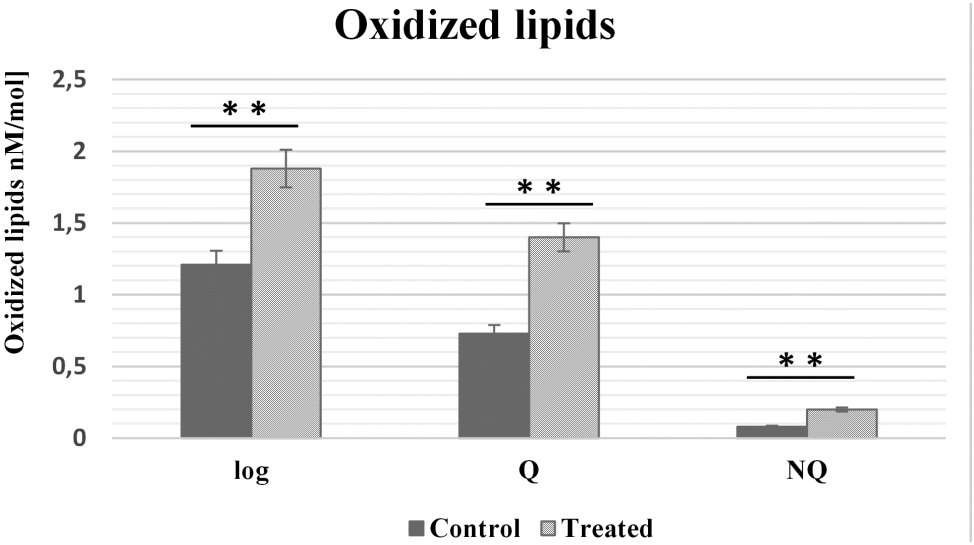

Figure 3. Comparative analysis of oxidized lipids in untreated (control) and Zeocin-treated Log, Q and NQ cells of *S. cerevisiae* BY4741. Each value represents the mean ± SEM (n = 3). Significant differences (* $p < 0.05$; ** $p < 0.001$) are presented.

Intracellular glutathione levels

We also sought to investigate the effect of the radiomimetic Zeocin on the non-enzymatic defence mechanisms, in particular, glutathione. Obtained results revealed that the total levels of this tripeptide in all the treated yeast cell populations were higher compared to the control ones. The most significant increase was observed in proliferating cells - about 2.5 times (Fig. 4).

Spontaneous levels of double-strand breaks

The spontaneous DSB levels were found to depend on the growth phase. Around 1.5-fold higher DSBs levels were measured in non-quiescent cells compared to the logarithmic and quiescent ones (Fig. 5). No statistically significant difference was calculated between the DSB levels in logarithmic and quiescent cells.

Zeocin induced double-strand breaks

Furthermore, the growth phase was estimated as a very important factor for the DNA susceptibility of *S. cerevisiae* to Zeocin (Fig. 6). Approximately similar statistically significant response measured as DSB induction was measured in both logarithmic (Fig. 6 A, D) and non-quiescent cells (Fig. 6 C, D). The DSBs in logarithmic cells did not differ statistically from those in the non-quiescent cells. However, no significant effect of this radiomimetic has been observed in quiescent cells, regardless of the applied concentration (Fig. 6 B, D). The DSB levels were comparable with those in untreated cells.

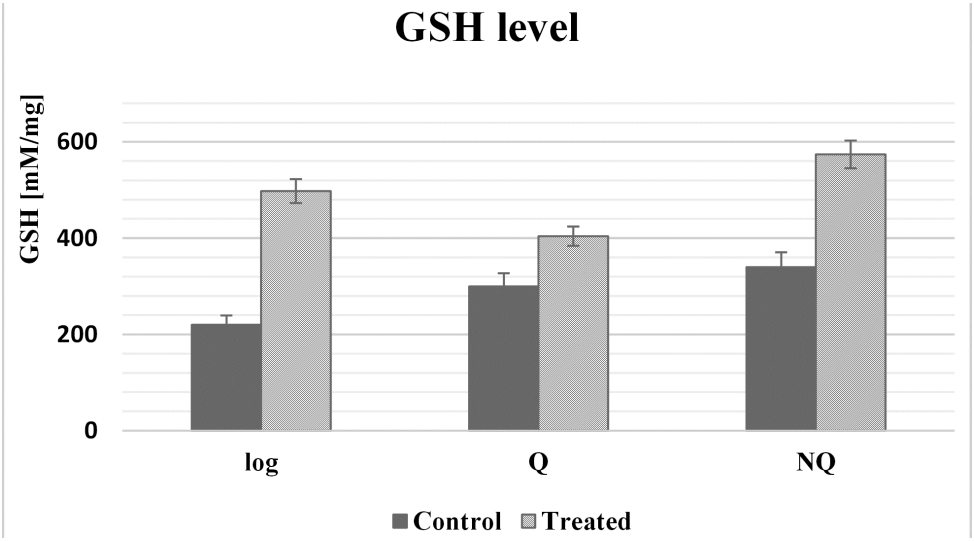

Figure 4. Comparative analysis of the amount of total glutathione in untreated (control) and Zeocin-treated Log, Q and NQ cell of *S. cerevisiae* BY4741. Each value represents the mean ± SEM (n = 3). Significant differences (* $p < 0.05$; ** $p < 0.001$) are presented.

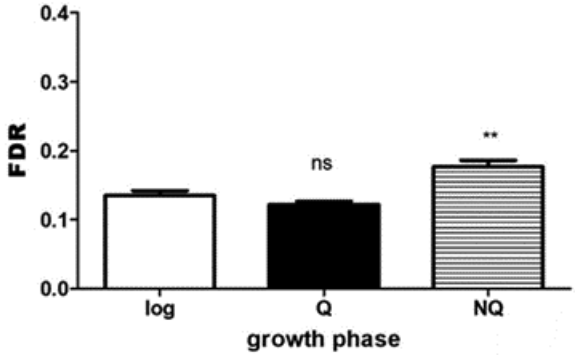

Figure 5. Spontaneous levels of DBSs depending on the growth phase. Error bars represent standard error of the mean from at least three independent experiments. Where no error bars are evident, they are equal of less than the symbols. Statistical significance of differences is indicated with an asterisk (** $p < 0.01$; ns $p > 0.05$).

Repair capacity depending on the growth phase

The best expressed repair capacity was calculated for the logarithmic cells when 60 min recovery time was given. On the other side, NQ cells were found unable to repair Zeocin induced DSBs despite the recovery time (Table 1). It was not possible to calculate repair capacity of Q cells, having in mind that no statistically significant increase of DSBs was measured after the treatment with different Zeocin concentrations.

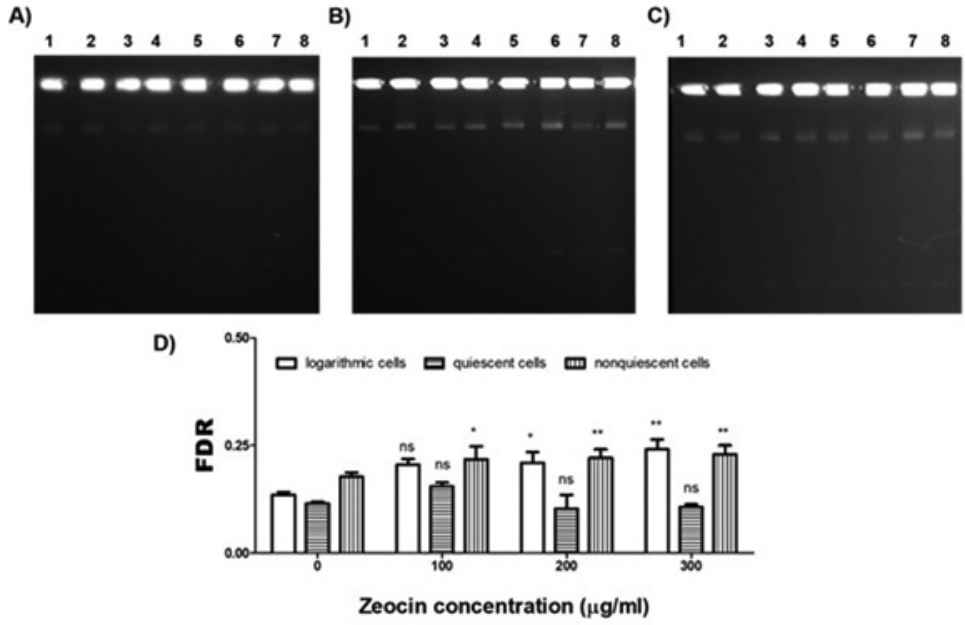

Figure 6. DSBs induced in *S. cerevisiae* BY4741, depending on growth phases after Zeocin treatment in a concentration range 100–300 μg/ml **A** cells in logarithmic phase **B** quiescent cells **C** non-quiescent cells. 1, 2 - control; 3, 4 - treatment with 100 μg/ml Zeocin; 5, 6 - treatment with 200 μg/ml Zeocin; 7, 8 - treatment with 300 μg/ml Zeocin **D** induction of DSBs after treatment with different concentrations of Zeocin calculated as FDR. The significance in the differences where ns $p > 0.05$, $^{**}p < 0.01$. Where no error bars are evident, they are equal or less than the symbols.

Table 1. Repair capacity of logarithmic and non-quiescent cells calculated after the treatment with Zeocin at concentrations 100, 200 and 300 μg/ml with 30- and 60-min recovery time.

	Logarithmic		Non-quiescent	
	Recovery time (min)		Recovery time (min)	
Zeocin concentration (μg/ml)	**30**	**60**	**30**	**60**
100	1.25	1.41	0.84	0.87
200	2.41	1.86	1.20	1.02
300	2.29	3.21	0.99	0.88

Discussion

Currently, the yeast *Saccharomyces cerevisiae* remains one of the highly important experimental models in the field of toxicogenomics. Studying the biology of yeast cells, especially those in quiescence, could reveal the potential of this microorganism for investigating the cellular response to a particular environment. The evaluation of toxicological response and stress mechanisms in this microorganism could further be helpful in the clarification of equivalent mechanisms in higher eukaryotes. The environmental conditions can change dramatically, which includes progressive depletion of nutrients, rising ambient temperatures, or sudden contamination with

xenobiotics. Regardless of their nature, such changes in the environment invariably cause stress to the organisms, to which they must effectively adapt in order to survive. This stress is often associated with ROS accumulation (Avery 2011). Results obtained through this study confirmed that the exposure to Zeocin led to disturbance of the cellular redox balance and induced an increase in the levels of toxic oxygen radicals independently of the growth phase. The fraction of NQ cells probably rapidly lost the ability to divide and accumulated almost 4 times higher levels of ROS (Fig.1). Meanwhile, the stationary cells which have entered in G_0 cell cycle, showed significantly higher resilience to the effect of Zeocin. The treatment with this toxic chemical led to only a 2-fold increase in the levels of ROS, which was probably due to their specific morphological and physiological properties. It was well-known that *S. cerevisiae* quiescent cells are characterised by thicken cell walls, condensed chromosomes and increased termo- and osmotolerance (Gray et al. 2004). On the other hand, the NQ cells differ significantly, possessing genomic instability, being easily lysed, and providing nutrients for those cells entering G_0 (Aragon et al. 2008). As regards the logarithmic cells, they are characterised by higher metabolic activity, elevated ROS levels and respectively increased susceptibility to toxic compounds, in that case Zeocin (Cabral et al. 2003).

A direct indicator of the onset of the redox balance disturbance is the appearance of carbonyl groups in the proteins. Oxidative damage to proteins affects the processes maintaining the cellular homeostasis, which compromises their viability (Farrugia and Balzan 2012). In this respect as the Q-cells were characterised by very low metabolic levels and lower ability to sequestered damaged molecules, the observed elevated oxidative modifications in their proteins was not surprising. This might be also due to the coordinated toxic effects of Zeocin, and stress caused by the lack of nutrients in the environment. In NQ cells, the amount of carbonylated proteins after treatment with Zeocin was comparable to that in control cells - 6.56 and 6.06 μM/mg, respectively (Fig. 2). This indicated that here the appearance of oxidized proteins is rather a consequence of the physiological state of the cell population and is not directly resulting from the Zeocin-induced oxidative stress.

The measured higher intracellular concentration of malonaldehyde in Zeocin exposed cells further confirmed that one of the cytotoxic effects of this antibiotic was related to the induction of oxidative stress in the cell. It resulted in impairment of membrane functionality and permeability, probably causing the release of the intracellular content (Hodges et al. 1999). Obviously, when the metabolic activity of the cell is higher and the transport across the membrane - dynamic, the yeast cells are more vulnerable to the action of xenobiotics, including the Zeocin. This eventually explains the measured excess levels of oxidized lipids in the logarithmic cells comparing to the Q and NQ ones (Fig. 3).

To prevent the unbalanced accumulation of ROS and consecutive cellular damages, yeast cells react with specific induction of both non-enzymatic and enzymatic antioxidant defence mechanisms. Key molecule acting as first line of defence against oxidative injuries is the glutathione. That is why the total intracellular amount of this tripeptide is an important parameter for measuring the oxidative stress levels. In this study we confirmed that after exposure to IC_{50} Zeocin the three types of yeast populations showed

increased intracellular levels of glutathione ranging from 1.5-fold (for Q cells) to 2.5-fold (for logarithmic cells) (Fig. 4). Having in mind that in actively proliferating cells the rate of transcription and translation are the highest, it is not surprising that in these yeast populations the biosynthesis of glutathione was augmented with more than 100%. By contrast, in quiescent yeast cells, characterised by very low metabolic profile, the measured increase in glutathione is only about 30%. These findings also correlated with the observed tendency in the intracellular ROS levels of the studied yeasts. Non-quiescent cells had the highest measured concentrations of glutathione possibly related with the excess levels of intracellular ROS in general (Aragon at al. 2008).

Furthermore, this study aimed to assess the potential of Zeocin to induce DNA double-strand breaks (DSB) in dependence to the growth phase. The highest levels of spontaneous DSBs were observed in non-quiescent cell. Such results are not surprising, considering the fact that they represent the fraction of stationary cells having very low ability to reproduce and tendency to enter apoptosis. Such differences in the spontaneous DSB levels being dependent on the growth phase were also observed previously in other strains of *S. cerevisiae* (Todorova et al. 2015b, 2019). The statistically significant induction of DSB in logarithmic cells after the treatment with concentrations equal or higher than 200 μg/ml Zeocin is in a good correspondence with the one obtained on strain D7ts1 (Todorova et al. 2019). In late stationary cells significantly higher levels of DSBs were detected in comparison with those in exponential and early stationary cells. However, the entrance into G_0 cell cycle considerably affects the levels of DSB, rendering quiescent cells with a low susceptibility to DNA damages. When Zeocin is used as an inducing agent for DSB formation the tendency has been preserved. Most robust to its action were the cells already entered the G_0 state. A possible explanation for their resistance to this radiomimetic chemical could be again assigned to their specific cellular features. As mentioned above they are characterised by thickened cell walls, highly condensed chromosomes and, as a result, restricted ability of the Zeocin to penetrate the cell and reach its target molecule (Gray et al. 2004; Allen et al. 2006).

Conclusions

The comparative analysis of different yeast cell populations – logarithmic, quiescent, and non-quiescent – revealed that the cellular physiological state is a critical factor determining the resistance to xenobiotics. More robust to Zeocin exposure were the quiescent cells showing lower levels of ROS and DSBs. By contrast, the logarithmically grown yeasts are more susceptible to the action of this compound with observed higher damages in DNA, proteins and lipids.

Dataset deposition

The data underpinning the analysis reported in this paper are deposited at Figshare at https://figshare.com/s/e89e88bfc6f1247a4755.

Acknowledgement

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