СОФИЙСКИ УНИВЕРСИТЕТ
„СВ. КЛИМЕНТ ОХРИДСКИ“
БИОЛОГИЧЕСКИ ФАКУЛТЕТ

SOFIA UNIVERSITY
St. KLIMENT OHRIDSKI
FACULTY OF BIOLOGY

ABSTRACT OF PhD THESIS

for acquiring the educational and science degree “Doctor“
in the professional field 4.3. Biological sciences, Scientific specialty: Microbiology

Quiescent state in yeast *Saccharomyces cerevisiae* – a model for studying toxicological and stress response

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Sofia, 2023

The full volume of the dissertation is 229 pages. It consists of an **Introduction** (1 page), **Review** (45 pages), **Purpose and main tasks** (2 page), **Materials and Methods** (15 pages), **Results and Disscussion** (90), **Conclusions** (2 pages), **Contributions** (1 page). The list of references contains **575 titles**. The text of the PhD thesis includes **61 figures** and **5 tables**. These lists are given at the beginning of the dissertation. The experimental work was carried out in the Laboratory of Applied Microbiology at the Department of General and Industrial Microbiology.

The PhD thesis was discussed and allowed to be defended during an extended session of the Department of General and Industrial Microbiology, Faculty of Biology, Sofia University, which was held on 03.07.2023. It is scheduled for defense before a scientific jury formed by order …… of the Rector of Sofia University "St. Kliment Ohridski".

Scientific jury:

Prof. Dr. Petya Koycheva Hristova

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Prof. Dr. Marya Bogomilova Angelova, DSc

Assoc. Prof. Tsvetelina Sashkova Paunova – Krasteva

The defense of the PhD thesis will be on …… at …… at the Faculty of Biology.

The materials related to the defense are available in the Department of General and Industrial Microbiology and on the website of the Faculty of Biology

INTRODUCTION

The yeast *Saccharomyces cerevisiae* is one of the most used model organisms in molecular biology, biotechnology and the study of several processes related to human health and disease (Petranovic and Nielsen., 2008). With its unique genetic characteristics and a high degree of conservatism with higher eukaryotic organisms, *S. cerevisiae* represents a suitable system for studying the mechanisms of chemical toxicity. The combined application of classical toxicological studies with genetic and bioinformatics analyses further provides a deeper understanding of the toxicological response at the molecular and cellular level in biological systems following exposure to toxic compounds - information that, if available, would facilitate analyses related to risk assessment. However, the processes' physiological complexity hampered the use of actively proliferating yeast cells. On the other hand, the more stable G_0 quiescent state, where the cells resemble those of mammals, is an advantage. This allows studying the action of similar compounds in other species, contributing to environmental risk assessment and developing detoxification strategies. Studying variations in gene expression or metabolite levels after exposure to a toxic agent can also help identify the cellular components and pathways involved and play a vital role in the toxicological response.

In this context, the PhD thesis aims to develop an alternative model based on quiescent *S. cerevisiae* cells for more accurate and sensitive toxicological analysis in higher eukaryotes.

This detailed study will expand knowledge of the conserved mechanisms underlying quiescence in the yeast *S. cerevisiae*. It will evaluate the possibility of using G_0 yeast cultures to screen and predict toxicological responses in higher eukaryotes.

AIM AND TASKS

The main aim of this PhD thesis was:

Studying the applicability of *Saccharomyces cerevisiae* cells in different life cycle phases as a eukaryotic model of cellular response to toxic and stressful agents.

To achieve the set aim, the following tasks were formulated:

1. To develop a model scheme for obtaining and isolating yeast cells from different phases of the cell cycle – logarithmic (Log), quiescent cells (Q) and stationary non-proliferative cells (NQ).

2. To investigate the redox status of the isolated yeast populations.

3. To study the effect of various medicinal (zeocin, ibuprofen) and toxic (H_2O_2, menadione) preparations on the survival of the studied yeast populations.

4. To evaluate the cytotoxic effect of the tested chemical agents on Log, Q and NQ yeast cells.

5. To analyse the genotoxic effect of the selected toxic compounds zeocin and menadione on the three yeast cell populations.

6. To study the role of different physical stress factors for the survival of yeast cells isolated at different stages of the cell cycle.

7. To apply an *in silico* analysis of the evolutionary strategies for entering dormancy in yeast cells.

8. To create a model "Functional Profiles" for the stress response of *S. cerevisiae* cells in different cell cycle phases.

MATERIALS AND METHODS

In the present PhD thesis, the object of study is the haploid yeast strain *Saccharomyces cerevisiae* BY4741 (MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0) (Harsch et al., 2010), provided by the German EUROSCARF collection.

YPD yeast agar medium with composition: glucose: 2%, yeast extract: 0.5%, peptone: 1%, agar: 2.5%, pH 6.5 was used to maintain the strain. To obtain biomass, a liquid nutrient medium YPD was used with the following composition: glucose 2%, peptone 1%, yeast extract 1%; pH 6.5.

Cultivation of *Saccharomyces cerevisiae* strain BY4741 was carried out in a batch system, with liquid nutrient YPD medium at the following parameters: 205 rpm, 30ºC, for 14 hours (to isolate cells in logarithmic growth phase) or 7 days (to obtain G_0 and NQ yeast cultures). Biomass was harvested by centrifugation at 5000 rpm for 10 min.

Stationary yeast cell populations were isolated according to the method of Allen et al. (2006). A cell-free extract of the experimental cells was obtained by mechanical disintegration in a Bullet Blender for 10 min at 8000 rpm.

A mitochondrial fraction was isolated by the method of Holtta et al. (1977). A colony assay was performed to determine the concentration range of the test toxic compounds (H_2O_2, menadione, ibuprofen and zeocin) and select the LD_{50}. The effect of the various toxic agents on the viability of yeast proliferating, Q and NQ cells (OD_{540}=1.0) was evaluated relative to untreated control cells.

The effect of physical parameters on the viability of cells in logarithmic, Q and NQ phase was determined after application of different temperature (-5°C, 4°C, 30°C, 50°C and 70°C), pH (2.0 and 10), hypoosmotic (with sterile water) and hyperosmotic (0.4 M and 0.7 M NaCl, 30%, 50% and 70% sucrose and 40%, 60% and 87% glycerol) pressure, radiation (UV254), gravitational force (3000g , 30000g and 50000g), mechanical force and ultrasound (40 kHz), for different periods, taking into account the percentage of survival compared to control cells.

Biochemical analyses were performed by the following methods: soluble protein (Lowry et al., 1951), carbonyl groups in proteins (Mesquita et al., 2014), ROS in the cell by NBT method, intracellular total glutathione (Tietze, 1969, modified by Zhang, 2000), lipid peroxidation (Hodges et al., 1999).

Double-strand breaks (DSBs) in the DNA of the three experimental cell populations of *S. cerevisiae* BY4741 strain were determined by constant electric field electrophoresis (CFGE) according to the procedure of Todorova et al. (2015) and Todorova et al. (2019).

Mitochondrial ROS accumulation and resulting DNA damage after zeocin treatment were observed by fluorescence microscopy using Rhodamine 123 and DAPI dyes, respectively.

The bioinformatics analyses retrieved sequences of the studied yeast genes and proteins from the *Saccharomyces Genome* Database (SGD: http://www.yeastgenome.org). They are compared using the program for progressive comparison of multiple sequences - ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalo/). The intracellular compartmentalisation of the studied proteins was analysed with PSORT II Prediction software (https://psort.hgc.jp/form2.html).

RESULTS AND DISCUSSION

1. Growth dynamics and cell differentiation in the yeast *S. cerevisiae*

The growth dynamics and cellular differentiation were monitored in the haploid *Saccharomyces cerevisiae* BY4741 strain during batch cultivation for 168h on the YPD medium. Thus, the cells were subjected to "starvation" due to the complete depletion of a carbon source from the medium. The obtained results showed intensive glucose metabolism in an exponential growth phase between the 3rd and 24th hour (Fig. 1). After depletion of glucose, between the 24th and 48th hour, the culture undergoes a diauxic shift. It was found

that between the 96th and 120th hour, ethanol and other non-fermentable carbon sources are entirely exhausted, the cells are subjected to "starvation", and the number of proliferating

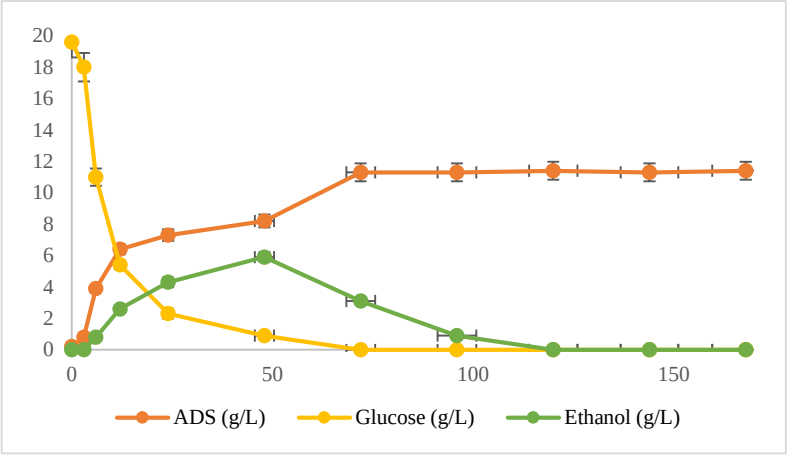

Figure 1: Dynamics of growth and carbon source uptake in batch culture of haploid strain *Saccharomyces cerevisiae* BY4741

cells dramatically decreases. and some of them enter the so-called “resting/quiescent state” (Gray et al., 2004).

In this regard, the next step in the performed research was to monitor cell differentiation and ageing in the haploid *Saccharomyces cerevisiae* BY4741 strain by determining the percentage of live, dead, budding cells and cells entering into the G_0 state (Fig. 2).

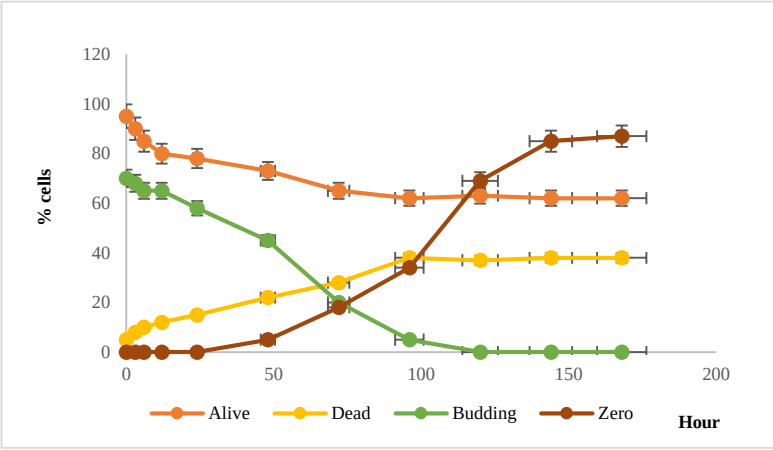

Figure 2: Cell differentiation and apoptosis in the haploid strain *S. cerevisiae* BY4741

In the period between the 3rd and 24th hour, the percentage of living cells remained high (~ 80%), but after the 24th hour, a relative decrease in budding cells was observed. In the period between the 48th and 96th hours of cultivation, a differentiation program is initiated, giving rise to a population of cells in a latent non-proliferative state - G_0 and several populations of cells in a non-latent state. The population of G_0 cells are non-budding, metabolically inactive and retains their proliferative capacity (50 – 90 % of cells). The non-dormant (NQ) cell population is heterogeneous. It consists of senescent cells that may be viable and reproductively competent, viable but reproductively incompetent, or cells that show characteristic features of apoptotic and/or necrotic states (10 – 50 % of cells) (Arlia-Ciommo et al., 2014).

Due to the high identity and conservatism of the processes between humans and yeast, the following experimental analyses aimed to investigate the effects of environmental stress factors (chemical and physical) on the different *S. cerevisiae* BY4741 cell populations.

2. Yeast as a model system for environmental impact assessment

2.1. Evaluation of the toxicological impact of chemical agents

2.1.1. Survival

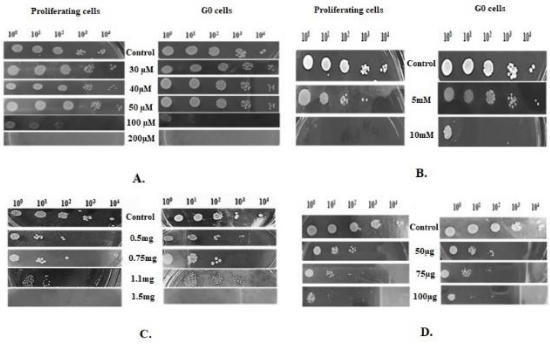

Figure 3: Colony analysis to assess the resistance of yeast cells to exogenous toxic agents – menadione (A.) H_2O_2 (B.); ibuprofen (C.) and zeocin (D.). Control - medium without the added toxic agent

The first step in studying the toxic effect of the selected chemicals was to assess the effect of different concentrations on the viability of logarithmically grown and G_0 cell populations of *S. cerevisiae* BY4741. The aim was to select a concentration at which 50% growth inhibition (LD_{50}) was observed (Fig. 3). To achieve this goal, a colony assay was performed on a culture medium with menadione, hydrogen peroxide, ibuprofen and zeocin added in different concentrations. As a result of the treatment, the following LD_{50} doses were determined: 5 mM/ml for hydrogen peroxide, 1.1 mg/ml for ibuprofen, 100 µM/ml for menadione and 50 µg/ml for zeocin.

The results obtained after treatment of Log, Q and NQ cell suspensions of *S. cerevisiae* BY4741 for 1 hour at room temperature with the described medicinal preparations showed that

when 100 µM menadione was added to the medium, a more substantial toxic effect was observed on the cells in the G_0 state, with 60% growth inhibition reported.

Subsequent studies aimed to determine the percentage of surviving cells after subjection to the determined LD_{50} doses of the four toxic chemicals. The percentage of surviving cells was determined based on the unit colonies formed (CFU/ml) (Fig. 4).

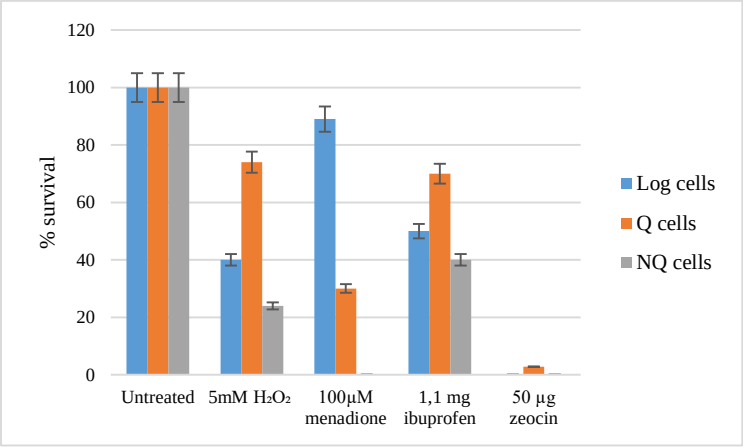

Figure 4: Survival of *S. cerevisiae* strain BY4741 after treatment with chemical agents

The assay performed with exogenous hydrogen peroxide showed that contrary to the results observed with menadione treatment, G_0 cells of *S. cerevisiae* strain BY4741 were less susceptible to hydrogen peroxide. Q cells are also characterised by much higher resistance to the harmful effects of ibuprofen (70% survival) and zeocin (2.8% survival). This is most likely because in stationary phase cells are characterised by increased thermostability, low metabolic activity, reduced rates of transcription and translation, resistance to applied environmental stress and poor plasma membrane permeability (Srivastava et al., 2016; de Sousa-Lopes et al., 2004).

2.1.2. Cytotoxicity

2.1.2.1. Hydrogen Peroxide (H_2O_2)

Amount of ROS

To follow the effect of severe oxidative stress on the three subcellular populations of *S. cerevisiae* BY4741, cell suspensions with OD_{540}=1.0 were treated with 5mM H_2O_2 for 1 hour at room temperature. After the removal of the stress agent, the cells were subjected to mechanical disruption and the resulting cell-free extracts were used to determine the levels of induced intracellular damage (Fig. 5). The obtained results showed a more than 2-fold higher concentration of generated ROS in NQ cells (470 µM/ml), compared to the proliferating ones (220 µM/ml). Virtually, no additional ROS generation was observed in Q cells compared to control untreated cells, which corresponds to literature data reporting that Q cells are more resistant to stress. Conversely, NQ cells are susceptible to heat and other types of stress and rapidly lose their ability to reproduce (Allen et al., 2006; Davidson et al., 2011), which fact is also reflected in the observed 20% increase in the generated toxic forms of oxygen in this yeast cell population.

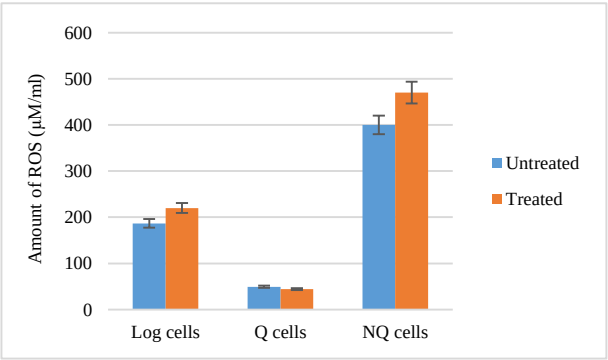

Figure 5: Amount of ROS in Log, Q and NQ cells of *S. cerevisiae* BY4741 after treatment with 5mM H_2O_2

Oxidized protein levels

This protein carbonylation, the most common type of ROS-induced protein modification, is considered irreversible and induces protein degradation in the cell. From the data presented in Fig. 6, NQ cells are characterised by a 1.5 times higher concentration of oxidised proteins after treatment with hydrogen peroxide (13 μM/mg) than the control cells (8.5 μM/mg). In Q cells, less susceptibility to the toxic effect of the hydrogen peroxide was again observed, which is consistent with the results obtained regarding the survival of the three cell populations after treatment with this agent (Fig. 4), where G_0 cells show the highest percentage of viability – 74%.

In proliferating cells, the increase in the concentration of oxidized proteins is approximatley 1.4 times higher (1.3 μM/mg) then the control ones (0.9 μM/mg). Entry of yeast cells into early stationary phase is known to increase their resistance to oxidants such as H_2O_2 (Jamieson., 1992; Izawa et al., 1996).

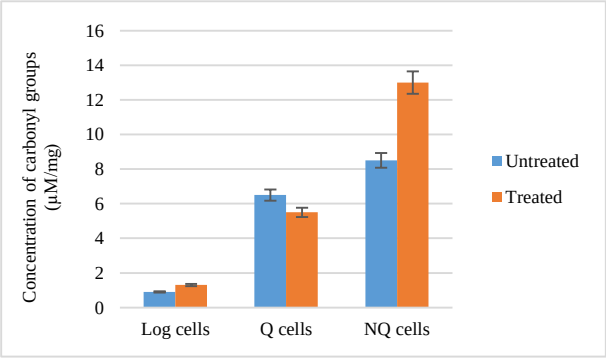

Figure 6: Oxidized protein levels in Log, Q and NQ cells of *S. cerevisiae* BY4741 after treatment with 5mM H_2O_2

Levels of oxidized lipids

Unsaturated fatty acyl groups in membranes are a major target for the hydroxyl radical and superoxide anion, and this attack initiates autocatalytic lipid peroxidation leading to the formation of reactive lipid radicals and lipid hydroperoxides (Wiseman et al., 1996; Gunstone., 1996). Degradation, in turn, of lipid hydroperoxides leads to the generation of a wide variety of highly reactive aldehydes (Levine., 2002). In connection with the above, the toxic effect of H_2O_2 on yeast lipids was also assessed, evaluating the change in the intracellular levels of their oxidised forms (Fig. 7). It was found that no increase in their levels was observed in any of the three cell populations.

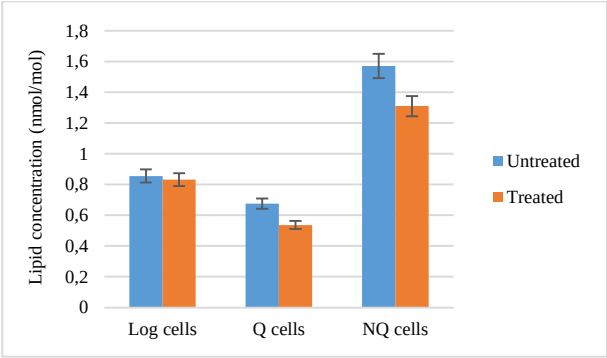

Figure 7: Oxidized lipid levels in Log, Q and NQ cells of *S. cerevisiae* BY4741 after treatment with 5mM H_2O_2

Total Glutathione

Upon exogenous exposure to toxic agents, one of the first lines of defence in the cell is the expression of the tripeptide glutathione (Grant et al., 1996; Jamieson., 1998; Dawes., 2004). Therefore, in the present studies, specific attention was paid to the change in the levels of this antioxidant after exposure to exogenously added 5 mM H_2O_2.

The obtained data showed that the highest levels of this antioxidant were observed in NQ cells (0.99 mM/mg), which probably represents a compensatory mechanism to overcome the

highly disturbed homeostasis (Fig. 8). In the remaining two cell populations – Q and Log cells, reported values did not differ from those measured in control yeast cultures.

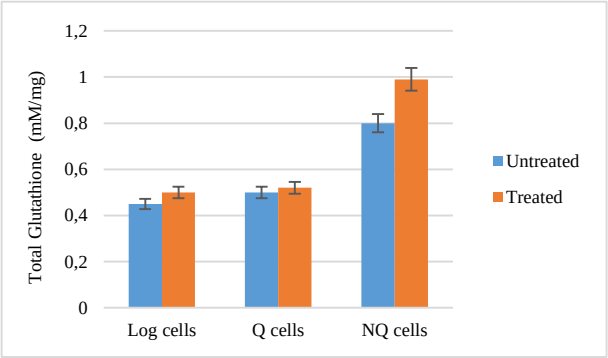

Figure 8: Amount of total glutathione in Log, Q and NQ cells of *S. cerevisiae* BY4741 after treatment with 5mM H_2O_2

2.1.2.2. Menadione

Amount of ROS

Menadione induces cell death through apoptosis and inhibits the growth of various cancer cells (Lee et al., 2016), which is also the main reason that one of the chemical agents used in the experiments was precisely this quinone derivative, widely used in medical practice. To study the effect of menadione on different cell populations of *S. cerevisiae* BY4741, they were treated with the LD_{50} = 100 μM established in the previous experiments.

After incubation for 1 hour at 25°C, the levels of generated ROS in the studied cells were measured. The results are consistent with the data reported for the survival of the three cell populations after treatment with this preparation. The cytotoxic effect of menadione in G_0 cells was found to be stronger (265 μM/ml) compared to those observed in logarithmic cells (186.6 μM/ml), a fact probably related to the process of

generating energy in Q cells through mitochondrial respiration and the associated higher cellular oxygen levels (Fig. 9) (Bonawitz et al., 2007).

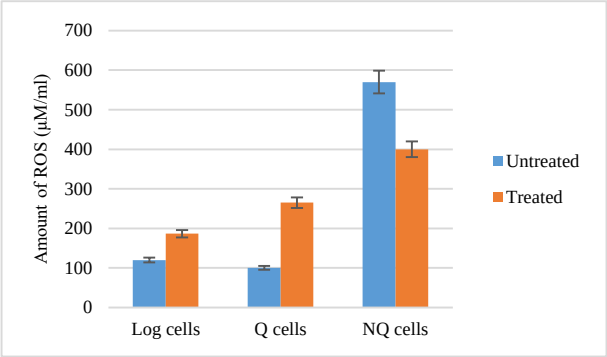

Figure 9: Amount of ROS in Log, Q and NQ cells of *S. cerevisiae* strain BY4741 after treatment with 100 µM menadione

Levels of oxidized proteins

In relation to the oxidative changes in the proteins, it was shown that the rise in the levels of formed carbonyl groups was about seven times higher in Q cells than in the logarithmic ones (Fig. 10).

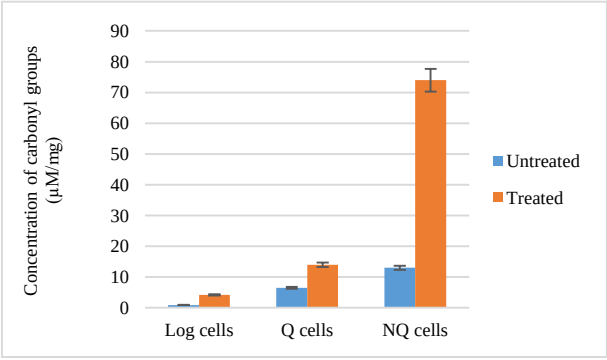

Figure 10: Oxidized protein levels in Log, Q and NQ cells of *S. cerevisiae* BY4741 after treatment with 100 µM menadione

This phenomenon is likely related to ROS accumulation and carbonylated protein formation (Coliva et al., 2019). The most significant amount of oxidised proteins, 74 µM/mg, was measured in NQ cells, likely due to their apoptotic nature. After treatment of the model cultures with the LD_{50} dose of menadione - 100µM, the value of carbonylated proteins increased twice in Q cells. It reached 14 µM/mg, 3 times higher than those measured in the proliferating cells (4, 2 µM/mg) (Fig.10).

Levels of oxidised lipids

Contrary to the results of the previous experiments, where the cells in the exponential phase showed higher resistance to the action of menadione, lower levels of oxidised lipids were observed here, specifically in the Q cells (Fig. 11). The results are comparable to the data from the literature, according to which, during the stationary phase, a large amount of trehalose accumulates in *S. cerevisiae* cells, which during oxidative stress protects lipids from oxidation (Herdeiro et al., 2005).

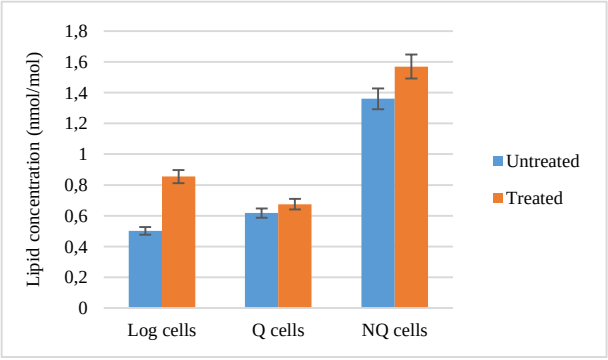

Figure 11: Oxidized lipid concentration in Log, Q and NQ cells of *S. cerevisiae* BY4741 after treatment with 100 µM menadione

Total Glutathione

The results showed that the GSH concentration in untreated Q cells was 3 times higher than that in Log cells. Interestingly, menadione treatment of yeast in the G_0 state did not significantly increase basal GSH levels in the cells. Its concentration increases no more than 2-fold due to exposure to this toxic agent. At the same time, Log cells treated with 100 μM menadione showed nearly 7-fold higher levels of GSH (2mM/mg) than control cells (0.3mM/mg). A dramatic increase in total glutathione levels was also reported in NQ cells (5.7 mM/mg) (Fig. 12).

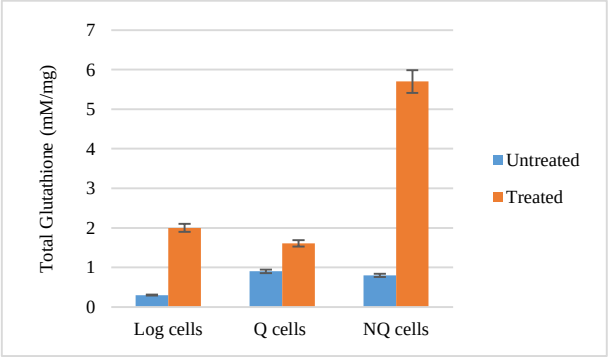

Figure 12: Total glutathione levels in Log, Q and NQ cells of *S. cerevisiae* BY4741 after treatment with 100 μM menadione

2.1.2.3. Ibuprofen

Amount of ROS

Due to the limited experimental data regarding the effect of ibuprofen on *S. cerevisiae* cells, in the present study, the level of intracellular damage in the three yeast cell populations – Q, NQ and proliferating, was evaluated after treatment with LD_{50} = 1mg/ml concentration of ibuprofen. The obtained experimental results showed an increase in the levels of ROS in all three types of yeast cultures studied. The highest values were

recorded in Q cells (400µM/ml), both compared to the control (300µM/ml) and the other two cell populations (Fig.13).

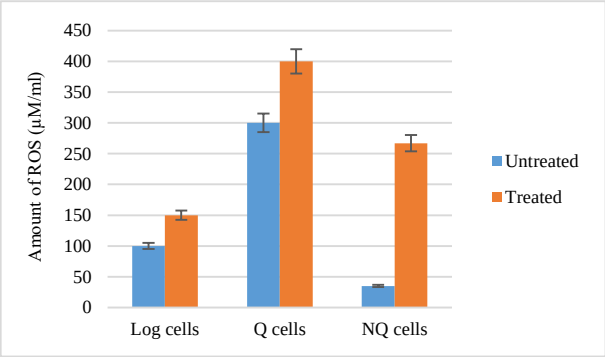

Figure 13: Amount of ROS in Log, Q and NQ cells of strain *S. cerevisiae* BY4741 after treatment with 1.1 mg/ml ibuprofen

The reason for the accumulation of reactive oxygen species is most likely because ibuprofen treatment leads to both Tat2 degradation and the associated starvation processes in the cell (He et al., 2014).

Oxidized proteins

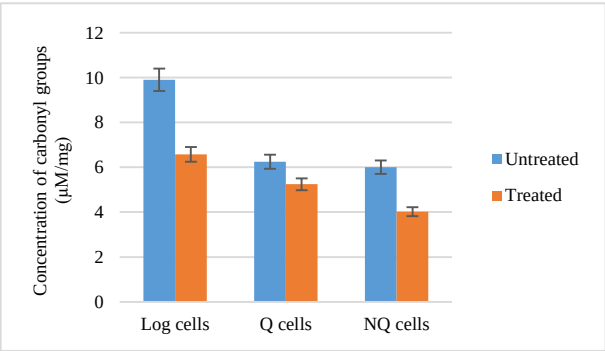

Figure 14: Oxidized protein levels in Log, Q and NQ cells of *S. cerevisiae* BY4741 after treatment with 1.1 mg/ml ibuprofen

The obtained results showed that despite the observed increase in the level of reactive oxygen species in the treated proliferating, G_0 and NQ cells, no changes were detected in the levels of carbonyl protein groups, which emphasises the absence of significant toxic effect after its application to the cell (Fig. 14).

Oxidised lipids

Exposure of the three cell types to the LD_{50} concentration of ibuprofen again showed no damaging effect on cell membranes. During the exponential growth phase, a nearly 7-fold lower concentration of oxidised lipids was observed.

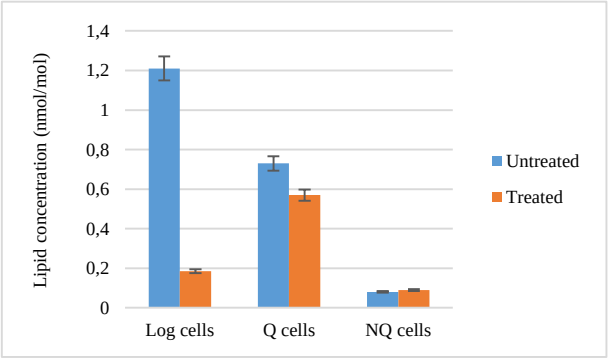

Figure 15: Amount of oxidized lipids in Log, Q and NQ cells of *S. cerevisiae* BY4741 after treatment with 1.1 mg/ml ibuprofen

On the other hand, in G_0 state, a 1.3 lower values of of oxidized lipids were measured compared to the control cells (Fig. 15). According to literature data, the addition of 0.2 mM ibuprofen resulted in a significant extension of the replicative lifespan in *S. cerevisiae*, which once again confirms the lack of toxic effect of this non-steroidal anti-inflammatory agent on yeast cells (Chong et al., 2014).

Total Glutathione

From the presented experimental results, it can be seen (Fig. 16) that after treatment with 1.1 mg/ml ibuprofen, the levels of total glutathione in all three cell populations increased by approximately 20% - 30%, which is in agreement with the obtained data for the ROS concentrations in the cells (Fig. 13). Fully expected, elevated GSH levels are a sign of oxidative processes in the cell, but compared to the other toxic agents used, ibuprofen, although poorly studied as a mechanism of action, has the weakest toxic effect, which is also consistent with the lowest increase in glutathione levels.

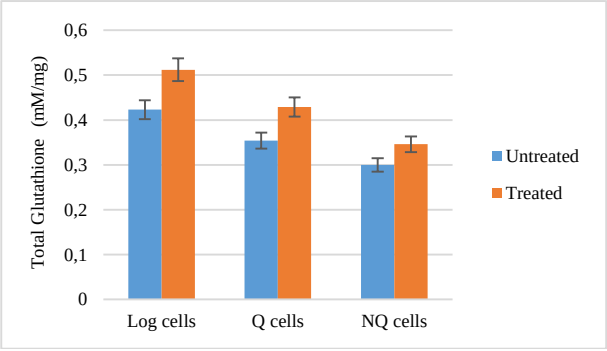

Figure 16: Total glutathione levels in Log, Q and NQ cells of *S. cerevisiae* BY4741 after treatment with 1.1 mg/ml ibuprofen

2.1.2.4. Zeocin

Double strand breaks in the DNA molecule are the most critical damages caused by ionising radiation or radiomimetics. One of the genotoxic agents, a significant source of double-strand breaks, is the zeocin, an antibiotic from the bleomycin family (Todorova et al., 2019).

Amount of ROS

Obtained data for the levels of accumulated ROS in the cells after exposure to 50 µg/ml zeocin in the Log, Q and NQ cell yeast populations agreed with the results obtained for the yeast survival (Fig. 4). 4-fold higher levels of ROS (400 µM/ml) were observed in logarithmically grown cells (100 µM/ml) (Fig. 17). The reason for this spike in oxidation levels is most likely due to the high rate of transcription and translation in actively dividing cells, which leads to the more frequent generation of mutations and the inability to compensate the oxidative damages generated.

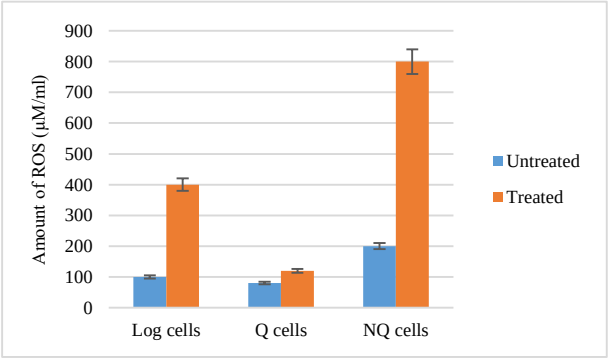

Figure 17: Amount of ROS in Log, Q and NQ cells of *S. cerevisiae* strain BY4741 after treatment with 50µg/ml zeocin

Oxidized proteins

The observed increased oxidative modifications in Q cell proteins upon zeocin treatment are unsurprising, as these cells are characterised by meagre metabolic rates and a lower ability to repair damaged molecules. This may also be due to the coordinated toxic effects of zeocin and the 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. 18).

This indicates that in this case the formation of oxidized proteins is rather a consequence of the physiological state of the cell population and not a direct result of zeocin-induced oxidative stress (Marinovska et al., 2022).

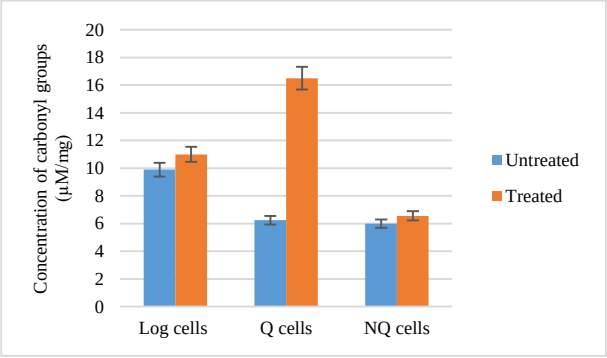

Figure 18: Oxidized protein levels in Log, Q and NQ cells of *S. cerevisiae* strain BY4741 after treatment with 50 µg/ml zeocin

Oxidized lipids

Another important indicator signaling a disruption of cellular homeostasis due to the damaging effect of zeocin is an increase in the levels of peroxidised lipids. In this regard, the measured higher intracellular concentration of malonaldehyde in the zeocin-treated cell populations further confirms that one of the cytotoxic effects of this antibiotic is related to the induction of oxidative stress in the cell.

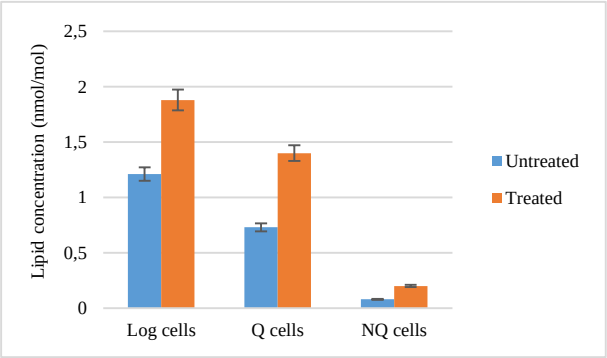

Figure 19: Oxidized lipid concentration in Log, Q and NQ cells of *S. cerevisiae* BY4741 after treatment with 50 µg/ml zeocin

This results in membrane functionality and permeability impairment, possibly causing a release of intracellular contents (Hodges et al., 1999). Obviously, when the cell's metabolic activity is higher, and the transport through the membrane is dynamic, yeast cells are more vulnerable to the action of xenobiotics, including zeocin. This ultimately explains the increased levels of oxidised lipids in Log cells compared to those of Q and NQ (Fig. 19).

Total Glutathione

The main molecule acting against oxidative damage is glutathione. Therefore, the total intracellular amount of this tripeptide is an essential parameter for measuring levels of oxidative damage. Not surprisingly, after exposure to the LD_{50} dose of zeocin, all three yeast populations showed increased intracellular levels of this antioxidant, with the highest values found in logarithmically grown cells (2.5-fold increase), where the level of impaired lipids is the highest (Fig. 20).

In the G_0 cells, an induction of glutathione biosynthesis was also reported. Still, it was only about 30%, probably due to this agent's less toxic effect on the yeast populations' cell membranes. These findings also correlate with the observed trend for the intracellular ROS levels of the studied yeasts. After zeocin treatment, high glutathione concentrations were

also observed in NQ cells, possibly related to the excess levels of intracellular ROS in general (Aragon et al., 2008).

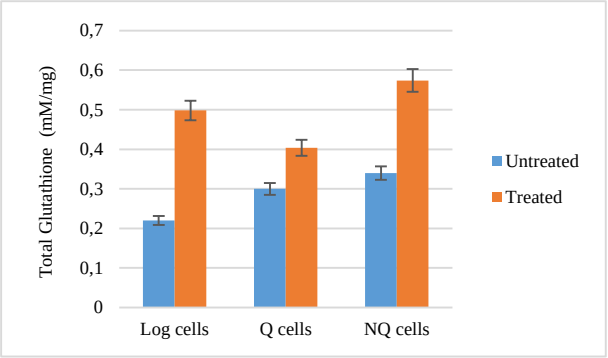

Figure 20: Total glutathione levels in Log, Q and NQ cells of *S. cerevisiae* strain BY4741 after treatment with 50 µg/ml zeocin

In connection with the toxic effect of zeocin observed in previous experiments and the widespread use of antibiotics of the bleomycin family in the last decade, some additional studies on the toxicity of this xenobiotic were also conducted. The survival of two of the yeast cell populations - the actively dividing and the quiescent - was followed after treatment with different, lower than the established LD_{50} dose of zeocin (5, 10, 15, 20, 25 µg/ml) for 1 hour (Fig. 21).

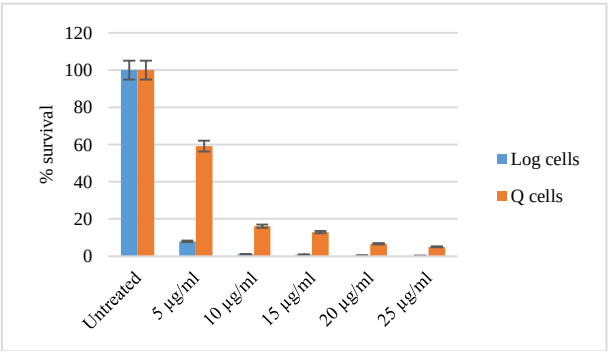

Figure 21: Log and Q cell survival of *S. cerevisiae* strain BY4741 after treatment with different concentrations of zeocin

Logically, a decrease in the survival of yeast microorganisms was observed after increasing the concentration of this toxic agent, with Q cells retaining relatively higher resistance compared to those in the exponential growth phase.

The susceptibility of the two yeast cell populations to zeocin was also monitored after administration of LD_{50} = 50µg/ml zeocin for different periods (1, 30 and 60 min). The data obtained showed that a sharp drop in survival was found after treatment, even for 1 minute, which once again emphasises the powerful toxic effect of this radiomimetic (Fig. 22).

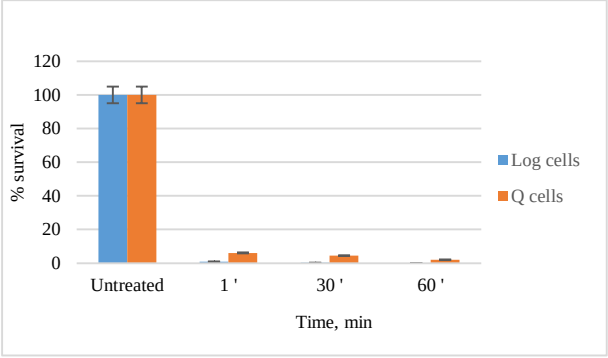

Figure 22: Log and Q cell survival of *S. cerevisiae* BY4741 after treatment with 50 µg/ml zeocin for different times

However, regardless of this observed effect, again Q cells show a significantly higher resistance to the action of this xenobiotic, which represents essential information from a medico-biological point of view - metabolically inactive eukaryotic cells in the G_0 cell cycle are much less susceptible to the action of glycopeptide antibiotics, which can cause single- or double-strand breaks in the DNA molecule and are used in clinical practice as anticancer agents.

Mitochondrial respiratory capacity and inner mitochondrial membrane potential influence ROS formation, cellular damage accumulation, and lifespan. Therefore, the next step in the study of the toxic effect of zeocin was to consider the damage induced by the LD_{50} dose on yeast mitochondria isolated from the exponential and stationary growth phase. For this purpose, the specific staining with the fluorescent dyes DAPI (4',6-diamino-2-phenylindole) and Rhodamine 123 was carried out.

DAPI is a DNA-sensitive fluorochrome, and mesearing the change in the fluorescence intensity can be successfully used to characterise the extent of DNA damage (Saadat et al., 2015) (Fig. 23).

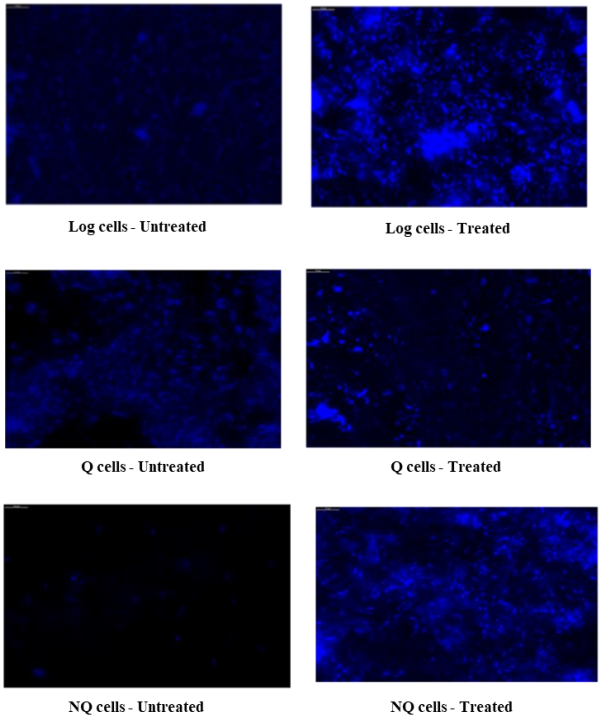

Figure 23: DAPI fluorescence analysis of mitochondrial DNA in Log, Q and NQ cells of *S. cerevisiae* BY4741 treated with 50 µg/ml zeocin

The results presented show that zeocin exerts a strong toxic effect in all three cell populations of *S. cerevisiae*. The highest reported DAPI fluorescence intensity was observed in NQ cells. The data obtained from the fluorescence microscopy confirmed the results from the previous experiments. The most resistant to the harmful effect of bleomycins, and in particular zeocin, are the Q cells, where the mtDNA damages are the lowest. In exponentially growing cells, the trend is maintained – double-stranded breaks are generated after exposure to this toxic agent.

Treatment of mitochondria from the three yeast populations with zeocin and their subsequent staining with Rhodamine 123 showed again that the lowest concentration of generated ROS was observed in Q cells (Fig. 24), a fact probably related to the higher resistance of these cell populations to different types of stress. The obtained data are closely correlated with those in the literature, according to which G_0 cells are resistant to several harmful effects (Gray et al., 2004).

The picture is entirely different in the mitochondria of the NQ cells, and the fluorescence intensity is several times stronger than that observed in the control, untreated cells. This once again confirms their poor resistance and rapid loss of viability. In the mitochondria of actively dividing logarithmic cells, a significant increase in fluorescence intensity was also observed, which further confirms the role of zeocin not only in damaging the structure of DNA but also in inducing oxidative stress in the cell and disrupting redox homeostasis.

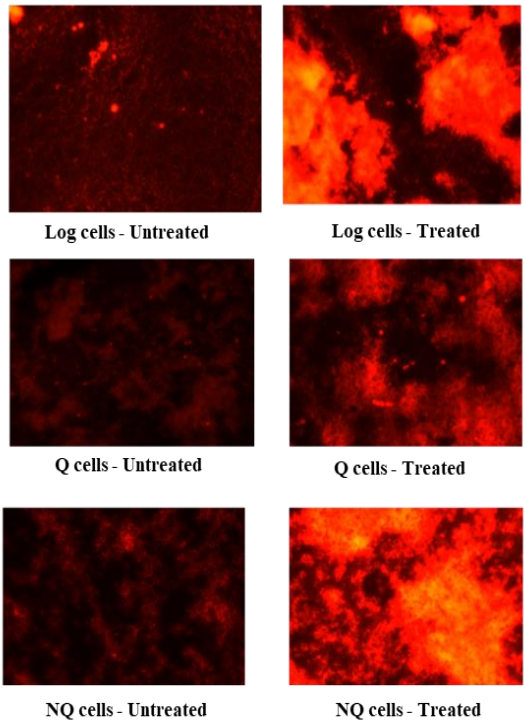

Figure 24: Determination of the levels of generated ROS after treatment of mitochondrial fractions of Log, Q and NQ cells of *S. cerevisiae* BY4741 with 50 μg/ml zeocin versus fluorescence intensity after staining with Rhodamine 123

2.1.3. Genotoxicity

In connection with the obtained results, in which the most enhanced toxic effect was observed after the administration of the antibiotic zeocin and the quinone derivative - menadione, as well as considering their mechanism of action, the next aspect in the present investigation was to measure the levels of induced DNA damage in the three yeast cell

populations (Log, Q and NQ of *S. cerevisiae* BY4741). Constant field gel electrophoresis was used to perform this analysis.

2.1.3.1. Double-strand breaks in DNA after zeocin treatment:

The levels of spontaneous double-strand breaks in the three yeast populations of *S. cerevisiae* strain BY4741 were investigated. About 1.5-fold higher levels of DSBs were measured in NQ cells compared to those found in exponentially growing yeast or the G_0 state. No significant difference was found between the levels of DSBs in log and Q cells (Fig. 25).

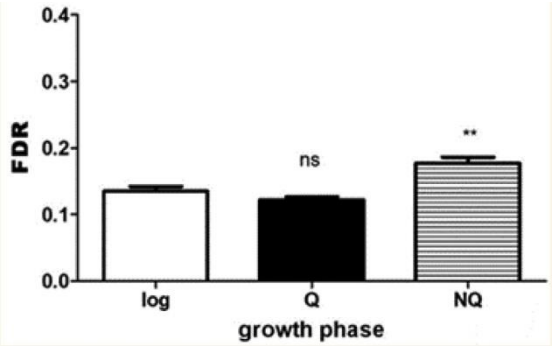

Figure 25: Spontaneous levels of DSBs dependent on growth phase in *S. cerevisiae* BY4741

Growth phase was also found to be a very important factor in the sensitivity of *S. cerevisiae* DNA to zeocin. When cells were subjected to different zeocin concentrations, roughly similar levels of induced DSBs were measured in both log (Fig. 26 A, D) and NQ cells (Fig. 26 B, D). No significant effect of this radiomimetic was observed in the cells entered in a quiescent state (Fig. 26 B, D). DSB levels were comparable to those in untreated cells.

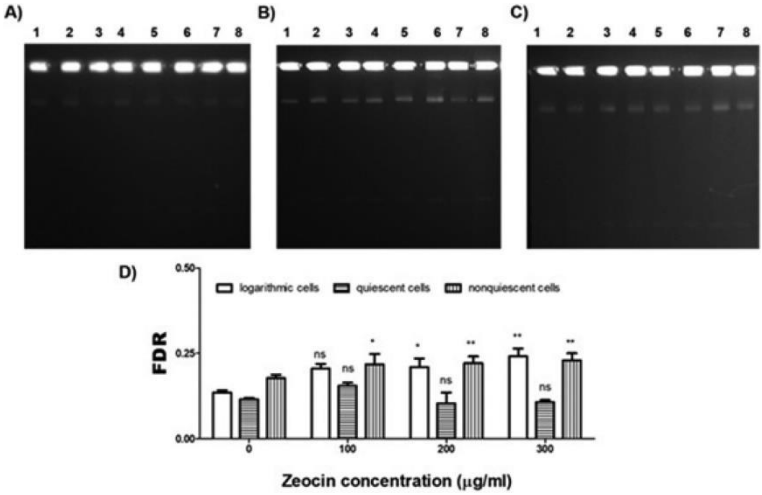

Figure 26: Induction of DSBs in *S. cerevisiae* BY4741, depending on the growth phase after treatment with zeocin at concentrations of 100-300µg/ml. A - cells in logarithmic phase, B - Q cells, B - NQ cells. 1, 2 – controls; 3, 4 - treated with 100 µg/ml zeocin; 5, 6 - treated with 200 µg/ml zeocin; 7, 8 - treated with 300 µg/ml zeocin. D - DSB induction after treatment with different concentrations of zeocin, calculated as FDR.

The repair capacity was also calculated for the tested yeast populations (Table 1). It was found to be the highest in logarithmically growing cells, which required only 60 minutes of recovery time. The logical reason for this lies in the fact that in cells in the exponential phase of growth, repair mechanisms are more pronounced. On the other hand, a loss of ability to repair zeocin-induced DSBs was reported in NQ cells despite the incubation time (1 h). Considering their nature (metabolically inactive), Q cells showed relatively little increase in the levels of DSBs after treatment with different concentrations of zeocin, and therefore, it was impossible to calculate the repair capacity.

Table: 1: Repair capacity of Log and NQ cells calculated after treatment with zeocin at concentrations of 100, 200 and 300 µg/ml with 30 and 60 min recovery time.

Zeocin concentration (µg/ml)	Log cells		NQ cells	
	Repair time (min)		Repair time (min)	
	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

2.1.3.2. Double strand breaks in DNA after menadione treatment:

Since menadione acts as a redox mediator and leads to the generation of reactive oxygen species that damage the cell, the following analyses were aimed at establishing the levels of induced DSBs in the three yeast cell populations after treatment with different concentrations of this toxic agent (Yamashoji et al., 2020).

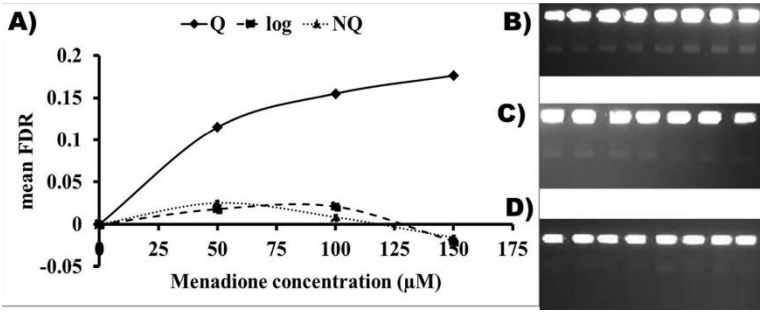

Figure 27: Induction of DSBs by different concentrations of menadione (50–150 µM). A – DSB induction presented as FDR; B – Q cells; C – Log cells; D – NQ cells

The data obtained showed that the increase in ROS levels in Q cells due to the action of menadione (Fig. 9) corresponds with the decrease in cell survival (Fig. 4) and leads to the well-marked induction of DSBs in DNA. Contrary to zeocin, the genotoxic effect of

menadione in Q cells is the strongest, most likely because they are generally characterized by high levels of oxygen and ROS and, accordingly, the level of additional generation of toxic radical forms is significantly increased. This contributes to more efficient DNA damage (Fig. 27) (Allen et al., 2008).

2.2. Evaluation of the toxicological effects of various physical factors on the growth of Log, Q and NQ yeast strains

The next step in the performed research was to study the effect of extreme values of several physical factors on the viability of different cell populations of *S. cerevisiae* strain BY4741.

2.2.1. Effect of temperature on the survival of *S. cerevisiae* BY4741 yeast cells

The survival rate of NQ cells when treated with low or high temperatures, in contrast to the other cell populations (Q and Log cells), was the lowest (Fig. 28). This effect is most likely because they possess genomic instability and easily autolysed (Aragon et al., 2008). On the other hand, G_0 cells were the most resistant at all temperatures tested. These data are also consistent with studies by Lu et al. (2009), who reported that sensitivity to heat shock is related to growth rate: cells in a stationary phase show higher resistance to temperature imbalance than those in an exponential growth phase.

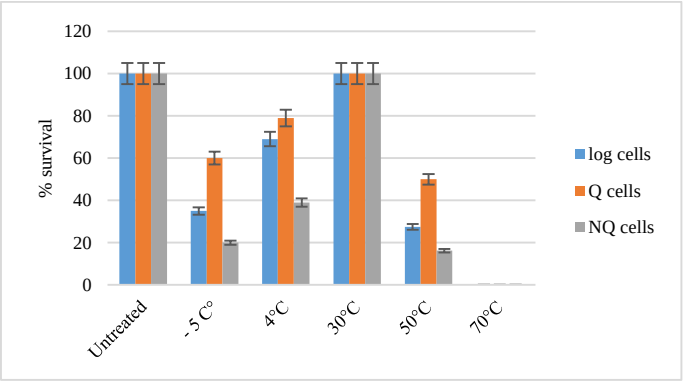

Figure 28: Effect of temperature on the survival of Log, Q and NQ cells of *S. cerevisiae* BY4741

2.2.2. Effect of pH on the survival of *S. cerevisiae* BY4741 yeast cells

After incubation in an acidic medium (pH 2.0) for 30 min, over 90% survival was reported for G_0 yeasts, in contrast to proliferating cells, where survival was below 80%. Increasing treatment time was associated with decreased proliferating cells' survival rate; at 60 min incubation, only 60% of the cells remained viable. Under the same conditions, G_0 cells show nearly 80% survival. NQ cells showed the highest susceptibility, most likely due to rapidly losing their reproductive capacity, as ~50% of these cells could not form daughter progeny (Fig. 29) (Aragon et al., 2008).

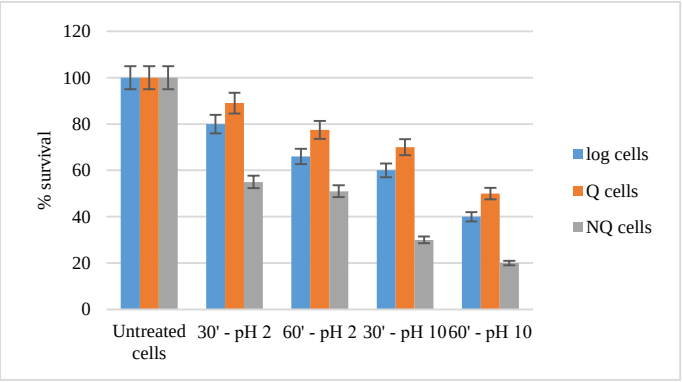

Figure 29: Effect of pH on the survival of Log, Q and NQ cells of *S. cerevisiae* BY4741

2.2.3. Effect of osmotic stress on the survival of yeast cells from strain *S. cerevisiae* BY4741

2.2.3.1. Effect of hypoosmotic stress

The effect of hypoosmotic stress on the survival of the three yeast cell populations was examined after suspending the cells in sterile distilled water (Fig. 30). Both actively growing and quiescent cells retained 100% viability even after 48 hours of treatment. Contrary, NQ cells rapidly lose their viability, and after 48 hours, only 20% of them retain their ability to reproduce.

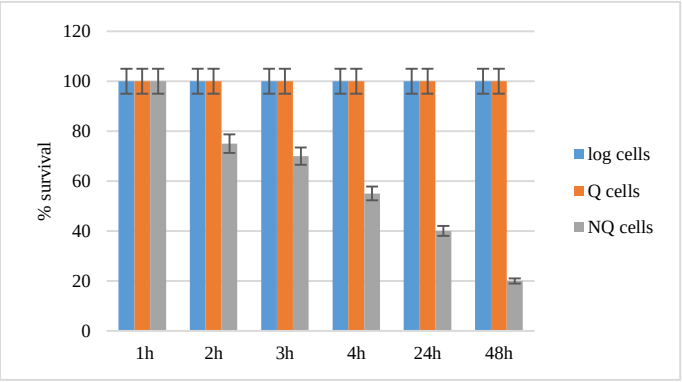

Figure 30: Effect of hypoosmotic medium conditions on the survival of Log, Q and NQ cells of *S. cerevisiae* BY4741

2.2.3.2. Effect of hyperosmotic stress

Sodium chloride

Exposing the yeast cells to a hyperosmotic condition rapidly releases water into the medium, dehydrating the cell. In this connection, the survival of populations of NQ, Q and logarithmically growing cells after applying hyperosmotic stress was investigated. For this purpose, the three types of cells were treated for 20 minutes at two concentrations of sodium chloride in the medium – 0.4 and 0.7 M. (Fig. 31). The obtained data showed that NQ cells rapidly lose their viability, which fully corresponds to the notion that they are the most resistant to action with toxic agents. Q cells are characterised by greater resistance to high concentrations of NaCl in the medium, as they accumulate trehalose during starvation and transition to the G_0 cell cycle (Lilie and Pringle., 1980). In contrast, actively dividing cells have not accumulated this metabolite, and therefore a smaller proportion of them can successfully respond effectively to exercise from high NaCl concentrations.

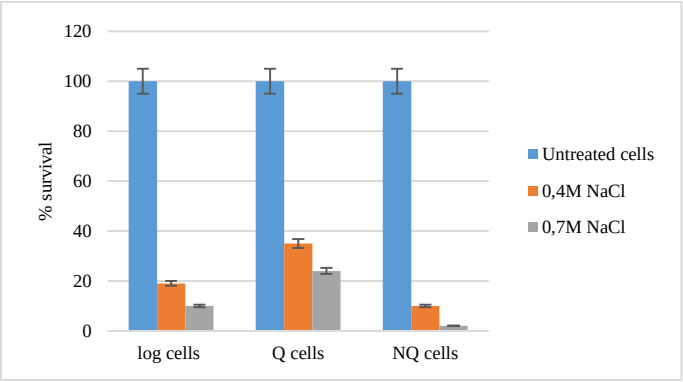

Figure 31: Effect of 0.4 and 0.7M NaCl on the survival of Log, Q and NQ cells of *S. cerevisiae* BY4741

Sucrose

Proliferating, G_0 and NQ cells of *Saccharomyces cerevisiae* BY4741 were also treated with sucrose for 4 h at room temperature.

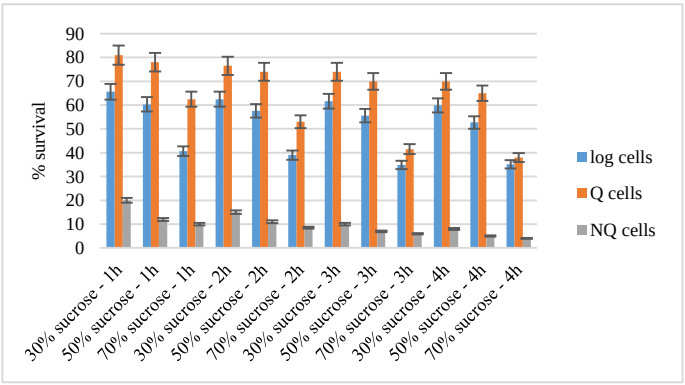

Figure 32: Effect of different concentrations of sucrose on the survival of Log, Q and NQ cells of *S. cerevisiae* BY4741

The sharpest decline in cell survival was reported at 1 hour from the start of the experiment, with about 35% of log cells dying in 30% sucrose solution and about 60%

dying in 70% sucrose solution (Fig. 32). G_0 cells maintained their viability within 81% and 62.5% in 30% and 70% sucrose solution, respectively. At 4 h, 59.9%, 8%, and 70% survival at 30% sucrose, and 35%, 4%, and 38% survival at 70% sucrose were reported for proliferating, NQ, and Q cells of *S. cerevisiae* BY4741, respectively. (Fig. 32).

Glycerol

In the experiments conducted, three different concentrations of glycerol (40%, 60%, 87%) were used to evaluate the percentage survival of proliferating, Q and NQ cells. The results are presented in Fig. 33 and again showed higher resistance to hyperosmotic stress in quiescent (Q) cells. In the first hour after treatment with 40% glycerol, the cellular viability drops sharply, with only 9.3% survival observed even in G_0 cells. A nearly 23-fold lower survival was found in logarithmically grown cells, while NQ cells were completely inhibited by the three concentrations of glycerol.

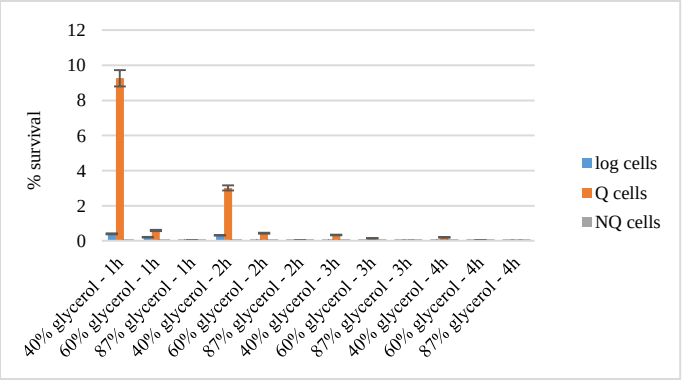

Figure 33: Effect of different concentrations of glycerol on the survival of Log, Q and NQ cells of *S. cerevisiae* BY4741

2.2.4. Effect of UV rays on the survival of yeast cells of *S. cerevisiae* strain BY4741

Direct exposure of yeast cells to UV-C_{254} rays exerts a powerful fungicidal effect on them. Even after the first minute, almost 100% growth inhibition was observed, except for cells in the G_0 state (6% survival) (Fig. 34).

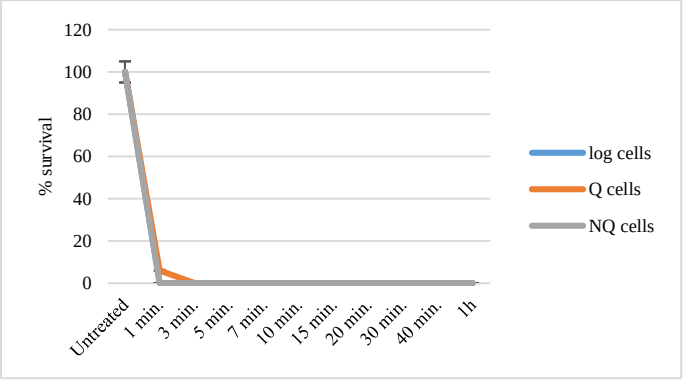

Figure 34: Effect of UV rays on the survival of Log, Q and NQ cells of *S. cerevisiae* BY4741

2.2.5. Effect of gravitational force on the survival of *S. cerevisiae* BY4741 yeast cells

The effect of hypergravity on the survival of proliferating, Q and NQ cells was investigated by centrifugation at different applied gravitational force g (g = 3000; g = 30000 and g = 50000) for different periods (1 to 4 hours). After the application of 3000g, no growth inhibition was detected in both cell populations (Q and Log), even after 4 h of centrifugation. In NQ cells, viability was more than 3-fold lower than the other two *S. cerevisiae* cell types. Increasing the gravitational force to 30000g and 50000g resulted in an almost complete loss of viability in NQ cells, 26% and 6%, respectively. The remaining two cell populations maintained their survival (100%) when high gravity was applied (Fig. 35).

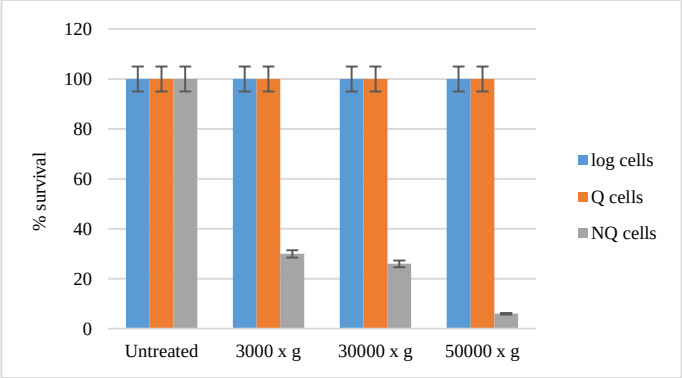

Figure 35: Effect of gravitational force on the survival of Log, Q and NQ cells of *S. cerevisiae* BY4741

2.2.6. Effect of mechanical force on the survival of *S. cerevisiae* BY4741 yeast cells

Mechanical disintegration is one of the most cost-effective methods for obtaining a cell-free extract from yeast cells. Therefore, different modes and conditions of disintegration for yeast microorganisms were investigated during the conducted experiments. Analysing the obtained results, it is evident that the quiescent *S. cerevisiae* BY4741 cells (Q) have a higher resistance to impact with mechanical force than the other two cell populations. A tiny percentage of destroyed cells from the Log and Q cell populations were found after 1 min of disintegration - 17.3% and 11.5%, in contrast to NQ cells, where 92% cell disintegration was observed. At each subsequent minute, up to 6 minutes, the percentage of disintegrated cells increased by approximately 7-10% for logarithmic, 5-7% for G_0 cells and 2-4% for NQ cells. At the end of the experiment (6 minutes), the percentage of surviving cells was 44.8%, 51.9 and 1% for log, Q and NQ cells, respectively (Fig. 36).

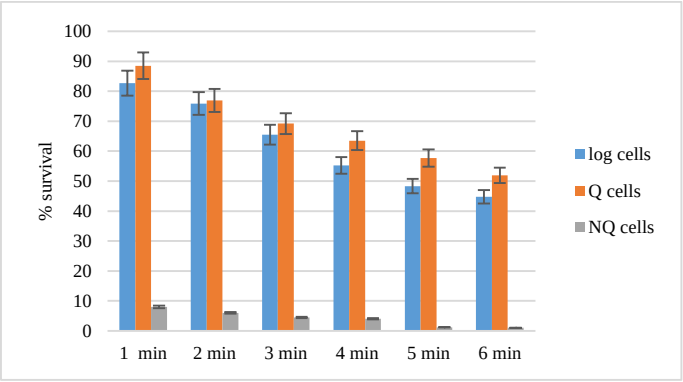

Figure 36: Effect of mechanical force on the survival of Log, Q and NQ cells of *S. cerevisiae* BY4741

2.2.7. Effect of ultrasound on proliferating, Q and NQ cells of *S. cerevisiae* strain BY4741

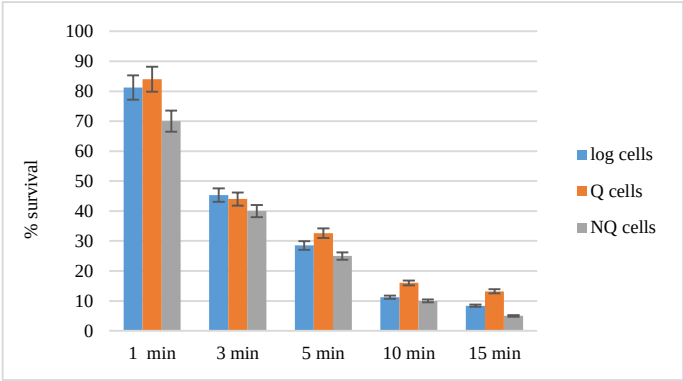

Figure 37: Effect of ultrasound on the survival of Log, Q and NQ cells of *S. cerevisiae* BY4741

Ultrasonic cavitation and high temperatures were found to act synergistically to suppress the growth of *S. cerevisiae* (Wordon et al., 2012).

Therefore, subsequent research was aimed at studying the effect of ultrasound waves on the survival rate of the three *S. cerevisiae* BY4741cell populations. The experimental data obtained are presented in Fig. 37. Log, Q and NQ yeast cells were sonicated for 15 min, and the per cent surviving cells were calculated with respect to the control non-sonicated cells. During the first minute of ultrasound application, the trend for highest survival was confirmed for Q cells, followed by Log and NQ, respectively (84%, 82.5% and 70%).

3. *In silico* analysis of genes from signalling pathways regulating quiescence and stress response

Given the existing interdependence between metabolic pathways and quiescence, it is appropriate to develop an *in silico* model to study the regulatory mechanisms important for the entry and maintenance of cellular homeostasis in the G_0 state in yeast. Therefore, a next step in the present investigation was to perform a bioinformatic analysis in order to characterize key genes responsible for entering in quescence in *S. cerevisiae*. Function and intracellular localization of four kinases have been studied - TOR, Protein kinase A, Protein kinase C and Snf1 protein kinase, both in yeast and humans.

3.1. TOR protein kinase

Analysis of the *Saccharomyces* Genome Database (SGD) confirmed that the yeast *Saccharomyces cerevisiae* possesses two genes, TOR1 and TOR2, which encode respective protein kinases:

• *TOR1* (YJR066W) – gene encoding the synthesis of Tor1p, a PIK-related protein kinase, a target of rapamycin.

• *TOR2* (YKL203C) – gene encoding the synthesis of Tor2p, a PIK-related protein kinase, a target of rapamycin.

TOR1 and *TOR2* encode two closely related factors that regulate cell growth in response to nutrient availability and stress (Cardenas et al., 1999; Loewith et al., 2002).

The percentage of identity generated by ClustalW between their amino acid sequences is 67.74 %, which indicates divergence in the evolution process (Figs. 38 and 39). They encode the synthesis of peripheral membrane proteins Tor1p and Tor2p – members of the family of phosphoinositide (PI) 3-kinase related kinases (PIKK) that act as serine/threonine kinases in response to the presence of nutrients or growth factors.

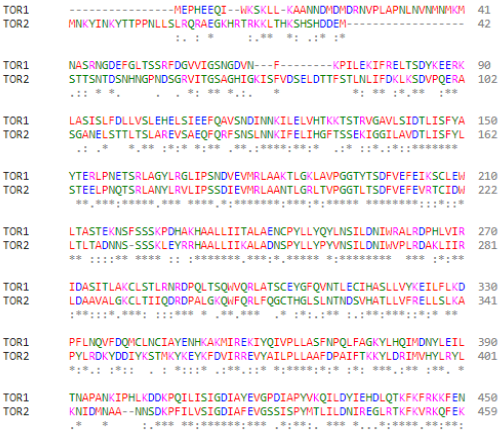

Figure 38: Sequence comparisons performed using ClustalW

Percent Identity Matrix - created by Clustal2.1

1: TOR1 100.00 67.74

2: TOR2 67.74 100.00

Figure 39: Percent identity matrix generated by ClustalW

In silico data on the intracellular distribution of Tor1p and Tor2p proteins in *S. cerevisiae* show that, in agreement with literature, both proteins are peripheral membrane proteins, and analysis showed that their C- and N-termini are located on the inside of the membrane (Table 1).

Table: 2: Investigation of the intracellular localization of the proteins involved in the TOR protein kinase

Protein	Gene	Directional sequence	% Identity
Tor1p	***TOR1 S. cerevisiae***	**Peroxisomes Nucleus Vacuole**	**42.2%**
Tor2p	***TOR2 S. cerevisiae***	**Peroxisomes Nucleus Vacuole Mitochondria**	
Protein kinase mTOR	***mTOR Homo sapiens***	**Nucleus Peroxisomes Detention in ER**	

ClustalW analysis of the primary structure of the two yeast proteins Tor1p and Tor2p with the human serine/threonine protein kinase - mTOR (protein ID NP_004949.1) showed 42.21% homology between them (Fig. 40).

```
Percent Identity  Matrix - created by Clustal2.1


   1: NP_004949.1  100.00   42.21   44.06
   2: TOR1          42.21  100.00   67.74
   3: TOR2          44.06   67.74  100.00
```

Figure 40: Percent identity matrix generated by ClustalW

3.2. Protein kinase A (PKA)

PKA is a component of a signaling pathway that controls a variety of cellular processes, including metabolism, cell cycle, stress response, stationary phase, and sporulation. PKA acts primarily as an inhibitor of quiescence (van Aelst et al., 1991; Gray et al., 2004; Virgilio., 2011). *In silico* analysis of the human genome revealed the presence of three genes, *PRKACA*, *PRKACB* and *PRKACG*, which encode the catalytic subunits of PKA. The *PRKAR1B* gene encodes a protein (381 a.a.) that is a regulatory subunit of PKA.

Figure 41: Comparison of the *S. cerevisiae* and human protein kinase A protein sequences

Four different regulatory and three catalytic subunits have been identified in human. The percentage of identity between the genes encoding the regulatory subunits of PKA in yeast and human is significant but relatively low (33.52 %) (Fig. 41 and Fig. 42).

```
Percent Identity  Matrix - created by Clustal2.1


  1: NP_001158230.1  100.00   33.52
  2: BCY1             33.52  100.00
```

Figure 42: Percent identity matrix generated by ClustalW

Analysis of the intracellular localization of human protein kinase A revealed the presence of pat4 and pat7 nuclear targeting signal sequences. Like the yeast protein (Bcy1p), regions rich in basic amino acid residues were found in this protein, suggesting the localization of PKA in the nucleus (Table 3).

Table: 3: Investigation of the intracellular localization of proteins involved in the protein kinase A (PKA) metabolic pathway

Protein	Gene	Directional sequence	% Identity
Cyclic AMP (cAMP)-dependent protein kinase (PKA) Trk1r, Trk2r, Trk3r Regulatory subunit	***TPK1, TPK2 и TPK3*** ***S. cerevisiae*** ***BCY1***	**Nucleus**	**33.52%**
Cyclic AMP (cAMP) - dependent protein kinase (PKA) 4 regulatory subunits	***PRKACA, PRKACB , PRKACG*** ***Homo sapiens*** ***PRKAR1B***	**Nucleus**	

3.3. Protein kinase C

Protein kinase C (PKC) is a family of serine and threonine protein kinases (Lew., 2000). The primary role of Pkc1p in the transition of a dividing cell to G_0 is related to the characteristic changes that occur at the cell surface (Krause and Gray., 2002). In contrast to *S. serevisiae*, in humans, *in silico* analysis showed the presence of five genes encoding proteins of the protein kinase C family – *PRKCA, PRKCB, PRKCD, PRKCE, PRKCZ*, located on different chromosomes. After performing a multiple sequence comparison of the corresponding five proteins with yeast protein kinase C by ClustalW, a similarity of 34.5% was found between them (Figs. 43 and 44).

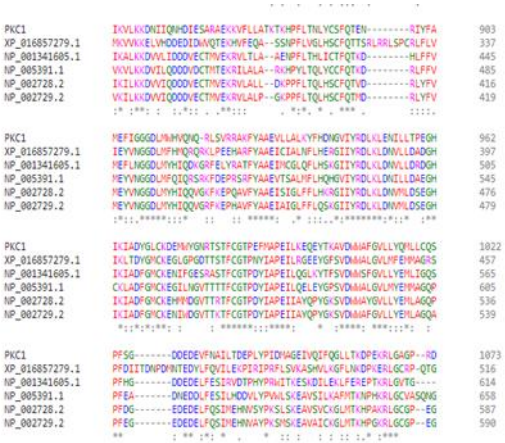

Figure 44: Comparative analysis of protein kinase C sequences in *S. cerevisiae* and human

1: PKC1	100.00	32.65	33.93	33.43	36.52	36.20
2: XP_016857279.1	32.65	100.00	36.30	43.33	44.19	44.33
3: NP_001341605.1	33.93	36.30	100.00	43.66	49.36	48.99
4: NP_005391.1	33.43	43.33	43.66	100.00	52.73	53.23
5: NP_002728.2	36.52	44.19	49.36	52.73	100.00	80.15
6: NP_002729.2	36.20	44.33	48.99	53.23	80.15	100.00

Figure 43: Percent identity matrix between human and yeast protein kinase C generated by ClustalW

Appling bioinformatics study of the genome of *S. cerevisiae*, revealed the presence of one *PKC1* gene encoding the synthesis of serine/threonine protein kinase C (1151 aa) (Table 4). Analysis of the amino acid sequence of Pkc1p in *S. cerevisiae* showed clear signals for nuclear localization of the protein (73.9% probability). A second type peroxisomal signal was also found at the N-terminus - RIQYMLQQL, at position 115, determining the probability of protein localisation in peroxisomes.

Table: 4: Investigation of the intracellular localization of proteins involved in the protein kinase C (PKC) metabolic pathway

Protein	Gene	Directional sequence	% Identity
Protein kinase C	*PKC1* *S. cerevisiae*	Nucleus Peroxisomes	34.5 %
Protein kinase C – α	*PRKCA* *Homo sapiens*	Nucleus	
Protein kinase C – β	*PRKCB* *Homo sapiens*	Nucleus ER	
Protein kinase C – δ	*PRKCD* *Homo sapiens*	ER	
Protein kinase C – ε	*PRKCE* *Homo sapiens*	Mitochondria Nucleus Peroxisomes	
Protein kinase C – ζ	*PRKCZ* *Homo sapiens*	Mitochondria ER	

3.4. Snf1p protein kinase

The *SNF1* gene encodes the yeast homologue of the human AMP-activated protein kinase (AMPK). AMPC in higher eukaryotes is activated through direct allosteric

changes by various stressors that alter the ATP/AMP ratio in the environment (Hardie et al., 1998). *In silico* analysis of the *S. cerevisiae* genome revealed the presence of one gene encoding the Snf1p protein kinase, which showed 46.93% amino acid sequence homology with the human 5'AMP-activated protein kinase (AMPK) (NP_006243.2) (Fig. 45 and 46). The significance and the relatively high similarity percentage indicates that the gene encoding the Snf1p protein kinase is the human AMPC ortholog. AMPC exists as a heterotrimeric protein complex that is made up of α, β, and γ subunits. The catalytic α-subunits are encoded by the genes *PRKAA1*, *PRKAA2*. The β and γ subunits are encoded by the *PRKAB1*, *PRKAB2* and *PRKAG1*, *PRKAG2*, *PRKAG3* genes, respectively (https://www.ncbi.nlm.nih.gov/gene/?term=human).

Figure 45: Comparison of *S. cerevisiae* Snf1p protein kinase sequences with human AMPK.

```
1: SNF1         100.00   46.93
2: AAB32732.1    46.93  100.00
```

Figure 46: Identity matrix between the investigated Snf1p and AMPK

The PSORT II study showed that in the translated DNA sequence of the human AMPK gene, no characteristic signals for localization to a particular cellular structure were present (Table 5). With the greatest probability, this enzyme is localized in the cytoplasm of the cell (65.2%).

Table: 5: Investigation of the intracellular localization of proteins involved in the Snf1p protein kinase pathway

Protein	Gene	Directional sequence	% Identity
Snf1p – protein kinase	***SNF1 S. cerevisiae***	**Nucleus Vacuole Cytoplasm ER**	**46.93%**
AMP-activated protein kinase - catalytic subunit 2 (AMPK)	***PRKAA2 Homo sapiens***	**Cytoplasm Nucleus**	

Based on the obtained scientific data and their analysis, "Functional profiles" were developed for the *S. cerevisiae* cellular response to stress in relation to the specific phase of the cell cycle - proliferative, G_0 and stationary non-proliferative phase (Fig. 47).

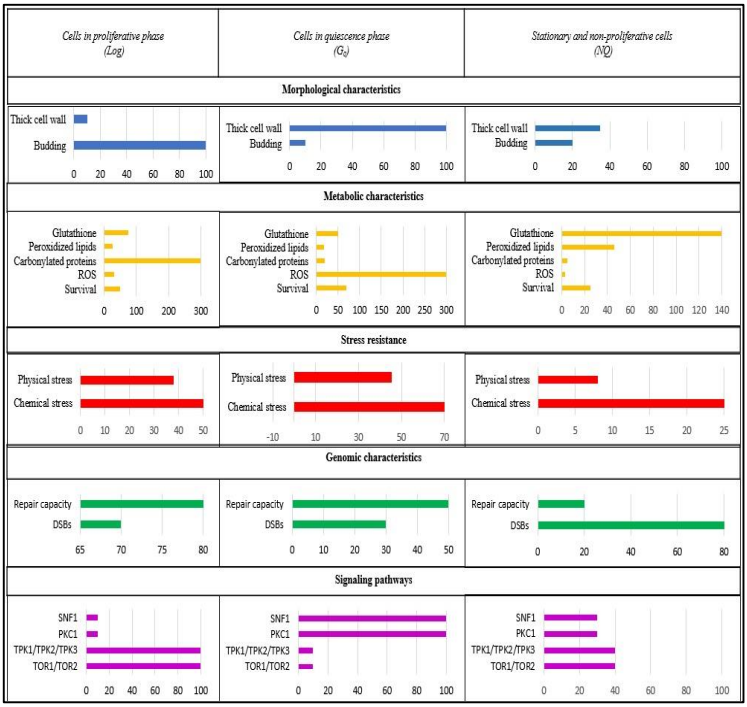

Figure 47: Functional profiles of stress response in Log, Q and NQ cell populations of *S. cerevisiae* BY4741. The presented data represent the change in % of the investigated indicators compared to untreated control cultures.

CONCLUSIONS

1. It was found that the entry of the cell into the G_0 cell cycle is provoked under conditions of limitation of one or several of the main biogenic elements in the medium and a high percentage of quiescent cells is reached when cells are grown for 168 hours on YPD medium.

2. The experimentally determined LD_{50} toxic doses for the chemical agents studied are 100 μM/ml for menadione, 5 mM/ml for hydrogen peroxide, 1.1 mg/ml for ibuprofen and 50 μg/ml for zeocin.

3. All tested toxic compounds were shown to suppress the growth of the NQ yeast cell population the most.

4. It was found that zeocin exerted the most potent toxic effect on the studied yeast populations, inhibiting 100% growth of NQ and Log cells and 95% - of yeast in G_0 state.

5. Ibuprofen slightly affected the cell viability of the studied yeast populations of *S. cerevisiae* BY4741.

6. It was observed that the effect of some of the applied toxic agents depends on the specific phase of the life cycle – H_2O_2 more strongly affects cells in the logarithmic growth phase, while menadione - cells in quiescence (G_0).

7. The stress response of G_0 cells of *Saccharomyces cerevisiae* BY4741 to various toxic agents significantly differs from that of actively proliferating and non-latent stationary cells. It depends not only on the cell cycle phase (logarithmic, G_0 and/or stationary) but also on the compound's specific mechanism of cytotoxic action.

8. The highest percentage of intracellular damage, when exposed to various medicinal (zeocin, ibuprofen) and toxic (H_2O_2, menadione) preparations, is observed in actively

dividing cells - up to 50% increase in the level of oxidized lipids and over 200% - in carbonylated proteins.

9. Quiescent G_0 cells of *S. cerevisiae* BY4741 are the most resistant to chemical effects and show the lowest levels of intracellular damage (< 25 %).

10. A lower DNA sensitivity of G_0 cells was found compared to that of cells in the logarithmic phase of growth.

11. The obtained experimental data do not show a dose-dependent increase in the amount of induced DSB depending on the concentration of zeocin.

12. The highest repair capacity was found in logarithmically growing cells (60 min recovery time).

13. Yeast cells in quiescence are significantly more resistant to physical stress and show higher survival rates at low and high temperatures, UV radiation and hyperosmotic conditions.

14. Genes and proteins that play key roles in entering and exiting the G_0 cell cycle in yeast have corresponding homologs in humans.

15. The yeast regulatory proteins TORp, PKAp, PKCp and Snf1p can be targeted to different intracellular compartments, which provides greater metabolic plasticity to yeast cells.

CONTRIBUTIONS

SCIENTIFIC CONTRIBUTIONS

1. The adaptive response of *S. serevisiae* model cultures, in different phases of the cell cycle, to the impact of different physical and toxic chemical factors was studied and key differences between them were demonstrated.

2. The genotoxic and DNA damaging potential of the widely used toxic medicinal preparations menadione and zeocin was determined depending on the applied concentration and the physiological conditions of the cell population.

3. Valuable information was obtained that G_0 cells of *S. cerevisiae*, like cancer cells, are characterized by highly increased intracellular levels of ROS.

4. Through the application of modern bioinformatics approaches, it has been shown that the genes and proteins that play a key role in the entry and exit of the G_0 cell cycle in yeast have corresponding homologues in humans, which in the process of evolution have undergone a different degree of divergence (homology between 32.65% - 46.93%).

5. For the first time, model comparative "Functional profiles of stress response" were developed in *S. cerevisiae* BY4741 cells isolated from different phases of the cell cycle - proliferative, G_0 and non-latent stationary.

APPLIED CONTRIBUTIONS

1. A successful methodological approach was developed for obtaining and isolating model cell cultures in a quiescent state (G_0) from *S. cerevisiae* BY4741 strain - a test system for studying cellular molecular mechanisms for toxic stress response and functional profiling of genetic requirements for chemical tolerance.

2. It was shown for the first time that G_0 yeast cells have a significantly higher resistance to the action of zeocin, which represents important information from a medico-biological point of view - metabolically inactive eukaryotic cells are much less susceptible to the action of antitumor antibiotics.

3. The reduced metabolic activity of yeast in the G_0 state determines their greater stability and resistance to the effects of various toxic agents. This makes them a suitable model system for studying the basic toxicological mechanisms of harmful substances in animals and humans.

4. G_0 cells are more resistant to chemical and physical stress than proliferating and stationary non-latent cells, making them suitable for potential industrial applications.

PUBLICATIONS

1. **Marinovska P.,** Todorova T., Tomova A., Pisareva E., Boyadzhiev K., Dimitrov M., Parvanova P., Dimitrova M., Chankova S., Petrova V., (2022). *Saccharomyces cerevisiae* yeast cells as a test system for assessing Zeocin toxicity. In: Chankova S., Peneva V., Metcheva R., Beltcheva M., Vassilev K., Radeva G., Danova K., (Eds) Current trends of ecology. BioRisk 17: 105–116. **Q2**, SJR 0.3, IF 0.167.

2. **Marinovska PG.,** Todorova TI., Boyadzhiev KP., Pisareva EI., Tomova AA., Parvanova PN., Dimitrova M., Chankova SG., Petrova VY., (2022). Cellular susceptibility and oxidative stress response to menadione of logarithmic, quiescent, and nonquiescent *Saccharomyces cerevisiae* cell populations. In: Chankova S., Peneva V., Metcheva R., Beltcheva M., Vassilev K., Radeva G., Danova K., (Eds) Current trends of ecology. BioRisk 17: 127–138. https://doi.org/10.3897/ biorisk.17.77320. **Q2**, SJR 0.3, IF 0.167.

PARTICIPATION IN SCIENTIFIC FORUMS

1. Posters:

- **Polya Marinovska**, Ventsislava Petrova. Zeocin induces mitochondrial DNA breaks and ROS accumulation in *S. cerevisiae* Log, Q and NQ cell populations. SCIENTIFIC CONFERENCE „KLIMENT'S DAYS" (04/11 2022).

- **Polya Marinovska**, Emilia Pisareva, Anna Tomova, Ventsislava Petrova. Influence of physical factors on logarithmic, quiescent and non-quiescent S. cerevisiae cells. „INTERNATIONAL SEMINAR OF ECOLOGY'' (29/09-30/09 2022).

- **Polya Marinovska**, Emiliya Pisareva, Anna Tomova, Ventsislava Petrova. *IN SILICO* analysis of genes from signalling pathways regulating quiescence in yeasts. „SCIENTIFIC CONFERENCE „KLIMENT'S DAYS" (05/11 2021).

- **Polya Marinovska**, Teodora Todorova, Anna Tomova, Emiliya Pisareva, Krassimir Boyadzhiev, Martin Dimitrov, Petya Parvanova, Maria Dimitrova, Stephka Chankova, Ventsislava Petrova. Quiescent yeast cells for assessing Zeocin toxicity. „13th INTERNATIONAL SEMINAR OF ECOLOGY'' (29/09-30/09 2021).

- Anna Tomova, **Polya Marinovska**, Ventsislava Petrova. Response of proliferating and quiscent Saccharomyces cerevisiae cells to menadion and H_2O_2 toxicity.„14th congress of microbiologists in Bulgaria with international participation" (10/10-13/10 2018).

2. Oral presentation:

- **Polya Marinovska**, Teodora Todorova, Krassimir Boyadzhiev, Emiliya Pisareva, Anna Tomova, Petya Parvanova, Maria Dimitrova, Ventsislava Petrova and Stephka Chankova. Cellular susceptibility and oxidative stress response to Menadione of logarithmic, quiescent and nonquiescent Saccharomyces cerevisiae cell populations. „13th INTERNATIONAL SEMINAR OF ECOLOGY'' (29/09-30/09 2021).

PROJECTS

National projects:

1. "Quiescence state in *Saccharomyces cerevisiae* - a model for studying toxicological and stress response", number: ДН11/10/15.12.2017, MES.

2. "Development of new biological nanoparticles from the mucus of the garden snail *Cornu aspersum* as antimicrobial agents", number: КР-06 ПН-61/8 of 2022. BG-175467353-2022-04-0065, MES.

International projects:

1. "Green Technology Foresight about challenges from biotechnology and ICT - Digit-BioTech" number: 2019-1-BG01-KA203-062371, ERASMUS + program;

2. "Smart Decision Tools for Reducing Hazards to Our Environment and Water Resources by Rehabilitating Open Dump" number: 2020-1-TR01-KA226-VET-098150;