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Oxidal® Ameliorates the Ty1 Retrotransposition Induced by Methyl Methanesulfonate in *Saccharomyces cerevisiae*

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Authors' contributions

This work was carried out in collaboration among all authors. Author TT designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors MD and GD managed the analyses of the study. Author II managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim: The aim of the study was to evaluate the potential of Oxidal® to decrease the Ty1 retrotransposition rate in a model system *Saccharomyces cerevisiae*.
Study Design: *Saccharomyces cerevisiae* cell suspensions were pre-treated with different concentrations Oxidal® and subsequently treated with 16mM methyl methanesulfonate. (MMS)
Methodology: The potential of various concentrations Oxidal® was evaluated based on "spot" test and Ty1 retro-transposition test.
Results: Data revealed that only 5% Oxidal® possesses some cytotoxic properties. Lack of Ty1 retro-transposition was observed after single treatment with 1, 2.5 and 5% Oxidal® concentrations.

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On the other hand, all the tested concentrations showed promising results against the standard carcinogen methyl methane sulfonate. The most pronounced anti-carcinogenic and cytoprotective effects were observed after pre-treatment with 2.5% Oxidal®, which could be attributed to the antioxidant properties of the combination of ingredients; methylene blue, salicylic acid and caffeine. Further studies could reveal the exact mechanism of action of the supplement and the role of the antioxidant potential.

Conclusion: New data is provided concerning the potential of Oxidal® at low concentrations to protect *Saccharomyces cerevisiae* cells from MMS-induced Ty1 retro-transposition. The cytoprotective properties of the supplement were also obtained. These results could be considered as a basis for further studies revealing the exact mechanisms of cell protection of the Oxidal®. Additionally, our data could also serve as an important step of the in-depth research of a potential antiviral activity.

Keywords: Oxidal®; methylene blue; cytotoxic/anticytotoxic; carcinogenic/anticarcinogenic effects; Saccharomyces cerevisiae.

ABBREVIATIONS

SARS-CoV-2: Severe Acute Respiratory Syndrome Coronavirus 2
COVID-19 : Coronavirus Disease-19
MMS : Methyl Methanesulfonate
ROS : Reactive Oxygen Species
Ty : Transposon of Yeast
YEPD : Yeast Extract, Peptone, Dextrose

1. INTRODUCTION

Environmental pollution is considered as one of the major causes for different types of human cancer – 70-90% [1]. Cancer is characterized with deregulation of the cellular energetics [2]. It is believed that cancer development could be described by three stages: Initiation, promotion and progression. Generation of reactive oxygen species (ROS) is proposed to act in all these stages of carcinogenesis [3,4]. Oxidative stress is reported as a crucial mechanism not only for cancer development but also in various pathologies such as cardiovascular diseases, diabetes, rheumatoid arthritis, Alzheimer or Parkinson disease [5]. As cancer is the second leading cause of mortality worldwide [6], many studies have been focused on the evaluation of substances, which may ameliorate the carcinogenic potential of different genotoxins. Thus, it is of a great importance to study compounds and products possessing antioxidant properties.

Oxidal®is a dietary supplement containing three major components – methylene blue, caffeine and salicylic acid. This product has already been reported to possess pronounced inhibitory activity on pathogenic bacterial strains [7,8]. Another study revealed the potential of Oxidal®, catholyte water and nano colloidal silver for prevention again SARS-CoV-2 and related disease COVID-19 [9]. It is believed that the combination of Oxidal® ingredients may increase oxygenation and improve metabolic processes.

Methylene blue is a widely applied dye, which is reported to compete with molecular oxygen for the transfer of electrons [10,11]. This is typical for the enzyme xanthine oxidase, where the dye diverts the electrons' flow from the metal sulphur center of the enzyme, a place where molecular oxygen is normally converted into superoxide radicals. By this mechanism of competition, the generation of the cytotoxic superoxide radicals is attenuated [10-12]. Aksu et al. [12] reported that methylene blue could attenuate hepatic damage by reduction of the oxidative stress.

Caffeine at very low concentrations has been reported to protect against sporadic Alzheimer's disease-like pathology by reduction of the cholesterol-induced increase in β-amyloid, phosphorylated tau and oxidative stress levels [13].

Salicylic acid is shown to exhibit anti-proliferative and antitumor activity *in vitro* and *in vivo* [14-16]. Additionally, salicylic acid possesses anticancer effect epithelial tissues most probably by inhibition of c-Myc [17].

One strong ROS inducer is methyl methane sulfonate (MMS). MMS is an alkylating agent causing mainly base mispairing and replication blocks [18]. Data exists that MMS possesses strong pro-oxidative effect by inducing high levels

of reactive oxygen species (ROS) and carcinogenic effect [19].

Our hypothesis is that the commercial product Oxidal® could reduce the carcinogenic potential of methyl methane sulfonate.

The aim of the study was to evaluate the potential of Oxidal® to decrease the Ty1 retro-transposition rate in a model system *Saccharomyces cerevisiae*.

Saccharomyces cerevisiae was chosen as a model system due to the genome similarities with this in mammals. The full genome sequence revealed that around 31% of the proteins coded by yeast genes have human homologues. Additionally, around 50% of the genes responsible for various hereditary diseases have yeast orthologues [20,21].

2. MATERIALS AND METHODS

2.1 Strains

Four strains were used for a preliminary evaluation of the potential cytotoxic effect. The strains and their characteristics are described in Table 1.

2.2 Compounds

The dietary supplement Oxidal® (IdeaLabs, LLC, Washington, USA; author GeorgiDinkov) was studied in the present work. The compound contains methylene blue ([7-(dimethylamino) phenothiazine-3-ylidene]-dimethylazanium chloride; $C_{16}H_{18}ClN_3S$) – at concentration 1% (by mass), salicylic acid (2-hydroxibenzoic acid; $C_7H_6O_3$) - 1% (by mass) and caffeine (1,3,7-trimethylpurine-2,6-dione; $C_8H_{10}N_4O_2$) - 1% (by mass).

2.3 Methods

Cell suspensions were treated with Oxidal®, dissolved in deionized water at various range of concentrations, mentioned in the methodological descriptions, for 60 min at optimal for cell growth conditions (30°C, aeration). Methyl methane sulfonate (Sigma Aldrich), used as a positive control and carcinogenic agent was applied at concentration 16 mM for 30 min.

2.3.1 "Spot" test

The concentrations range was evaluated based on spot test. The cell suspensions of four strains were treated with Oxidal® in the following concentrations: 1, 2, 3, 4 and 5% as described above. After the treatment, cells were washed, diluted to a concentration $1x10^5$ cells/ml and spotted on a solid YEPD (yeast extract, peptone, dextrose) medium. The intensity of the spots was used as preliminary information concerning the potential cytotoxic effect of the compound.

2.3.2 Ty1 transposition assay

Ty1 transposition assay was performed as described by [19,23,26]. Briefly, after single treatment with 1, 2.5 and 5% Oxidal®, or subsequent treatment with MMS, cell suspensions were washed with YEPD medium, and cultivated at t=20°C (optimal conditions for Ty1 transposition) for 24 hours. Appropriate dilutions of cells were then placed on YEPD to evaluate cell survival and on selective medium lacking histidine – for His^+transposants. Yeast media were prepared as described by [27].

Table 1. Different strains and their characteristics

D7ts1	diploid strain with genotype *MATa/α ade2-119/ade2-40 trp5-27/trp5-12 ilv1-92/ilv1-92 ts1/ts1*	[22]
551 rho+	genotype *MATα ura3-167 his3Δ200:TymHIS3AI sec53 rho*$^+$ (National Bank for Industrial Microorganisms and Cell Cultures, Sofia, Bulgaria, Cat № 8719)	[19,23,24]
551 rho-	mutant strain 551 with disrupted or completely missing mitochondrial DNA, developed after treatment with ethidium bromide	This study
551 yap1Δ	has a disrupted YAP1 gene and was constructed by transformation of 551 cells with the yap1:: hisG-URA3-hisG cassette; the strain is characterized with a deletion of the yap1 gene, which makes this strain incapable of synthesizing yap1 protein	[25]

Mean transposition rates were determined and results presented as "fold increase of Ty1 transposition rate" related to control sample, taken as 1.00. A fold increase in treated cultures equal to or higher than 2.00 is considered as positive response of the Ty1 assay.

2.4 Statistical Analysis

All results were presented as mean±SEM from at least 3 independent experiments. The statistical analysis was performed by Graphpad Prism 5 software and includes an application of One-way ANOVA with Bonferroni's *post hoc* test. P<0.05 was accepted as thelowest level of statistical significance.

3. RESULTS AND DISCUSSION

3.1 Potential Cytotoxicity and Carcinogenicity

3.1.1 "Spot" test

Concerning the potential cytotoxicity, spot test was performed on four strains. Data obtained revealed lack of cytotoxicity despite the concentration applied (Fig. 1). The spot intensity was similar in all the treatments. None of the strains was affected by the supplement.

Such result suggests that Oxidal® does not possess cytotoxic effect on *Saccharomyces cerevisiae*.

It is known that when entering mitochondria, methylene blue (MB) acts like an additional electron source. More importantly, it prevents electron leakage for oxidants formation, the toxic side products in mitochondria (19). Overall, these data support that MB promotes mitochondrial function and reduces ROS production [28,29].

In present case, lack of cytotoxicity in the strains 551 rho- and 551 yap1Δ, may provide evidence for the activity of Oxidal even when the mitochondrial function is impaired.

3.1.2 Survival

Further studies were performed on strain 551rho+. Treatment with the lowest tested concentration – 1% Oxidal® did not result in reduction of the cell survival. The percentage of survived cells was comparable with the control – untreated cells. Statistically significant decrease was observed after the treatment with the highest tested concentration Oxidal® (5%) - 45% (*P<0.0001*). Statistically significant but biologically insignificant difference between the control - untreated cells and those treated with 2.5% Oxidal® (*P<0.01*) was also calculated.

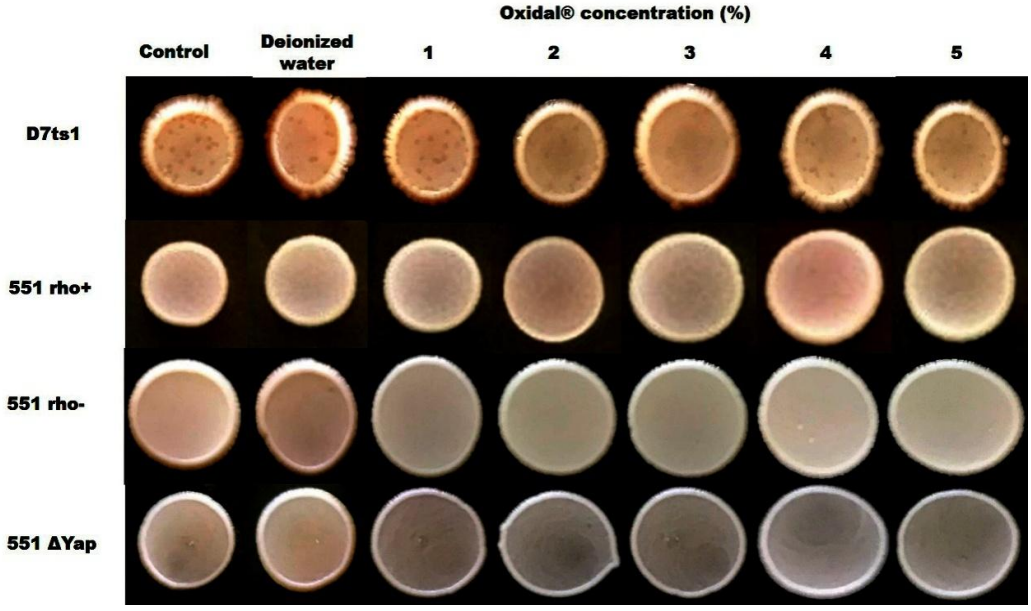

Fig. 1. Spot test for preliminary evaluation of the cytotoxic potential of Oxidal® in a concentrations' range 1-5%

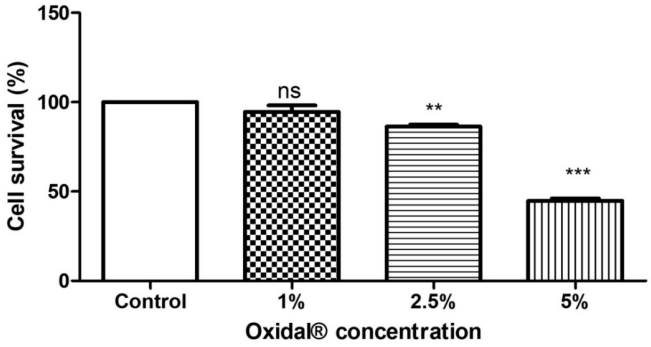

Fig. 2.Cell survival (%) after the treatment with 1, 2.5 and 5% Oxidal®. Average values ± SEM from at least 3 independent experiments. The significance in differences between positive control – single treatment with MMS and treatment with the different concentrations was calculated by ANOVA with post-hoc test- Bonferroni's Multiple Comparison Test. Where no error bars are evident, they are equal or less than the symbols

Based on these results, it could be suggested that 5% Oxidal® possesses cytotoxic effect on *Saccharomyces cerevisiae* strain 551 rho+ (Fig. 2).

These data do not correspond with the spot test. Such discrepancy could be explained with the specificity of the tests. Spot test is performed for preliminary evaluation of the potential cytotoxicity while cell survival is a more precise method.

3.1.3 Ty1 retro-transposition

The results obtained in the present work showed that none of the concentrations tested increase the Ty1 retrotransposition rate (Fig. 3). Thus, suggesting lack of carcinogenic potential.

It is well known that carcinogens induce the transposition of Ty1 retro-transposon [26]. The specific activation of Ty1 is due to increased synthesis of ROS [19,23,24,30-33]. Based on the results reported here, it could be speculated that Oxidal is not able to induce ROS. Interestingly, the transposition rate after single treatment with 2.5 and 5% resulted in Ty1 retro-transposition rate significantly lower than the spontaneous one. This may indicate full block of the event.

3.2 Potential Cytoprotective Activity and Anti-carcinogenic Effect

3.2.1 Survival

The results obtained revealed that treatment with the standard carcinogen MMS lead to 58% cell survival.

Interesting results were obtained when cells were pre-treated with Oxidal® and then treated with MMS. Data clearly reveal that pre-treatment with all the tested concentrations of Oxidal® and subsequent treatment with MMS resulted in around 82% cell survival. The cell survival was comparable among the three samples (fig. 4). These results suggest that Oxidal® at the tested concentrations protects the cells from the cytotoxic activity of MMS.

3.2.2 Transposition rate

The transposition rate measured after single treatment with 16mM MMS was 18 (Fig. 5).

Pre-treatment with different concentrations Oxidal® resulted in a dose-dependent reduction of the MMS-induced transposition. Pre-treatment with 2.5% Oxidal® showed around 5-fold decrease in the Ty1 retro-transposition rate while the pre-treatment with 5% Oxidal® resulted in around 6-fold reduction.

Taken into account the survival data, although, 5% Oxidal® possessed well-expressed anti-carcinogenic activity, single treatment of *Saccharomyces cerevisiae* revealed cytotoxic effect.

Based on these results, it could be suggested that Oxidal® at low concentrations possesses anti-carcinogenic properties. The exact mechanism of protection is not fully known. It could be related to a modulation of the oxidative stress by the combination of the three ingredients – methylene blue, salicylic acid and caffeine. It is well known that not all substances with reduction properties could be classified as antioxidants.

Antioxidants could be compounds, which are able to penetrate into the cells and protect the cellular compartments from the oxidative damage. As ROS are characterized with very short half-life and high reactivity, it always should be taken into account the fate of the potential antioxidants in live cells, instead of only in cell lysates or extracts [19]. The present study reveals that Oxidal® protect the cells, which means that it is able to penetrate into the cells.

In the present study, the dietary supplement Oxidal was found to protect the cells from the carcinogenic pro-oxidant methyl methane sulfonate through amelioration of the Ty1 retro-transposition events. This result could also serve as an important step in indepth research of the potential antiviral activity because it is well known that Ty1 retro-transposons are similar to retroviruses such as equine anemia virus, human immunodeficiency virus type 1 (HIV-1) [discussed in 26].

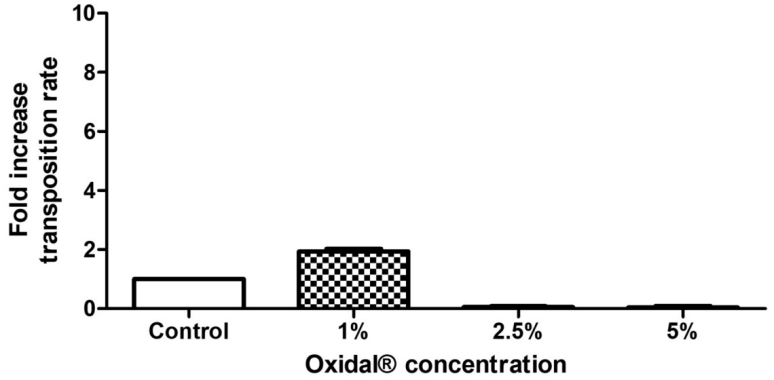

Fig. 3. Potential carcinogenic effect of different concentrations Oxidal® - 1, 2.5 and 5%, on *S. cerevisiae* 551rho$^+$ measured as Fold increase transposition rate. Average values ± SEM from at least 3 independent experiments. The significance in differences between negative control - untreated cells and treatment with the different concentrations was calculated by ANOVA with post-hoc test- Bonferroni's Multiple Comparison Test. Where no error bars are evident, they are equal or less than the symbols

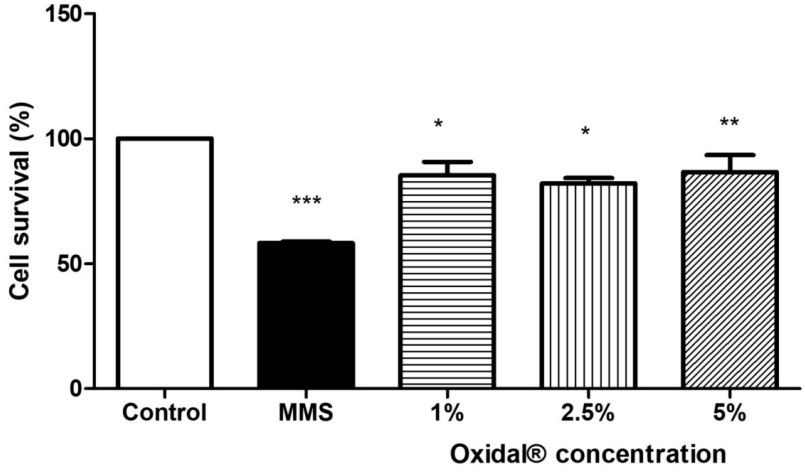

Fig. 4. Cell survival (%) after pre-treatment with 1, 2.5 and 5% Oxidal® and subsequent treatment with the standard carcinogen methyl methane sulfonate. Average values ± SEM from at least 3 independent experiments. The significance in differences between positive control – single treatment with MMS and treatment with the different concentrations was calculated by ANOVA with post-hoc test- Bonferroni's Multiple Comparison Test. Where no error bars are evident, they are equal or less than the symbols

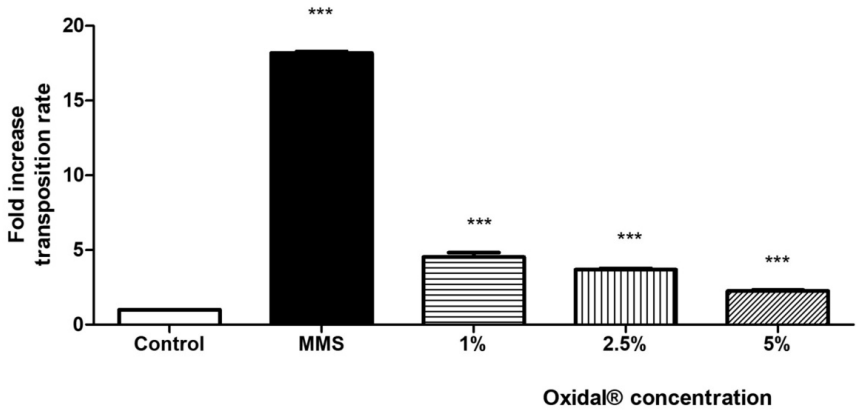

Fig. 5. Potential anti-carcinogenic effect of different concentrations Oxidal® - 1, 2.5 and 5% against the standard carcinogen methyl methane sulfonate, on*S. cerevisiae* 551rho$^+$ measured as Fold increase transposition rate. Average values ± SEM from at least 3 independent experiments. The significance in differences between positive control – single treatment with MMS and treatment with the different concentrations was calculated by ANOVA with post-hoc test- Bonferroni's Multiple Comparison Test. Where no error bars are evident, they are equal or less than the symbols

4. CONCLUSION

New data is provided concerning the potential of Oxidal® at low concentrations to protect *Saccharomyces cerevisiae* cells from MMS-induced Ty1 retro-transposition. The cytoprotective properties of the supplement were also obtained. These results could be considered as a basis for further studies revealing the exact mechanisms of cell protection of the Oxidal®.

DISCLAIMAR

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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