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LINALOOL-INDUCED OXIDATIVE STRESS PROCESSES IN THE HUMAN PATHOGEN *CANDIDA ALBICANS*

GÁBOR MÁTÉ,[1]* DOMINIKA KOVÁCS,[1] ZOLTÁN GAZDAG,[1]
MIKLÓS PESTI[1] and ÁRPÁD SZÁNTÓ[2]

[1]Department of General and Environmental Microbiology, Faculty of Sciences, University of Pécs, Pécs, Hungary
[2]Department of Urology, Medical School, University of Pécs, Pécs, Hungary

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The present study investigated the linalool (Lol)-induced effects in acute toxicity tests in the human pathogen *Candida albicans* (*C. albicans*). Lol treatments induced reduced germ tube formation of the pathogen, which plays a crucial role in the virulence. In comparison with the untreated control, the exposure of 10^7 cells ml^{-1} to 0.7 mM or 1.4 mM Lol for one hour induced 20% and 30% decrements, respectively, in the colony-forming ability. At the same time, these treatments caused dose-dependent decrease in the levels of superoxide anion radical and total reactive oxygen species, while there was 1.5 and 1.8-fold increases in the concentrations of peroxides and lipid peroxides, respectively, indicating oxidative stress induction in the presence of Lol. Lol treatments resulted in different adaptive modifications of the antioxidant system. In 0.7 mM-treated cells, decreased specific activities of superoxide dismutase and catalase were detected, while exposure to 1.4 mM Lol resulted in the up-regulation of catalase, glutathione reductase and glutathione peroxidases.

Keywords: Antioxidant enzyme – *Candida albicans* – linalool – reactive oxygen species – oxidative stress

INTRODUCTION

In the last few decades the number of candidiasis with fatal outcome has been increasing continuously as a consequence of an increment in antifungal resistance and the limited number of effective drugs available. At least 20 *Candida* species can cause infections in humans. A remarkable amount of invasive infections is caused by *C. albicans*, a diploid yeast exhibits dimorphism (e.g. pseudohyphae or hyphae formation via germ tubes from blastospore/vegetative cells) and induces several types of candidiasis [28, 40].

Essential plant oils and their main components, used for centuries in folk-medicine, may serve as an alternative solution to cure *Candida* infections. Linalool (Lol, $C_{10}H_{18}O$, IUPAC: 3,7-dimethylocta-1,6-dien-3-ol), a monoterpene alcohol, has two optically active (D and L) forms; it is insoluble in glycerol, but soluble in alcohol, ether and water (up to 10.3 mM). It is one of the major components of more than 200

*Corresponding author; e-mail address: magoaat@gamma.ttk.pte.hu

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essential oils obtained from various aromatic plants, and is applied worldwide as a fragrance ingredient in various types of cosmetics, household cleaners and detergents. Lol has a dose-dependent cytotoxicity and antimicrobial activity at relatively lower concentration against both Gram-negative and Gram-positive bacteria, yeasts and filamentous fungi [3, 4, 14, 26, 46].

The toxicity and mode of action of Lol has been described in a number of papers in which both acute and chronic tests were applied, but the findings are controversial. In chronic toxicity tests, Lol affected the plasma membrane composition through the inhibition of the expression of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, which catalyses the conversion of HMG-CoA to mevalonic acid, a crucial step in the biosynthesis of cholesterol/ergosterol. In this way, Lol disturbs the synthesis of cholesterol and ergosterol in HepG2 and fungal cells, leading to hypocholesterolaemia [10, 22, 24]. Exposure to Lol has also been found to induce alterations in fatty acid composition via increased levels of polyunsaturated and unsaturated fatty acids. Unfortunately, the background processes were not investigated [8]. Gazdag et al. [16] recently demonstrated that the lack of ergosterol in *Saccharomyces cerevisiae* (*S. cerevisiae)* induced an adaptive process at the plasma membrane level by increasing the proportion of unsaturated fatty acids. As a result of the presumed plasma membrane disorganization, Lol exhibited synergistic activity with fluconazole against a fluconazole-resistant *C. albicans* strain; in a long-term (48-h) acute test, broth microdilution assays demonstrated that the minimal inhibitory concentration (MIC) of fluconazole decreased by 64-fold [46].

Lol-induced cytotoxicity may also be a consequence of the accumulation of reactive oxygen species (ROS), including superoxide radical ($O_2^{\bullet-}$), peroxides (H_2O_2, lipid peroxides, etc.), hydroxyl radicals ($^{\bullet}OH$), etc. The oxidative stress-inducing ability of Lol has been characterized *in vitro* by the dysfunction of the mitochondria isolated from HepG2 cells via the inhibition of mitochondrial complexes I and II. The activities of enzymes linked to the respiratory chain were inhibited in a concentration-dependent manner resulting in a decreased ATP level which may have contributed to the loss of cell viability [43]. Dose-dependent decreases in glutathione (GSH) level and the reduction of nitroblue tetrazolium (NBT) to formazan, suggested an increase in ROS [43]. However, no data are available from direct measurements of total ROS or ROS species separately. Dose-dependent increase in the concentration of malondialdehyde (MDA, the end-product of lipid peroxidation) was observed in H1299 tumour parental cell line and its drug-resistant line after Lol treatments. In the same experiment, an increment was observed in the level of 8-oxo-2'-deoxyguanosine (an indicator of oxidative stress-induced DNA damage) [12]. These results suggested that Lol treatment induces an unbalanced redox state in the cells, independently of the type of the applied cell line, but information relating to regulation of the antioxidant system would be useful for an understanding of the biological interaction of Lol with cell components *in vivo*.

The aims of the present study were to acquire information about the Lol-induced changes in ROS, and the responses of the antioxidant system to these changes in *C. albicans* cells. To gain a more sophisticated picture of the stress response of

C. albicans to Lol at the level of cell physiology, we quantified the total ROS, the individual ROS, GSH and the specific activities of several important antioxidant enzymes under strictly controlled conditions.

MATERIALS AND METHODS

Strain and culture conditions, germ tube induction assay and determination of survival rates

A well-characterized adenine auxotroph *C. albicans* strain *33erg*$^+$ (ATCC 44829, American Type Culture Collection Maryland, USA) was selected for the experiments [33]. This strain was the same as we applied earlier to investigate the modes of action of clary sage oil, and its main components Lol and linalyl acetate [5]. Mid-exponential phase cultures were obtained on a shaker operating at a shaking frequency of 33.3 Hz in liquid minimal medium (MM) containing 1% dextrose, 0.5% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.05% $MgSO_4$ (w/v) and 50 μg ml^{-1} adenine, 2 μg ml^{-1} biotin, 4 μg ml^{-1} thiamine, pH 4.1, at 30 °C. The strain was maintained on MM supplemented with 2% agar. Lol was diluted in ethanol and vortex mixing for 10 s was applied before treatment.

For germ tube formation assays, *C. albicans* cells were collected by centrifugation (1017 g, 5 min) from 18-h early stationary cultures, washed three times with sterile distilled water and resuspended in horse serum (supplemented with 50 μg ml^{-1} adenine) containing 10^7 cells ml^{-1}. These cultures were incubated in a shaking incubator at 33.3 Hz at 37 °C. The germ tube formation of *C. albicans* cells was counted microscopically after incubation for 180 min [23].

The survival rates of cells were estimated according to Lee et al. [25]. Briefly, suspensions containing 10^7 mid-log phase cells ml^{-1} in MM were exposed to 0, 0.7, 1.4 or 5.6 mM Lol for one hour, and at 0, 30 and 60 min samples were taken and spread onto Petri dishes. After three days of incubation at 30 °C, colonies were counted.

Measurements of ROS and antioxidant enzyme activities

To estimate intracellular peroxides, $O_2^{\bullet-}$ and total ROS, the dyes dihydrorhodamine 123 (DHR 123; 10 μM), dihydroethidium (DHE; 10 μM) and 2',7'-dichlorofluorescein diacetate (DCFDA; 25 μM) were used, respectively [29, 39]. The 10^7 cells ml^{-1} were treated with 0.7 mM or 1.4 mM Lol for one hour, then collected by centrifugation and washed with fresh MM. The extents of rhodamine, ethidium and dichlorofluorescein formation were measured with a fluorescence spectrophotometer (Hitachi F-7000). The data were calculated in (mg dry biomass)$^{-1}$. The rate of lipid peroxidation was determined by using the test for thiobarbituric acid (TBA)-reactive substances (TBARS) [41].

The specific activities of CuZn superoxide dismutase (SOD_{CuZn}), Mn superoxide dismutase (SOD_{Mn}) [32], glutathione S-transferase (GST) [44], glutathione reductase (GR) [35], glutathione peroxidase (GPx) [9], glucose-6-phosphate dehydrogenase (G6PD) [11], catalase (CAT) [38] and the intracellular concentrations of GSH and GSSG [1] were determined by means of well-established colorimetric assays. The protein content of the cell-free extract was measured by a modified Lowry method [34].

In vitro *interactions between Lol and* $O_2^{\bullet-}$

The rate of reduction of NBT chloride was monitored by utilizing the xanthine–xanthine oxidase system according to the method of Oberley and Spitz [32]. Through the reaction of xanthine and xanthine oxidase, $O_2^{\bullet-}$ was generated (xanthine + O_2 + H_2O → uric acid + $O_2^{\bullet-}$ + H^+), which reacts with NBT to give a coloured formazan dye (NBT^{2+} + 2Cl^- + 4 $O_2^{\bullet-}$ + 4 H^+ → diformazan + 4 O_2 + 2 HCl) [7]. After the addition of different concentrations of Lol and the well-characterized antioxidant molecule GSH, the reduction in the intensity of formazan was monitored at 560 nm for one minute with a 30 s delay with a Hitachi U2910 spectrophotometer.

Statistical analysis

Unless otherwise indicated, the data presented here are means ± standard deviations (S.D.) calculated from at least three independent experiments. Statistical analysis was performed with a two-tailed Student *t*-test using PAST v2.17c software.

Chemicals

All of the chemicals used in this study were of analytical grade and were obtained from Sigma-Aldrich Ltd. (Budapest, Hungary), except that DHE was purchased from Fluka (Buchs, Switzerland).

RESULTS AND DISCUSSION

In comparison with the control, by exposure to 0.7 mM or 1.4 mM Lol, *C. albicans* cells achieved 33% and 70% decrease in germ tube formation, respectively (Fig. 1). The formation of germ tubes and hyphae and/or pseudohyphae via germ tubes is regarded as a virulence factor of *C. albicans* and is necessary for successful penetration from the mucosal surface into the deeper tissues and for biofilm formation [28]. The benefit of essential oil/monoterpenes/Lol/antifungal drug cotreatment may be a decreased virulence.

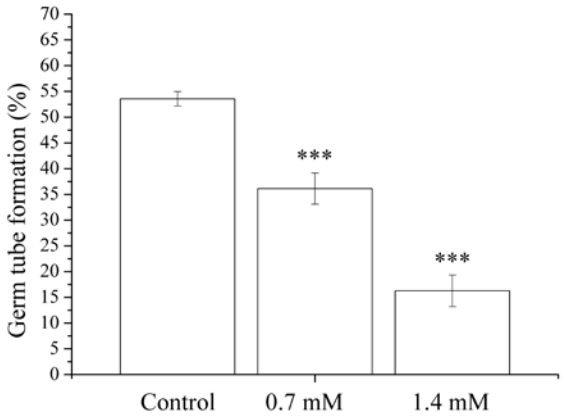

Fig. 1. Effects of treatment with various concentrations of Lol on the germ tube formation of *C. albicans* cells after cultivation for 180 min at 37 °C. ***$p<0.1\%$. p values were calculated via the Student t-test

When the survival rates of cultures were determined following subinhibitory treatments, 0.7 mM and 1.4 mM Lol caused 20% and 30% decreases, respectively, in the colony-forming ability of the cells after one hour (Fig. 2). In each of the subsequent acute investigations of the oxidative stress-inducing impact of Lol, strictly controlled conditions were applied, e.g. mid-log-phase cells (10^7 cells ml^{-1}) in MM (to eliminate the antioxidant effect of a complete medium) [15] with a 70–80% survival rate. In the evaluations of the mode of action of Lol, its plasma membrane-modifying effect was excluded, as the one-hour acute Lol treatment was unlikely enough to induce detectable alterations in either the fatty acid composition or the ergosterol content of *C. albicans*, which requires a generation time of 2.35 h (Fig. 2). After pretreatments

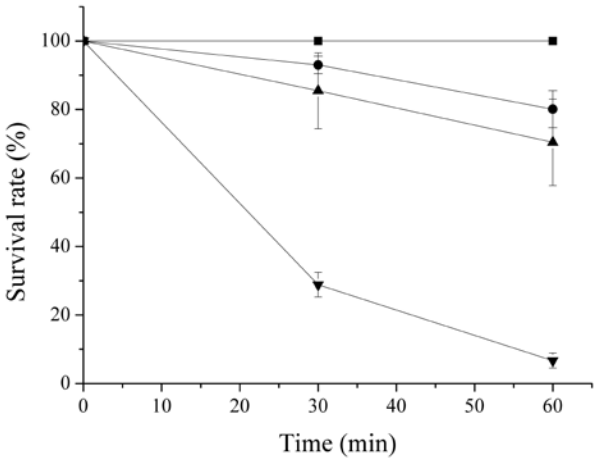

Fig. 2. Plots of survival rates of *C. albicans* cells in the presence of different concentrations of Lol (■: control, ●: 0.7 mM, ▲: 1.4 mM, ▼: 5.6 mM Lol)

with 0.7 or 1.4 mM Lol for one hour, no increase in colony-forming ability was observed, indicating the absence of adaptation processes against Lol at a cell level (data not presented) [25].

The oxidative stress-inducing ability of Lol was investigated under these conditions. An increase in the content of ROS had been suggested earlier by Usta et al. [43]. In contrast with their experiments, we applied treatment with subinhibitory concentrations of Lol (Fig. 2); the resulting 26% or 76% decrease in the level of total ROS was a consequence of a decreased $O_2^{\bullet-}$ level (Table 1). We have proved earlier that longer (24–72-h) acute tests and chronic tests yield similar results, at least in investigations of the redox state of cells [29]. At the same time, results obtained without determination of the number of living cells or with low cell viability (lower than 70%) can be misleading, as a result of apoptotic or necrotic processes which are not a direct consequence of oxidative stress: gene expression is usually measured 20–30 min and enzyme activities 60 min after the beginning of treatment [29]. To confirm the above, 5.6 mM Lol (a 91% decrease in the colony-forming ability) induced a 14.4-fold increment in the total ROS content, which might be a consequence of apoptotic/necrotic processes. Interestingly, Lol-treated *C. albicans* cells exhibited an elevated content of peroxides (Table 1). A 0.7 mM Lol did not influence the peroxide content significantly, but 1.4 mM Lol induced a 1.79-fold increase. Interestingly, an increased peroxide content was not experienced in the level of total ROS. The intense decrease in $O_2^{\bullet-}$, the predominant ROS in cells (in mitochondrial respiration, at least 1% of the oxygen is converted into $O_2^{\bullet-}$) [20], is probably compensated by the increase observed in the smaller quantity of peroxides, and hence there is no overall change in the total ROS compensation (Table 1). However, the plasma membrane-dependent effects of Lol were excluded in view of the fatty acid composition of the strain [33]: the high unsaturated fatty acid ratio permits lipid peroxidation even in a 1-hour treatment. Lol has been reported to promote a 2.5-4.0-fold increase in the amount of MDA in H1299 cells [12]. It is known from *in vitro* studies that Lol inhibits the respiratory chain. Our experiments did not detect an ability of Lol to induce mitochondrial petite mutants when a petite-positive *Saccharomyces cerevisiae* strain was used (data not presented) [13]. These results suggested that in our experimental system Lol did not affect the mitochondrial DNA or the mitochondrial function.

Table 1

Intracellular contents of total ROS, $O_2^{\bullet-}$, peroxides and lipid peroxides in control *C. albicans* cells, and in cells treated with 0.7 mM or 1.4 mM Lol for one hour

Samples	Total ROS[a]	$O_2^{\bullet-}$ [b]	Peroxides[c]	Lipid peroxides[d]
Control	9.76 ± 0.94	0.26±0.05	4.51±0.89	0.033±0.001
0.7 mM Lol	7.20±0.70**	0.08±0.01***	6.77±1.51	0.038±0.004
1.4 mM Lol	2.30±0.72***	0.07±0.00***	8.07±1.63*	0.039±0.002**

[a]Contents are given in nmol DCF (mg dry biomass)$^{-1}$; [b]Contents are given in nmol ethidium bromide (mg dry biomass)$^{-1}$; [c]Contents are given in nmol rhodamine (mg dry biomass)$^{-1}$; [d]Contents are given in nmol malondialdehyde. *$p<5\%$; **$p<1\%$; ***$p<0.1\%$. p values were calculated via the Student t-test.

The intense decrease in total ROS and $O_2^{\bullet-}$ are indicative of the affinity of Lol for biological radicals, especially for $O_2^{\bullet-}$. In order to investigate this, the xanthine–xanthine oxidase reaction was applied. In a Lol concentration range of 0.7–1.4 mM no difference was detected in the diformazan intensity and the same phenomenon was observed in case of GSH (Fig. 3). This can be explained in that GSH (and also Lol) reacts with $O_2^{\bullet-}$ with lower affinity than NBT^{2+} [20]. Jones et al. [21] found that only 22% of mitochondrial $O_2^{\bullet-}$ is transformed by GSH. A Lol or GSH concentration of 5.6 mM would be required for the possibility of the Lol (or GSH)–$O_2^{\bullet-}$ interaction to exceed that of the NBT^{2+}–$O_2^{\bullet-}$ interaction (Fig. 3). This interaction presumes some antioxidant properties to Lol, as was published earlier [2, 31, 42, 45]. Naturally, this 1-min kinetic measurement is unable to reflect the antioxidant effects of Lol in the 1-hour cell treatments, and no information is available about its uptake and biotransformation. Only indirect data are available, based on 9-day experiments, on its fungal biotransformation [30].

A novel aspect of our study was the investigation of the specific activities of the most important antioxidant enzymes in *C. albicans* cells exposed to Lol. On 0.7 mM Lol treatment, the specific activities of the SODs (the enzymes responsible for the reduction of $O_2^{\bullet-}$ to O_2 and H_2O_2) [17] decreased by 53% and that of CAT (the enzyme of H_2O_2 detoxification) [37] by 22% (Table 2). Máté et al. [29] demonstrated the kinetics of $O_2^{\bullet-}$ and peroxide production. In exposures for one hour, $O_2^{\bullet-}$ was often converted to H_2O_2. The decreases in the intracellular concentration of $O_2^{\bullet-}$ and increases in that of peroxide after exposure to 0.7 mM or 1.4 mM Lol, and the losses in the activities of the SODs and CAT after 0.7 mM Lol treatment present clear evidence of this phenomenon (Tables 1 and 2). Other possibilities: (i) the SOD levels are down-regulated in response to the decreasing ROS in order that the intracellular ROS content should not fall below its physiological concentration, thought this assumption

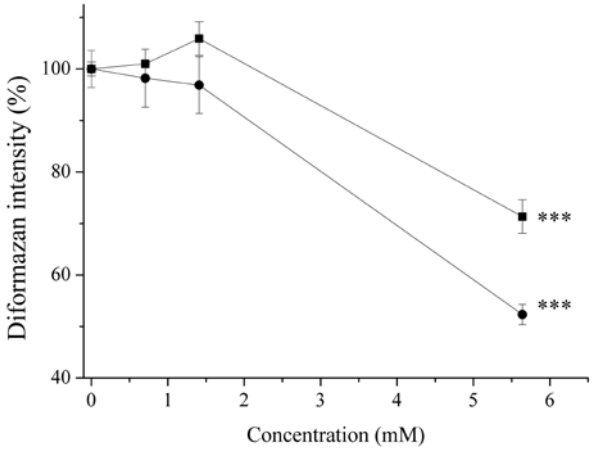

Fig. 3. In vitro effects of Lol (■) and GSH (●) on the NBT → diformazan conversion. $O_2^{\bullet-}$ was generated via the xanthine–xanthine oxidase reaction and was consumed in the reaction with NBT, in which diformazan was produced. ***$p<0.1\%$. p values were calculated via the Student t-test

Table 2
GSH and GSSG concentrations and specific activities of SODs, CAT, GPx, GR, G6PD and GST in *C. albicans* control cells and cells treated with 0.7 mM or 1.4 mM Lol for one hour

	Control	0.7 mM	1.4 mM
GSH[a]	2.431 ± 0.609	2.490 ± 0.897	2.034 ± 0.215
GSSG[a]	0.078 ± 0.004	0.092 ± 0.056	0.106 ± 0.007***
GSH/GSSG	31.27	29.44	19.07
Total SOD[b]	22.15 ± 6.46	11.21 ± 1.80*	21.61 ± 3.75
SOD_{Mn}[b]	11.25 ± 2.98	10.42 ± 1.74	12.69 ± 3.40
SOD_{CuZn}[b]	12.78 ± 3.65	2.02 ± 2.04**	15.95 ± 4.12
CAT[c]	77.68 ± 1.68	60.51 ± 11.95*	125.38 ± 13.62***
GPx[d]	7.74 ± 1.06	5.43 ± 1.31	24.48 ± 6.98**
GR[d]	129.92 ± 15.83	156.95 ± 24.38	302.61 ± 19.28***
G6PD[d]	645.85 ± 65.38	579.84 ± 32.88	676.26 ± 60.65
GST[d]	11.86 ± 0.38	13.15 ± 1.18	10.08 ± 0.88*

Abbreviations: CAT, catalase; G6PD, glucose-6-phosphate dehydrogenase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; GSSG, glutathione disulfide; GST, glutathione S-transferase; SOD, superoxide dismutase; SOD_{CuZn}, CuZn superoxide dismutase; SOD_{Mn}, Mn superoxide dismutase. [a]Contents are given in µM (mg dry biomass)$^{-1}$; [b]Specific activities are given in unit (min mg protein)$^{-1}$; [c]Specific activities are given in µmol (min mg protein)$^{-1}$; [d]Specific activities are given in nmol (min mg protein)$^{-1}$; *$p < 5\%$; **$p < 1\%$, ***$p < 0.1\%$. p values were calculated via the Student t-test.

requires further investigation; (ii) a direct interaction between Lol and $O_2^{\bullet-}$ is responsible for this decrement, as has been demonstrated (Fig. 3); or (iii) the inhibitory effect of Lol on xanthine oxidase manifested in the inhibition of other cellular functions leading to $O_2^{\bullet-}$ decreased level. Although 1.4 mM Lol caused only a 10% lower survival rate than that with 0.7 mM Lol (Fig. 2), these two concentrations provoked different antioxidant responses. As a consequence of the accumulation of peroxides and the occurrence of lipid peroxidation processes, significantly elevated activities of GPx (GSH-mediated neutralization of organic and inorganic peroxides, with the generation of GSSG) [6, 27] and CAT were observed (Table 2). As an indicator of oxidative stress, an elevated concentration of GSSG was measured after treatment by 1.4 mM Lol (Table 2). The increased GSSG content may play a crucial role in the induction of the antioxidant defense system [36], including the elevated specific activity of GR, the decreased specific activity of GST and the decreased GSH/GSSG ratio, as consequences of the unbalanced redox state of *C. albicans* cells exposed to 1.4 mM Lol (Table 2). GR is involved in the reduction of an increased amount of GSSG to GSH, and GST is responsible for balancing the GSH/GSSG ratio by exporting GSSG from the cells [20]. Interestingly, in contrast with our results, Usta et al. [43] reported a decrease in the content of GSH. Feasible causes of the difference may be the lower cell viability (50–0%) and longer exposure time (24 h) in the experiments of Usta et al. [43]. Gónzalez-Párraga et al. [18] found that the treatment of *C. albicans* for 60

min with a subinhibitory concentration of H_2O_2 (0.5 mM) increased the activities of the main antioxidants CAT, GR and SOD relative to the control, 50 mM H_2O_2 at 1% cell viability decreased the activities of these enzymes.

CONCLUSIONS

In conclusion, our results suggest that via mitochondrial-dependent $O_2^{\bullet-}$ generation and lipid peroxidation, Lol-induced dose- and time-dependent oxidative stress processes in one-hour-long acute tests may be the sources of its antimicrobial activity, manifested in significant decreases in germ tube and hence pseudohyphae and hyphae formation and survival rate. As a result of its SOD-dependent dismutation, the Lol–$O_2^{\bullet-}$ interaction and the partial inhibition of cellular functions, $O_2^{\bullet-}$ accumulation could not be detected. Our results clearly indicate the existence of oxidative stress intensity-dependent regulation of the antioxidant system.

To summarize the findings related to the adverse acute toxicity effects of Lol at high (70–80%) survival rate (in the present study), these can be explained in terms of the following processes at the molecular level: (i) Lol induces damage in the plasma membrane structure, parallel with increase in membrane fluidity [5, 22], (ii) Lol depresses the respiratory rate through interference with mitochondrial complexes I and II, resulting in a decrease in ATP level and cell viability [43; this study], (iii) Lol induces the accumulation of peroxides through mitochondrial-dependent $O_2^{\bullet-}$ generation and membrane-dependent lipid peroxidation [12, 43; this study), which (iv) alters the concentration of the crucial antioxidant GSH and results increased GSSG content [43; this study] and (v) up- or down-regulates the activities of certain antioxidant enzymes (GR, GPx and CAT) in a concentration-dependent manner (the present study).

In chronic and long-term acute tests, (i) Lol inhibits one of the key enzymes of sterol biosynthesis [10, 22, 24], (ii) leading to disorganization of the plasma membrane through changes in the composition of the plasma membrane and loss of essential cell components [5, 8], and (iii) the ROS-induced unbalanced redox state may contribute to a cell cycle arrest and DNA damage, resulting in apoptosis or necrosis [19, 46].

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