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Vitamin C Modulates Oxidative Stress Related Enzyme Activities in *Candida albicans*

[*Candida albicans*'da Oksidatif Stres ile İlişkili Enzim Aktivitelerini Vitamin C Düzenler]

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ABSTRACT

Aim: Dimorphism and virulence in *Candida albicans* is associated with reactive oxygen species and other oxidative stress related markers. Neutrophils are known to accumulate high ascorbate to combat *Candida*. In the present study, we have investigated *in vitro* effect of varying concentrations of vitamin-C on oxidative stress related enzyme activities in *Candida albicans.*

Methods: Cells were exposed to increasing concentrations of vitamin-C till mid-log phase of growth. Cell free extract and crude membrane of *Candida* cells were prepared. Levels of reduced glutathione and of defensive enzymes; catalase, glutathione peroxidase, glutathione reductase, glutathione S-transferase and superoxide dismutase were measured from cell free extract employing standard protocols. Crude membrane preparation was used to analyze lipid peroxidation level by estimating thiobarbituric acid-reactive substances.

Results: With increase in vitamin-C lipid peroxidation was found to decrease, whereas superoxide dismutase activity increased. Cytosolic reduced glutathione and enzymes activities of catalase, glutathione peroxidase, glutathione reductase and glutathione S-transferase decreased with increase in vitamin-C.

Conclusion: Results suggest that vitamin-C acts as antioxidant with respect to superoxide dismutase activity and lipid peroxidation. In respect of cytosolic reduced glutathione, enzymes activities of catalase, glutathione peroxidase, glutathione reductase and glutathione S-transferase vitamin C acts as pro-oxidant. Results may have strong bearing in treatment of candidiasis in presence of vitamin-C.

Keywords: *Candida albicans*, vitamin-C, oxidative stress

ÖZET

Amaç: *Candida albicans* da virülans ve dimorfizm reaktif oksijen türleri ve diğer oksidatif stres ilişkili belirteçler ile ilgilidir. Nötrofillerin, *Candida* ile mücadele edebilmek için yüksek askorbatı biriktirdiği bilinmektedir. Bu çalışmada değişen konsantrasyonlardaki vitamin-C'in *Candida albicans'*da oksidatif stres ile ilişkili enzim aktivitelerindeki *in vitro* etkileri incelendi.

Yöntemler: Hücreler büyümenin mid-log fazına kadar artan konsantrasyonlarda vitamin-C'ye maruz bırakıldı. *Candida* hücrelerinden, hücreden yoksun ekstre ve işlenmemiş membranlar hazırlandı. Standart protokoller uygulanarak bu hücreden yoksun ekstreden, azalmış glutatyon, katalaz, glutatyon peroksidaz, glutatyon redüktaz, glutatyon S-transferaz ve superoksit dismutaz gibi koruyucu (antioksidan) enzim düzeyleri ölçüldü. İşlenmemiş membran ise tiobarbitürik asit-reaktif madde miktarı ölçülerek lipid peroksidasyon düzeylerinin belirlenmesinde kullanıldı.

Bulgular: Vitamin-C'deki artış ile lipid peroksidasyonda azalma ve superoksit dismutaz aktivitesinde artma bulundu. Vitamin-C'deki artış ile katalaz, glutatyon peroksidaz, glutatyon redüktaz ve glutatyon S-transferaz enzim aktiviteleri ve sitozolik glutatyon düzeyi azaldı.

Sonuç: Lipid peroksidasyon ve superoksit dismutaz enzim aktivitesi sonuçlarına göre vitamin-C'nin antioksidan olarak etki gösterdiği öne sürülür. Azalan sitozolik glutatyon düzeyleri, katalaz, glutatyon peroksidaz, glutatyon redüktaz ve glutatyon S-transferaz enzim aktiviteleri ise vitamin-C'nin pro-oxidant olarak davrandığını gösterir. Sonuçlar candidiasisin tedavisinde vitamin-C varlığının unutulmaması yönünde sağlam kanıtlar oluşturmaktadır.

Anahtar Kelimeler: *Candida albicans*, vitamin-C, oksidatif stres

Introduction

Candida albicans (C. albicans) is an opportunistic pathogen that causes superficial and systemic infections (1). HIV infection and widespread use of immunosuppressive therapy has made infections by *C. albicans* more common in recent years and more studies about this pathogen are needed (2, 3). Vitamin-C boosts immunity by keeping disease-fighting white blood cells increased, so that the body is better able to stave off infections, especially opportunistic ones such as *Candida* that take advantage of a weak immune system. Ascorbic acid has been shown to enhance the lethal effects of amphotericin B on *C. albicans*. It is assumed that ascorbic acid acting as a pro-oxidant augmented the oxidation-dependent killing of fungal cells induced by amphotericin B (4). Locally applied ascorbic acid, while being not active or worsening the time course of acute vaginal candidiasis or other fungal infections, is very active in preventing fungal reinfection or super infection when applied after the completion of a successful standard antimycotic or antibiotic treatment to patients (5). Treatment with antibiotics, poor oral hygiene, and vitamin-C deficiency appeared as the most significant independent risk factors associated with topical candidiasis (6). Neutrophils which offer first line of defense, against systemic candidiasis rapidly transport dehydroascorbic acid inside and reduce it to ascorbic acid. Ascorbic acid concentration may rise up to 10 mM when neutrophils are incubated with *C. albicans* (7). Functional role of this increased ascorbate is still an enigma. The current recommended dietary allowance (RDA) of vitamin-C is 75 mg/day for women and 90 mg/day for men, based on the vitamin's role as an antioxidant as well as protection from deficiency. High intakes of the vitamin are generally well tolerated; however, a Tolerable Upper Level (TUL) was recently set at 2000 mg/day **(8).** Vaginal suppositories of amount up to 250 mg vitamin-C are routinely used to control candidal vaginitis (9). Like other living cells, *C. albicans* generate various oxidative agents, such as reactive oxygen species (ROS), from the mitochondrial respiratory chain in the normal aerobic metabolism process. ROS, such as superoxide anions, hydrogen peroxide, and hydroxyl radicals, can damage many of the cellular components (10-12). Antioxidant nutrients and enzyme defenses are fundamental protectors against all forms of stress. Since vitamin-C can act both as anti and prooxidant, in the present study; we have investigated the effect of different concentrations of vitamin-C on oxidative stress related enzyme activities in *C. albicans*.

Materials and methods

Candida albicans strain ATCC 10261 was used in this study. All media constituents, (yeast extract, peptone and dextrose), 5,5'- dithiobis-2-nitrobenzene (DTNB), were of analytical grade and procured from E. Merck (India). Other chemicals like vitamin-C, NADP, NADPH, oxidized and reduced forms of glutathione, were purchased from Sigma Chemical, USA. All assays were performed with varying concentrations of vitamin-C (2, 5, 10 and 20 mg/ml of culture media).

Growth conditions

Stock culture of *C. albicans* (ATCC 10261) was maintained on slants of nutrient agar (yeast extract 1%, peptone 2%, dextrose 2% and agar 2.5%) at 4ºC. To initiate growth for experimental purposes, one loop full of cells from an agar culture was inoculated into 25ml of YEPD (Yeast Extract Peptone Dextrose) nutrient medium and incubated at 30ºC for 24 h i.e. up to stationary phase (primary culture). The cells from primary culture (10^8 cells ml^{-1}) were re-inoculated into 100 ml fresh YEPD medium and grown for 8-10 h i.e., up to mid-log phase (10^6 cells ml^{-1}).

Preparation of cell free extract of Candida culture

Cells free extract of *Candida* culture was prepared according to described method Jethwaney *et al.* 1997 (13) with minor modifications. Cells grown to mid exponential phase along with different concentrations of vitamin-C in YEPD medium (1 g wet wt) were suspended in 2 ml grinding medium (250 mM sucrose, 10 mM Tris HCl, pH 7.5, 1 mM PMSF) and 2 g glass beads (0.45-0.50 mm diameter). The suspension was mechanically disrupted in an MSK Braun cell homogenizer (Sartorius AG, Germany) by agitating it for a total of nine cycles of 10s with a gap of 3s at 4000 vibrations per minute. The homogenate was collected and centrifuged (Sigma 3K30 USA) at 1000 g for 5 min at 4°C to remove unbroken cells and glass beads. The homogenate was then centrifuged for 40 min at 15 000 g at 4°C and the resulting pellet treated as crude membrane and final supernatant was treated as cell free extract. Crude membrane later on dissolved in 1 mM of Tris HCl.

Determination of vitamin-C solution: Stock vitamin-C solution was made in distilled water. It was filtered through sterile Millipore filter and then added in autoclaved sterile media to achieve appropriate concentration. Ascorbic acid concentration was determined by oxidizing it to dehydroascorbic acid by shaking with activated charcoal in presence of acetic acid. After coupling with 2,4 dinitrophenyl hydrazine the solution was treated with sulphuric acid to produce color which was measured at 540 nm. Ascorbic acid was determined by difference between oxidized and unoxidized samples. Concentration of vitamin-C was determined as per standard protocol (14).

Enzymatic assay for measuring oxidative stress

The following defensive enzymes related with oxidative stress were assayed according to the reported methods. For lipid peroxidation crude membrane was used and for the rest of enzyme assays cell free extract was used.

Lipid Peroxidation

For lipid peroxidation the production of thiobarbituric acid reactive substances (TBARS) was measured by the method of Bernheim *et al*. 1948 (15). This modified method uses trichloroacetic acid to eliminate interference caused by malondialdehyde precursors. To a reaction mixture in a total volume of 2.0 ml containing 1.8 ml phosphate buffer (0.1M, pH 7.4), 0.2 ml of crude membrane of *C. albicans* was added. The reaction mixture was incubated at 37ºC in water bath shaker for 1 hour. The reaction was terminated by adding 1.0 ml of 10% trichloroacetic acid followed by the addition of 1.0 ml of 0.67 % thiobarbituric acid. All the tubes were kept in boiling water bath for 20 minutes. The tubes were then cooled in ice and centrifuged at 2500 g for 10 minutes. The resulting supernatant containing TBARS was measured spectrophotometrically (Systronics uv-vis 117, India) by taking the absorbance at 432 nm against a reagent blank at 25ºC.

Reduced glutathione

Cytosolic reduced glutathione was determined by the method of Jollow *et al*. 1974 (16) with slight modifications. In this method 1.0 ml of the cell free extract was precipitate with 1.0 ml of 4 % sulphosalicylic acid. The samples were then kept for 1 hour at 4ºC and centrifuged at 1200 g for 15 minutes at 40ºC. The assay mixture consisted of 0.1 ml of above supernatant, 2.7 ml of Phosphate buffer (0.01 M, pH 7.4) and 0.2 ml of freshly prepared DTNB in a total volume of 3.0 ml. The color developed due to the formation of a yellow colored complex, 5-thio-2-nitrobenzoate, was measured immediately at 412 nm at 25ºC.

Catalase

Catalase activity was assayed by the method of Claiborne *et al.* 1985 (17). The assay mixture consisted of 1.99 ml phosphate buffer (0.05 M, pH 7.0), 1.0 ml of H_2O_2 (0.0019 M) and 10 µl cell free extract in a total volume of 3.0 ml in a quartz cuvette. Decrease in absorbance due to the disappearance of H_2O_2 was recorded by spectrophotometer at an interval of 30 seconds up to 3 minutes at 230 nm at 25ºC.

Glutathione peroxidase

Specific activity of the enzyme glutathione peroxidase was measured according to the procedure described by Mohandas *et al*. 1984 (18) .The reaction mixture in a 3.0 ml cuvette consisted of 1.53 ml of phosphate buffer (0.05M, pH 7.0) 0.1 ml of 1 mM EDTA, 0.1 ml of 1 mM NaN_3, 0.1 ml of 1 mM reduced glutathione, 0.1 ml of 0.2 mM NADPH, 0.01 ml of 0.25 mM H_2O_2 and 100 µl cell free extract in a final volume of 2.0 ml. The activity was measured in terms of decrease in absorbance at 340 nm suggestive of disappearance of NADPH at an interval of 30 sec for 3 min at 25ºC.

Glutathione reductase

Activity of glutathione reductase was assayed by the method of Carlberg et al. 1975 (19). The assay mixture taken in a 3.0 ml cuvette consists of 1.68 ml of phosphate buffer (0.1 M, pH 7.4), 0.1 ml of 0.1 mM NADPH (freshly prepared by dissolving 0.833 mg in 10 ml of 0.1 M phosphate buffer pH 7.4), 0.1 ml of 0.5mM EDTA (1.86 mg in 10 ml of distilled water), 0.05 ml of 1mM oxidized glutathione (freshly prepared) and 70 µl of cell free extract in a final volume of 2.0 ml. The activity was measured in terms of decrease in absorbance at 340 nm at an interval of 30 sec for 3.0 minutes at 25ºC.

Glutathione-S-transferase

Cytosolic glutathione–S-transferase activity was determined by the method of Habig *et al*. 1974 (20). The assay mixture taken in a 3.0 ml cuvette consists of 1.65 ml of phosphate buffer (0.1M, pH 6.5), 0.2 ml of 1 mM reduced glutathione (freshly prepared), 100 µl of cell free extract, 50µl of 1 mM freshly prepared 1-chloro-2,4-dinitrobenzene (CDNB) in a final volume of 2.0 ml. The increase in absorbance corresponding to an increase in CDNB conjugate formed was recorded at an interval of 30 sec for 3.0 minutes at 340 nm at 25ºC.

Superoxide dismutase

Superoxide dismutase (SOD) activity was measured as per protocol adopted from Marklund and Marklund 1974 (21). For preparation of tris buffer 50 mM Tris and 1 mM EDTA dissolved in distilled water, pH adjusted to 8.5. In control 2.9 ml tris buffer and 0.1 ml pyrogallol was taken and in test sample 2.8 ml tris buffer, 0.1 ml pyrogallol and 0.1 ml cell free extract was taken. After induction period of 90 seconds, absorbance was recorded first in control and then in test every 30 seconds for 3 minutes at 420 nm at 25ºC. The induction periods was allowed to achieve a steady state of authorization of pyrogallol. A rate of change of absorbance per minute in the control as well as test sample was noted to calculate the SOD activity.

For estimation of protein standard Bradford Assay method employed (22) and the activities of the enzymes were standardized on the basis of unit amount of protein.

Statistical analysis

The results obtained were given as “mean value ± standard error” (x ± SE). Statistical analyses were performed with the “graph pad instat software” and P value as < 0.001 was accepted as statistically significant.

Results

In the present study, we have investigated the effect of different concentration of vitamin-C on the activities of various enzymes, which are involved in the defense mechanisms of oxidative stress in *C. albicans*. All enzyme activities are well illustrated in Table 1.

Lipid peroxidation results showed gradual decline in rate of formation of TBARS with increasing concentra-

Table 1. Oxidative stress related enzyme activity in presence of Vitamin-C

Enzymes	Control	Different concentration of vitamin-C		
		2mg	10mg	20mg
GR	8.150 ± 0.117	7.680 ± 0.164	7.030 ± 0.117	4.600 ± 0.158
(nmol of NADPH oxidized /min/mg protein)				
GST	2.22 ± 0.03	2.11 ± 0.15	0.98 ± 0.01	0.86 ± 0.05
(nmol of CDNB conjugate formed/ min/mg protein)				
GPx	2.770 ± 0.003	1.500 ± 0.008	1.500 ± 0.008	1.350 ± 0.007
(nmol of NADPH oxidized /min/mg protein)				
GSH	851.46 ± 2.79	840.44 ± 1.39	789.70 ± 1.39	710.29 ± 0.69
(μmol GSH/ g cells)				
CAT	2393.0 ± 65.8	2170.0 ± 20.0	2098.0 ± 22.8	2021.0 ± 20.3
(n mol H_2O_2 consumed /min/ mg protein				
LPO	4394.00 ± 9.11	2902.00 ± 3.68	2348.00 ± 0.01	2113.00 ± 5.71
(nmol TBARS/mg protein)				
SOD	2.69 ± 0.24	9.99 ± 0.24	13.07 ± 0.24	19.26 ± 0.24
(Units/mg protein)				

Data represents mean value of experiment ± SE which are performed in triplicate. GR: glutathione reductase; GST: glutathione-S-transferase; GPx: glutathione peroxidase; and GSH: Reduced glutathione CAT: Catalase; SOD: superoxide dismutase; and LPO: lipid peroxidation

tion of vitamin-C. TBARS is an indicator of lipid peroxidation which increases in various diseases. Control value indicated 4394 nmoles of TBARS formed/mg protein, which eventually decreases with increasing concentrations of vitamin-C. At 2, 10 and 20 mg/ml values dropped to 2902, 2348 and 2113 nmoles of TBARS formed/mg protein respectively. Thus 53.53% decline in rate of formation of TBARS was found at 20 mg/ml vitamin-C concentration.

Activity of reduced glutathione in control cells was found to be 851 µmole GSH/g cells. As compared to above mentioned enzymes there was not much decline in enzyme activity with increasing concentrations of vitamin-C. At 2, 10 and 20 mg/ml concentrations values observed were 840, 789.70 and 710 µmole GSH/g cells respectively. Thus 16.9% reduction in activity observed with 20 mg/ml vitamin C.

Enzyme activity of catalase (one of the important defense enzymes) was 2393 nmoles of H_2O_2 consumed/min/mg protein. At 2, 10 and 20 mg/ml vitamin-C concentration this value dropped to 2170, 2098 and 2021.38 nmoles of H_2O_2consumed/min/mg protein respectively. Thus 15.71% reduction in enzyme activity was observed at higher concentration (20 mg/ml vitamin-C).

Control glutathione peroxidase activity observed was 2.77 nmoles of NADPH oxidized/min/mg protein. There was gradual fall in activity with 2, 10 and 20 mg/ml vitamin-C concentration indicated by values of 1.50, 1.50 and 1.35 nmoles of NADPH oxidized/min/mg protein respectively. Thus 50.98% inhibition in activity was found at higher (20mg/ml vitamin C) concentration.

In case of glutathione reductase activity, control cell showed value of 8.15 nmoles of NADPH oxidized/min/mg protein. Along with 2, 10 and 20 mg/ml vitamin-C concentration values dropped to 7.68, 7.03 and 4.6 nmoles of NADPH oxidized/min/mg protein respectively. Thus percent inhibition in enzyme activity with higher concentration (20mg/ml) of vitamin C was 43.56.

Control glutathione–S-transferase activity was found to be 2.22 nmoles of CDNB conjugate formed/min/mg protein. Along with 2, 10 and 20mg/ml vitamin C, activity declined as indicated by values of 2.11, 0.976 and 0.865 nmoles of CDNB conjugate formed/min/mg protein respectively, thus 61.04% inhibition in activity was at 20mg/ml vitamin-C concentration.

Superoxide dismutase assay showed increase in activity of enzyme with increasing concentration of vitamin-C. Control SOD enzyme activity was 2.69 units/mg protein, which gradually enhanced with increasing concentration of vitamin-C. At lowest concentration activity observed was 9.99 units/mg protein which increased to 13 units/mg protein at 10 mg/ml and finally at 20 mg/ml vitamin-C activity was found to be 19.26 units/mg protein. Almost 600% increment in SOD value observed at 20 mg/ml concentration.

Discussion

Aerobic organisms possess antioxidant defense systems to deal with ROS, which is a result of aerobic respiration and substrate oxidation. Normally their production is low and the low levels of ROS are necessary for several biological processes, including intracellular differentiation and cell progression, arrest of growth, apoptosis, immunity, and defense against microorganisms. Increased formation of ROS and/or decreased antioxidant defense can be defined as oxidative stress, which may damage biological macromolecules. To protect against damage, cells contain a number of defense mechanisms including endogenous well-characterized antioxidant enzymes, such as catalase, superoxide dismutase, glutathione peroxidase, glutathione reductase, glutathione S-transferase and low molecular weight antioxidant such as glutathione (GSH) (23, 24,12). Sulfhydryl groups (–SH) play a key role in response to oxidative stress, GSH/glutaredoxin and thioredoxin systems are crucial components in maintaining redox homeostasis of the cell (25).

Pathogens have co-opted these well-conserved antioxidation mechanisms to evade phagocyte defenses, production of these enzymes is, therefore, directly related to virulence.

Candida albicans is dimorphic yeast capable of producing alternate morphological forms (yeast or mycelium) in response to environmental changes. The dimorphic behavior of *C. albicans* has been implicated to play a significant role in pathophysiology of this fungus (26). Thomas et al. (27) have reported a dramatic decline of the level of intracellular GSH concomitant with yeast to mycelial conversion in *C. albicans*. The decline of GSH with the transition of yeast cells to mycelial forms in *C. albicans* indicates that the intracellular level of GSH is highly regulated. The level of free intracellular GSH level may be a biological cue for the initiation of a cascade of events that eventually lead to the conversion of yeast cell to mycelial forms. The reason for the decline of glutathione in the mycelial form is not understood. One or more enzymes involved in *C. albicans* GSH metabolism may be directly or indirectly responsible for maintaining a certain intracellular level of GSH. The presumed regulatory mechanism may operate at the level of synthesis or degradation of GSH. Major reactions that may lead to depletion of GSH are decreased levels of GSSG reductase, increased activity of glutathione-S-transferase and oxidation of GSH by glutathione peroxidase, including the formation of GSSG (28).

It has been reported that in acidic medium levels of glutathione reductase and glutathione transferase increased moderately whereas no significant alteration of glutathione peroxidase was observed. For the cultures grown in basic medium, the levels of these enzymes remain unchanged (29).

In this study the intracellular level of glutathione, which helps to maintain the redox potential of the cell was decreased with the increased concentration of vitamin-C. Activities of other enzymes related to glutathione metabolism such as glutathione reductase, glutathione peroxidase and glutathione-S-transferase were also decreased. It could be assumed that increasing concentration of vitamin C might be interfering with antioxidant system or defensive enzymes of *Candida* thus lowering level of reduced glutathione as well as activities of glutathione metabolic enzymes.

Ohmori et al 1999 (30) have examined the activity of enzymes that directly protect the cell against toxic oxygen radicals as well as enzyme activities involved in the metabolism of glutathione and precursor molecules under aerobic and anaerobic conditions. Total glutathione level (reduced and oxidized forms) was found to be 2.4 fold higher in aerobically grown yeast cells as compared to anaerobically grown cells. Aerobic growth conditions did not remarkably affect the enzyme activity of glutathione reductase but the activities of glutathione-S-transferase and glutathione peroxidase which catalyze consuming the reduced form of glutathione as well as catalase and superoxide dismutase were increased under aerobic conditions, strongly supporting the concept that yeast cells grown in aerobic condition were exposed to an oxygen stress. Supplemented cells of *Ashbya gossypii* a filamentous fungus showed increased levels of catalase, glutathione peroxidase, lipid peroxides and decreased glutathione indicating that vitamin E, a well-known antioxidant, had acted as a pro-oxidant at low levels of 2.5 μM and had increased the oxidative stress (31).

In accordance with our results, exposure of *C. albicans* to increased concentration of vitamin-C shows antioxidant effects in case of SOD and lipid peroxidation, where enzyme activity of SOD was found to be increased and there was a decrease in TBARS. Contrary to above effects, increasing concentrations of vitamin-C exerted pro-oxidant effect on catalase, reduced glutathione and reduced enzyme activities of glutathione peroxidase, glutathione reductase and glutathione-S-transferase related to its metabolism. Under physiological conditions, vitamin-C has a predominantly antioxidant role (32). Pro-oxidant and pro-apoptotic effects of vitamin-C may be related to hydroxylation and/or formation of ascorbyl radicals (33). There is report of ascorbic acid exhibiting its pro-oxidant nature in *C. albicans* where **it** enhanced **the** l**et**hal but not **the** permeabilizing **effects of amphotericin** B **on** *C. albicans* (4). Thus two sets of conclusions can be withdrawn from present work. First set indicating natural antioxidant nature of vitamin-C in SOD and lipid peroxidation levels in *Candida,* being beneficial for the organism and second set indicating its more controversial pro-oxidant role in catalase, glutathione metabolizing enzymes and reduced glutathione level having detrimental or adverse effects on fungus itself. Whether vitamin-C functions as an antioxidant or pro-oxidant is determined by at least 3 factors: 1) the re-

dox potential of the cellular environment; 2) the presence/absence of transition metals; and 3) the local concentrations of ascorbate (34). The last factor is particularly relevant in treatments that depend on the antioxidant/pro-oxidant property of vitamin-C, because it can be readily manipulated and controlled *in vivo* to achieve desired effects. We could assume that its pro-oxidant nature on *Candida* can be further investigated.

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