Astaxanthin supplementation reduces dichlorvos-induced cytotoxicity in *Saccharomyces cerevisiae*

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Abstract

This study evaluates the protective effect of astaxanthin against dichlorvos cytotoxicity in yeast *Saccharomyces cerevisiae*. Dichlorvos induce a dose-dependent cytotoxicity in yeast cells, which is mediated by oxidative stress. Our experimental results showed pre-treatment with astaxanthin enhances cell viability by 20–30% in yeast cells exposed to dichlorvos. A decrease in DCF fluorescence intensity and lipid peroxidation, increased SOD activity, and glutathione levels in astaxanthin-treated cells indicate that astaxanthin protected the cells against dichlorvos-induced oxidative stress. Reduced chromatin condensation and nuclear fragmentation in astaxanthin pre-treated cells also indicate that astaxanthin rescued the cells from dichlorvos-induced apoptosis. Our overall results suggest that dichlorvos induces oxidative stress-mediated cytotoxicity in yeast cells, and that was rescued by astaxanthin pre-treatment.

Keywords Cytotoxicity · Yeast · Astaxanthin · Dichlorvos · ROS · Apoptosis

Introduction

Organophosphate pesticides are widely used in agriculture, household applications, and storage of food products to protect against pests and insects (Das 2013). Dichlorvos is an organophosphate that inhibits acetylcholinesterases in the nervous system of insects and causes toxicity (Pancetti et al. 2007; Booth et al. 2007). Prolonged exposure to dichlorvos leads to an increase in the oxidative stress and depletion of antioxidant mechanism, which is associated with adverse effects in non-target organisms. People get exposed to dichlorvos through breathing, swallowing, and skin and eye contact. Symptoms of dichlorvos exposure include weakness, headache, blurred vision, tightness in chest, vomiting, skin irritation, paralysis, convulsions, and dizziness (France 1991). The association between dichlorvos and clinical syndromes has attracted an extensive concern on human health, and therefore, elimination of dichlorvos toxicity on humans has become a subject of intensive research.

Model systems have proved to be useful in understanding the complex regulatory pathways and networks that are involved in oxidative stress-mediated diseases (Botstein et al. 1997; Farrugia and Balzan 2012). Yeast *Saccharomyces cerevisiae* is a widely used eukaryotic model organism because of its numerous benefits. Yeast is genetically well defined, its entire genome sequence has been elucidated, and the corresponding databases are generally accessible. It has relatively simple growth requirements and can grow as dispersed cells in liquid or as colonies on solid media. Out of ~6000 genes in *S. cerevisiae*, it is estimated that ~30% have a homolog in humans. The gene expression analysis of *S. cerevisiae* in response to lindane and flutriafol toxicity revealed that oxidative stress, mitochondrial dysfunction, and respiratory quotient are almost similar to human. These studies indicate that yeast is a good model organism to study pesticide toxicity and the protection mechanisms (Koch et al. 1993; Parveen et al. 2003).

Searching for the natural compounds rich in antioxidants is escalating because of their importance in reducing the risks of many chronic disorders such as cancer and neurodegenerative diseases (Shahidi et al. 2012; Godic et al. 2014). Natural antioxidants are widely used ingredients in dietary supplements to prevent oxidative stress-mediated diseases. Carotenoids are family of natural compounds which are synthesized by plants and microorganisms (Mangels et al.

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1993). Astaxanthin is a red-pigmented carotenoid shown to have various biological activities including oxidative damage protection. Astaxanthin is a carotenoid, produced by microscopic algae, *Haematococcus pluvialis*, which is known to scavenge reactive oxygen species such as singlet molecular oxygen ($^1\text{O}_2$) and peroxy radicals. The interaction of carotenoids with $^1\text{O}_2$ depends largely on physical quenching, which involves direct energy transfer between the molecules (Higuera-Ciupara et al. 2006). The free radical scavenging activity of astaxanthin protects lipids from peroxidation and reduces oxidative damage in the cell and mitochondrial membranes (Ambati et al. 2014). Many studies have shown that astaxanthin is a potential candidate to treat cancer and neurodegenerative diseases such as Alzheimer's and Parkinson's diseases (Fassett and Coombes 2011). The current study was focused on the oxidative stress-mediated toxic effect of dichlorvos on *S. cerevisiae* and its protection by astaxanthin.

Materials and methods

The yeast *S. cerevisiae* BY4741 (MAT α his3 Δ :leu2 Δ :met15 Δ :ura3 Δ) was obtained from Thermo Fisher Scientific, USA. Yeast strain was grown in YPD medium-containing 2% bacteriological peptone, 1% yeast extract, and 2% dextrose. For solid YPD agar medium, 2% Bacto agar was used in addition to YPD liquid medium. Dichlorvos and astaxanthin were purchased from Sigma-Aldrich, USA. All the other chemicals were purchased from Himedia Laboratories, India.

Dichlorvos toxicity in yeast

The yeast (BY4741) strain was inoculated into 3 ml YPD broth and grown to exponential phase at 30 °C in a shaker incubator. The culture was diluted to get 1×10^4 cells/ml in YPD broth and 200 μl of cell suspension was dispensed into a flat-bottomed 96-well plate and treated with different concentrations of dichlorvos (0.1–1.3 mM), and incubated along with untreated control in triplicates at 30 °C for 18–24 h. Optical density (OD) of the culture was read at 600 nm in a microplate reader. The dichlorvos concentration at which yeast strain showed 50% growth inhibition (IC_{50}) was used for further experiment.

Dichlorvos sensitivity and growth protection by astaxanthin

Exponentially grown *S. cerevisiae* culture was treated with astaxanthin (30 μM) for 2 h followed by an exposure to dichlorvos (0.8 and 0.9 mM). The cultures were then incubated at 30 °C for 18–24 h in a shaker incubator. After the incubation, cells were tenfold serially diluted and 5 μl of

each dilution was spotted on the YPD agar plate. Plates were incubated at 30 °C for 2 days and the images were captured. In colony-forming unit (CFU) assay, cultures were serially diluted and an appropriate dilution was spread on YPD plates in triplicate. After incubating the plates for 2 days, CFU were counted and expressed as relative percent cell survival compared to control (de Sá et al. 2013).

Measurement of antioxidant biomarkers

To measure the antioxidant biomarkers, exponentially grown yeast cells were pre-treated with or without astaxanthin for 2 h followed by exposure to dichlorvos (0.8 mM) for 1 h at 30 °C in a shaker incubator. The cells were harvested by centrifugation for 5 min at 5000 rpm, and the cell pellet was used for the measurement of intracellular ROS, superoxide dismutase activity (SOD), lipid peroxidation, and reduced glutathione (GSH).

Intracellular ROS

Dichlorvos-induced ROS was measured in yeast cells using 2,7-dichlorofluorescein-diacetate ($\text{H}_2\text{DCF-DA}$). The cell pellet was washed in PBS (phosphate buffer saline pH 7.4) and incubated with $\text{H}_2\text{DCF-DA}$ (20 μM) for 10–15 min in the dark at room temperature. Immediately after incubation, cells were harvested by centrifugation and washed thrice with PBS. Cells were observed under a fluorescent microscope, and the fluorescence intensity was measured in a spectrofluorometer (Pereira et al. 2001).

Superoxide dismutase activity (SOD)

Cell extract was prepared in phosphate buffer (pH 7.2) by vigorous shaking in the presence of glass beads for 15–20 min on ice with an interval of 2 min. SOD activity was measured by treating the enzyme extract with 0.1 mM ethylenediaminetetraacetic acid (EDTA), 75 μM nitro blue tetrazolium chloride (NBT), 2 μM riboflavin, and 13 mM methionine. After the treatment, extracts were exposed to visible light for 15 min for NBT reduction and then absorbance was measured at 560 nm (Beauchamp and Fridovich 1971; Madamanchi et al. 1994). Protein content was measured by Bradford method (Marshall and Williams 1993), and the enzyme activity was expressed as units/mg of protein.

Lipid peroxidation

Lipid peroxidation in *S. cerevisiae* was quantified by determining the thiobarbituric acid reactive substance (TBARS) malondialdehyde. The cell lysate was prepared in phosphate buffer (pH 7.2), by vigorous shaking in the presence of glass beads for 15–20 min on ice with an interval of 2 min and

centrifuged. To the supernatant, 1 ml of TBARS reagent (0.25 M hydrochloric acid, 15% trichloroacetic acid, and 0.375% thiobarbituric acid) were added and heated for 15 min in a boiling water bath. After cooling, the absorbance was measured at 535 nm using a UV spectrophotometer. The concentrations of malondialdehyde in the samples were calculated by comparing 1,1,3,3-tetramethoxypropane, and the result was expressed as nano moles of MDA/mg of protein (Howlett and Avery 1997; Ghani et al. 2017).

Reduced glutathione

The cell lysate was prepared in phosphate buffer and centrifuged at 4000 rpm for 5 min. The supernatant was mixed with an equal volume of ice-cold perchloric acid (2 M) containing 4 mM EDTA. After 15 min of incubation, the cell extract was centrifuged at 4000 rpm for 5 min and the supernatant was neutralized by adding 2 ml of 100 mM phosphate buffer (pH 8.0) containing 3 M potassium hydroxide and 50 μ l 10 mM 5,5'-dithiobis-(2-nitrobenzoic acid) on ice. After 5 min of incubation on ice, absorbance was measured at 412 nm. Protein content was measured in the cell extract before treatment with perchloric acid, and the final results were expressed as μ M of GSH/mg of protein (Jamnik et al. 2006).

Effect of astaxanthin on the growth defects induced by dichlorvos

The exponentially grown culture was treated with astaxanthin for 2 h and then exposed to dichlorvos at 30 °C in a shaker incubator along with untreated and dichlorvos only treated controls. Optical density (OD₆₀₀) of the cultures was measured at regular intervals for 18 h and the readings were plotted against time. The growth was compared between control, dichlorvos-treated, and astaxanthin pre-treated cultures (Marešová and Sychrová 2007). To measure the nature of cell death induced by dichlorvos during growth, apoptosis-specific DAPI (4',6-diamidino-2-phenylindole), and acridine orange and ethidium bromide (AO/EB), staining was carried out on 8 h grown cultures. For DAPI staining, cells were fixed with 4% paraformaldehyde for 10–15 min, washed and suspended in sorbitol buffer (pH 7.4), and treated with DAPI (1 μ g/ml) for 5–10 min in dark at room temperature. Cells were washed twice and observed under a fluorescence microscope (Olympus IX71) for nuclear fragmentation (Probes 2008). For AO/EB staining, cells were washed and stained with AO/EB (100 μ g/ml) for 5 min at room temperature. The stained cells were washed twice with PBS and observed under fluorescence microscope for chromatin condensation (Kiruthika and Padma 2013).

Statistical analysis

The IC₅₀ value was determined from inhibitor v/s response curve in Graph Pad Prism 7 (Graph Pad Software) using the non-linear regression analysis. For comparison between dichlorvos-treated (DC) and astaxanthin + dichlorvos-treated (AXT + DC) groups, one-way analysis of variance (ANOVA) was carried out followed by Tukey's test. Significant changes are indicated as $p^* < 0.0001$.

Results

Dichlorvos induces cytotoxicity in *Saccharomyces cerevisiae*

The cytotoxic effect of dichlorvos was measured by the growth inhibition of *S. cerevisiae* (BY4741) cells. The result showed that an increase in the concentration of dichlorvos (0–1.3 mM) in the growth medium resulted in an increased percentage of cell death, indicating that dichlorvos induced a dose-dependent cytotoxicity in yeast cells. The cells showed 50% growth inhibition (IC₅₀) at 0.8 mM dichlorvos-treated culture (Fig. 1a). Furthermore, we tested whether astaxanthin can rescue *S. cerevisiae* cells from the cytotoxicity induced by dichlorvos using spot and CFU assays. The spot assay result (Fig. 1b) revealed that dichlorvos-treated cells were sensitive, and the sensitivity was rescued by astaxanthin pre-treatment. Similar to spot results, dichlorvos-treated cells showed 38.52% and 49.70% growth survival at 0.8 mM and 0.9 mM concentrations, respectively, compared to untreated control (100%) (Fig. 1c) in CFU counts. In contrast, astaxanthin (30 μ M) pre-treatment followed by an exposure to dichlorvos showed two-to-threefold increase in viability of *S. cerevisiae* cells. The protection assay results indicated that astaxanthin rescued *S. cerevisiae* cells from dichlorvos-induced cytotoxicity.

Astaxanthin reduces oxidative stress markers

To investigate the mode of cytotoxicity induced by dichlorvos and its protection by astaxanthin, oxidative stress markers such as intracellular ROS, SOD, lipid peroxidation, and reduced glutathione levels were measured in astaxanthin pre-treated cultures exposed to dichlorvos. The levels of intracellular dichlorofluorescein (DCF) in the cells were measured by fluorescence microscope and fluorometer. The fluorescence microscopic results showed an increased number of green fluorescent cells in dichlorvos-treated culture, and decreased when cells pre-treated with astaxanthin (Fig. 2a). Approximately, 50% increase in the DCF fluorescence level in the dichlorvos-treated samples was observed compared to control cells, whereas astaxanthin pre-treatment reduced

Fig. 1 Astaxanthin (AXT) protects yeast *Saccharomyces cerevisiae* from dichlorvos (DC) toxicity **a** Dichlorvos toxicity assay. Exponentially grown yeast (BY4741) cells were treated with different concentrations of dichlorvos for overnight. **b** Spot and **c** CFU assay. Cells were treated with or without AXT for 2 h followed by exposed to with or without dichlorvos (0.8 and 0.9 mM) for overnight. Cells were serially diluted, and spot and CFU assay were carried out. The resulted CFU of treated cells were expressed as relative percentage of survival in relation to untreated control (100%) which was obtained from the average standard deviation of three independent experiments. Values are mean $\pm$ SD; asterisk represents $p < 0.0001$ significant increase/decrease in AXT+DC-treated sample compare to DC-treated alone

the DCF fluorescence level to $\sim 21\%$ compared to control. These results suggest that the cytotoxic effect of dichlorvos was mediated by ROS-induced oxidative stress, and it was reduced by astaxanthin by scavenging ROS.

Astaxanthin induces SOD enzyme activity

Superoxide dismutases (SODs) are important enzymes involved in the cellular protection against oxidative stress. Our experimental results showed the reduced levels of SOD

activity in dichlorvos-treated cells compared to control, whereas astaxanthin pre-treated cells showed 30% increase in the SOD activity, compared to control. The SOD activity results suggested that astaxanthin might work by both scavenging ROS and inducing SOD enzyme activity to protect the yeast cells from oxidative stress-mediated killing by dichlorvos (Fig. 2b).

Astaxanthin reduces lipid peroxidation level

Lipid peroxidation occurs in cells when ROS damage the lipid membrane, resulting in the release of malondialdehyde (MDA) that causes damage to DNA and proteins. Our results showed that increased lipid peroxidation was observed in dichlorvos-treated cells. In contrast, the cells pre-treated with astaxanthin followed by dichlorvos treatment showed ~ 1.5 -fold decrease in lipid peroxidation (Fig. 2c). These results indicated that astaxanthin treatment reduced the lipid peroxidation induced by dichlorvos.

Astaxanthin induces reduced glutathione

Glutathione is one of the major non-enzymatic antioxidant molecules which plays a vital role in buffering the cells under oxidative stress (Jamnik et al. 2006). The result showed that twofold GSH induction in astaxanthin pre-treated samples when compared to untreated or dichlorvos control (Fig. 2d). These results indicate that the induction of GSH by astaxanthin helps in survival of yeast cells from oxidative stress-mediated cell death induced by dichlorvos.

Astaxanthin prevents growth defects caused by dichlorvos

The budding yeast *S. cerevisiae* is best known for its contribution in understanding eukaryotic cell cycle control. Growth curve experiment was carried out in the presence and absence of dichlorvos and astaxanthin in the yeast cells. Our results showed a 50-fold reduction in the growth of yeast cells treated with dichlorvos at 18 h. In contrast, astaxanthin pre-treated cells showed ~ 20 -fold increased growth, indicating astaxanthin protects yeast cells from the growth defects caused by dichlorvos treatment (Fig. 3a).

Astaxanthin reduces dichlorvos-induced apoptosis

Apoptotic cell death is characterized by controlled autodigestion of the cells which differs from necrosis with a distinct morphological and biochemical features such as chromatin condensation and DNA fragmentation. We measured DNA/nuclear fragmentation using DAPI, and chromatin condensation by AO/EB staining in the cells treated with dichlorvos and pre-treated with astaxanthin along with

Fig. 2 Measurement of oxidative stress markers. Exponentially grown yeast cells were pre-treated with or without AXT for 2 h followed by exposed with or without dichlorvos (0.8 mM) for 1 h. **a** ROS detection by $H_2DCF\text{-}DA$. Cells were treated with $H_2DCF\text{-}DA$ for 10–15 min in dark and washed, and images were taken in a fluorescent microscope and the level of fluorescence intensity was measured using spectrofluorometer. **b** SOD activity: SOD activity was measured by NBT reduction method. **c** Lipid peroxidation level: lipid peroxidation was measured by TBARS method; **d** reduced glutathione measurement. Reduced glutathione content was measured as described in materials and methods. Images are shown from at least three independent experiments. Values are mean $\pm$ SD; asterisk represents $p < 0.0001$ significant increase/decrease in AXT+DC-treated sample compared to DC-treated alone. Images are shown from at least three independent experiments

appropriate controls. DAPI-stained cells showed a bright blue color with higher fluorescence intensity indicating nuclear fragmentation and also that showed the cells are undergoing apoptosis. Our results showed that dichlorvos treatment exhibited more nuclear fragmented cells, whereas astaxanthin pre-treatment showed low fluorescence intensity and reduced nuclear fragmentation, indicating that astaxanthin protects the cells from nuclear fragmentation caused by dichlorvos (Fig. 3b).

AO/EB staining has been used as a reliable apoptosis marker for detecting cell death in yeast and mammalian

cells. The use of AO/EB staining to yeast cells allows the distinction of three types of cells, viable cells (uniform green nuclei with organized structure and green cytoplasm), early apoptotic cells (chromatin condensation, visible as a bright green spot that stands out in the green cytoplasm), and late stage of apoptotic cells (yellow–orange to red-condensed chromatin that stands out in the orange cytoplasm). In our study, dichlorvos treatment showed more number of late apoptotic cells with yellow–orange to red-condensed chromatin, whereas astaxanthin pre-treatment showed less chromatin condensation as bright green spot, indicating that

Fig. 3 Measurement of apoptotic markers. Exponentially grown yeast cells were pre-treated with or without AXT for 2 h followed by exposed with or without dichlorvos (0.8 mM). **a** Growth curve. **b** DAPI staining. Effect of AXT on nuclear fragmentation in dichlorvos-treated cells was carried out by DAPI staining. **c** AO/EB staining. Effect of AXT on the chromatin condensation was carried out by AO/EB staining. Values are mean $\pm$ SD; asterisk represents $p < 0.0001$ significant increase/decrease in AXT+DC-treated sample compared to DC-treated alone. Images are shown from at least three independent experiments

astaxanthin protects the cells from chromatin condensation caused by dichlorvos (Fig. 3c).

Discussion

Population explosion potentiated the green revolution to produce more food. As a result, pesticides of different groups were produced to support green revolution in the agriculture sector (Pingali 2012). The beneficial aspect of pesticides was hunger alleviation, which is largely met, but, on the other hand, the long-term usage of pesticides resulted in severe health issues. Pesticides are broadly classified into insecticides, herbicides, and fungicides based on their target pests. Dichlorvos is one of the pesticides commonly used for the protection of stored food grains. The core mechanism of dichlorvos is the inhibition of acetylcholinesterase (AChE); however, it induces the production of ROS (Booth et al.

2007; Das 2013; Ben Salem et al. 2015), which causes oxidative modification of proteins, DNA, and lipids (Espeland et al. 2010). Dichlorvos brings down the level of endogenous non-enzymatic (glutathione) and enzymatic (SOD and CAT) antioxidant levels (Singh and Sharma 2012; Das 2013) that leads to cell death. The use of cytoprotective compounds in the form of exogenous antioxidant can scavenge excess ROS which, in turn, helps in the cell survival and reduce diseases. A number of natural compounds including quercetin and rutin are reported to possess ROS scavenging and cytoprotective activity (Amari et al. 2008; Ben Salem et al. 2016). In the current study, we tested the cytotoxic effect of dichlorvos, and its protection by astaxanthin. Astaxanthin is a powerful antioxidant isolated from marine algae, *H. pluvialis* with broad health implications. Unlike other antioxidants such as beta carotene, vitamin E, C, D, and selenium, astaxanthin was never shown to be a pro-oxidant in the human body (Higuera-Ciapara et al. 2006; Papa et al. 2015). In this study, we have demonstrated that dichlorvos induces oxidative and apoptotic stress-mediated cell death in *S. cerevisiae* and that can be alleviated by astaxanthin supplementation.

Dichlorvos is toxic to *S. cerevisiae* cells and induces dose-dependent cytotoxicity (Fig. 1). Furthermore, dichlorvos-induced cell death was prevented by astaxanthin supplementation. A decrease in intracellular ROS, SOD, glutathione, and an increase in lipid peroxidation level in dichlorvos-treated cultures suggested that dichlorvos induced oxidative stress that results in cell death in yeast cells. A similar type of result was observed in dichlorvos induced oxidative stress in fish (Singh and Sharma 2012; Das 2013). Dichlorvos and lindane treatment have been reported to significantly decrease the SOD, catalases, and glutathione transferase activity in rat brain tissues and lipid peroxidation in human erythrocytes, which were reversed by ginger, vitamin C and E (Singh and Sharma 2012; Das 2013; Ojo et al. 2014). Increased levels of SOD activity, reduced glutathione, and decreased lipid peroxidation in astaxanthin pre-treated cells (Fig. 2) support spot and CFU results in which astaxanthin protected the yeast cells from dichlorvos-induced cytotoxicity. These results suggest that astaxanthin protects yeast cells from dichlorvos-induced oxidative stress. Other reports on these lines have also shown similar to our results where astaxanthin supplemented in the diet of rabbits showed enhanced SOD and thioredoxin reductase in the oxidative stress-induced rabbits (Augusti et al. 2012; Ambati et al. 2014). A well-known antioxidant, quercetin, has been shown to induce oxidative stress resistance not only by scavenging ROS but also by inducing endogenous antioxidant genes under oxidative stress conditions in *S. cerevisiae* and rat kidney.

ROS accumulation plays an important role in mediating the apoptosis mode of cell death in *S. cerevisiae* and

various mammalian cell lines. The yeast cells suffer from a prolonged exposure to ROS leads to the initiation of apoptosis because of the failure of pro-survival and antioxidant mechanism (Circu and Aw 2010). Apoptotic features such as increased chromatin condensation, increased nuclear fragmentation, and growth defects under dichlorvos-induced stress conditions (Fig. 3) suggest that dichlorvos induces apoptosis in yeast cells. In contrast, decreased chromatin condensation, decreased nuclear fragmentation, and protected cell growth defects in the astaxanthin pre-treated cultures suggested that astaxanthin protects against dichlorvos-induced apoptosis. The implication of oxidative stress and apoptosis in the etiology of many chronic and degenerative diseases suggests that antioxidant like astaxanthin dietary supplementation represents a promising avenue for antioxidant therapy (Kim and Kim 2018). In conclusion, our study shows that dichlorvos induces oxidative stress and apoptotic cytotoxicity, and astaxanthin supplementation protects the yeast cells largely by reducing oxidative stress and apoptosis.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interest.

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