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Beneficial effects of vitamin E supplementation against the oxidative stress on Cylindrospermopsin-exposed tilapia (*Oreochromis niloticus*)

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ABSTRACT

Cylindrospermopsin (CYN) is known to produce changes in some oxidative stress biomarkers in fish acutely and subchronically exposed to the toxin. The present study investigated the effects of vitamin E supplementation against the oxidative stress induced by pure CYN in tilapia (*Oreochromis niloticus*). Fish were pretreated with 700 mg vitamin E/kg fish body weight (bw)/day for 7 days by oral route, and on day seven, they received a single oral dose of 400 μg pure CYN/kg fish bw, and were killed after 24 h. The biomarkers evaluated included lipid peroxidation (LPO), protein and DNA oxidation, glutathione-S-transferase (GST), glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT) and γ-glutamyl-cysteine synthetase (γ-GCS) activities, and ratio of reduced glutathione-oxidized glutathione (GSH/GSSG). This is the first study showing that vitamin E supplementation is effective at reducing the toxicity induced by CYN, recovering the biomarkers assayed to basal levels. Therefore, vitamin E can be considered a useful chemoprotectant that reduces hepatic and renal oxidative stress and can be used in the prophylaxis and treatment of CYN-related intoxication in fish.

1. Introduction

Cyanobacterial harmful blooms (CyanoHABs) represent an environmental, public health and economic concern worldwide. Cylindrospermopsin (CYN) is an emerging cyanotoxin and one of the most important in terms of human health and environmental quality (Rogers et al., 2007). Currently, thirteen species are known to produce CYN (Anabaena bergii, Anabaena lapponica, Anabaena planctonica, Aphanizomenon flos-aquae, Aphanizomenon flos-aquae var. *klebahnii, Aphanizomenon gracile, Aphanizomenon ovalisporum, Cylindrospermopsis raciborskii*, *Lyngbya wollei, Oscillatoria* sp. PCC 6506, *Raphidiopsis curvata, Raphidiopsis mediterranea* and *Umezakia natans*) in four of the five continents: Oceania, Asia, America and Europe (Moreira et al., 2012). The variety of the CYN producers clearly indicates that the production is not species-specific and that the list of potential CYN producers may still remain incomplete. The toxicological studies to date have led to the establishment of a provisional Tolerable Daily Intake of 0.03 μg CYN/Kg/day, and a proposed guideline value of 1 μg CYN/L in drinking water by Humpage and Falconer (2003).

Field and laboratory studies demonstrated the effects, presence and transference of CYN in aquatic species including bivalves (Puerto et al., 2011a), crustaceans, snails and fish harvested from several water sources, as well as in vegetables and cereals (Freitas et al., 2015; Prieto et al., 2011), posing a serious concern especially if the organisms are destined for human consumption, fact that has been reviewed by some authors (Gutiérrez-Praena et al., 2013; Kinnear, 2010; Moreira et al., 2012). Moreover, it is known that the availability of extracellular CYN (>99% of total CYN) facilitates this exposure (Kinnear, 2010).

Although CYN mechanism of toxicity is not fully elucidated yet, it is considered a cytotoxin, affecting various organs and metabolic pathways, either directly or after some metabolic modification. CYN causes inhibition of protein (Terao et al., 1994) and glutathione (GSH) synthesis (Runnegar et al., 1994, 2002), as well as genotoxicity (Zegura et al., 2011) and endocrine disruption (Young et al., 2008). Some *in vitro* assays (Gutiérrez-Praena et al., 2011a, 2012a,b) and *in vivo* studies carried out in tilapia (*Oreochromis niloticus*) (Gutiérrez-Praena et al., 2011b; Guzmán-Guillén et al.,

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2013a; Puerto et al., 2011b; Puerto et al., 2014) have shown that oxidative stress is involved in CYN mechanism of toxicity, measured by levels of lipid peroxidation (LPO), protein and DNA oxidation, reduced-oxidized glutathione ratio (GSH/GSSG), and changes in the activity of different enzymes such as glutathione-S-transferase (GST), glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT), and γ-Glutamyl-cysteine synthetase (γ-GCS). To counteract these alterations, there are some antioxidants that act repairing the normal redox status or as preventive substances against reactive oxygen species (ROS). While it is possible that exposure to CYN may stress or overwhelm enzymatic defenses, there remains the possibility that prophylactic treatment with non-enzymatic substances may afford protection.

To date, some studies have documented the potential protection of antioxidants against the oxidative stress induced by other cyanotoxins as microcystins (MCs), including vitamin E (Pinho et al., 2005a,b; Prieto et al., 2008, 2009), selenium (Atencio et al., 2009) and N-acetylcysteine (NAC) (Puerto et al., 2009), most of them in tilapia (*O. niloticus*). Nevertheless, to our knowledge, the substances that have proved to protect fish against CYN intoxication from the point of view of oxidative stress are NAC (Gutiérrez-Praena et al., 2012d) and L-carnitine (LC) (ES 2460391A1; Guzmán-Guillén et al., 2013b). Moreover, we have recently demonstrated that vitamin E pretreatment (700 mg vit E/kg fish bw/day, for 7 days) prevented or ameliorated the histopathological lesions in liver, kidney, heart, intestinal tract, gills and brain of tilapia (*O. niloticus*) acutely exposed to a single oral dose of 400 μg pure CYN/kg fish bw (Guzmán-Guillén et al., in press).

Vitamin E is a generic descriptor for all molecules with α-tocopherol activity. All natural forms of vitamin E are d-stereoisomers formed by a substituted aromatic ring and a long isoprenoid side chain and, of the eight naturally occurring compounds with vitamin E activity, α-tocopherol has the highest biopotency. Vitamin E functions as a lipid-soluble chain-breaking antioxidant which protects polyunsaturated fatty acids (PUFAs) from lipid peroxidation by quenching and scavenging of various ROS, thus it helps to preserve the integrity of biological membranes, lipoproteins and lipid stores against oxidation (Hamre et al., 1998; Hung et al., 2007; Peuthert and Pflugmacher, 2010). Due to the stability of the resonance structure, the oxidized α-tocopherol is not reactive and therefore not able to continue to spread chain reactions (Chen et al., 1993).

Despite the known beneficial effects of vitamin E, little is published about its role in the modulation of the antioxidant defense in aquatic organisms exposed to MCs, such as crab species and tilapia, being effective in preventing some of the changes in oxidative stress markers (Pinho et al., 2005a,b; Prieto et al., 2008, 2009). However, since CYN is an emerging toxin, the potential effect of vitamin E supplementation on preventing the oxidative damage induced by this toxin in fish has not been previously investigated.

Taking into account the oxidative damage that CYN causes in fish, together with the increased occurrence of this toxin in freshwater systems, and the commercial importance of tilapia in aquaculture, it would be of interest to find compounds with the ability of preventing that damage without causing additional toxicity. In this context, the aim of the present work was to evaluate the protective role of a vitamin E pretreatment (700 mg vitamin E/kg fish bw/day, for 7 days) on the toxicity induced in liver and kidney of tilapia (*O. niloticus*) acutely exposed to a single oral dose of 400 μg pure CYN/kg fish bw. The biomarkers evaluated included LPO, protein and DNA oxidation, GST, GPx, SOD, CAT and γ-GCS activities, and GSH/GSSG ratio. To the extent of our knowledge and based on the existing literature, this is the first study that demonstrates the potential use of vitamin E in the prevention of oxidative stress damages in fish intoxicated with CYN.

2. Materials and methods

2.1. Chemicals

Vitamin E, as Trolox (±6-hydroxy- 2,5,7,8-tetramethylchromane-2-carboxylic acid), was obtained from Sigma–Aldrich (Madrid, Spain). Pure cylindrospermopsin (purity ≥ 95%) was supplied by Alexis Corporation (Lausen, Switzerland). All chemicals and reagents used in this study were analytical grade materials.

2.2. Experimental setup, acclimation of fish and exposure

Thirty-two male *O. niloticus* (Nile tilapia, Perciformes: *Cichlidae*; average weight 35 ± 7 g, and length 10 ± 2 cm) were obtained from Valenciana de Acuicultura (Valencia, Spain) and maintained at the laboratory in the same conditions as Guzmán-Guillén et al. (2013a). Before the beginning of the experiment fish were acclimatized for 15 days. Afterward, four experimental groups (n = 8) were considered and exposed both to vitamin E and CYN as in Guzmán-Guillén et al. (in press). Briefly, each group of fish was fed by a mixture of these components (fish food, vitamin E and/or pure CYN), according to the treatment, resulting in small pellets that were placed in the aquaria for fish to eat them:

(1) Control group: tilapia were daily fed only with commercial fish food for 7 days;
(2) Vitamin E control group: fish were daily fed with commercial fish food plus 700 mg vitamin E/kg fish, equivalent to 25 mg vitamin E/fish, for 7 days;
(3) Pure CYN control group: fish were daily fed with commercial fish food for 7 days and, on day 7, they received a single oral dose of 400 μg pure CYN/kg fish bw;
(4) Vitamin E + pure CYN group: fish were daily fed with commercial fish food plus 700 mg vitamin E/kg fish for 7 days and, on day 7, they received a single oral dose of 400 μg pure CYN/kg fish bw.

This dose of vitamin E was selected taking into account a previous study carried out in our laboratory with vitamin E on the same fish species intoxicated with CYN (Guzmán-Guillén et al., in press).

At the end of the experiment, 24 h after exposure to the toxin, all fish were anaesthetized in ice and euthanized by transection of the spinal cord. Liver and kidney were quickly removed, weighed, rinsed with ice-cold saline, frozen in liquid nitrogen and kept at −80 °C until further use.

2.3. Preparation of postmitochondrial supernatant (PMS) and protein estimation

Enzyme extracts from each tissue were prepared from each individual (not pooled) according to the method described by Puerto et al. (2009). Briefly, tissues were homogenized using 0.1 M potassium phosphate buffer (pH 6.5) containing 20% (v/v) glycerol, 1 mM ethylenediaminetetra-acetic acid, and 1.4 mM dithioerythritol. After removal of cell debris (10 min at 13,000*g*), the membrane fraction was separated by centrifugation at 105,000*g* for 60 min. The remaining supernatant, defined as the soluble (cytosolic) fraction, was used for subsequent determination of the oxidative stress parameters.

Protein contents in the samples were estimated by the method of Bradford (1976) using bovine γ-globulin as standard. Briefly, 5 μl of the diluted samples were mixed with 95 μl H_2O and 5 ml Coomassie Brilliant blue dye (Biorad Laboratories, Hercules, USA),