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Mutagenic and genotoxic potential of pure Cylindrospermopsin by a battery of *in vitro* tests

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ABSTRACT

Cylindrospermopsin (CYN) is a cyanobacterial toxin with an increasing world-wide occurrence. The main route of human exposure is through the ingestion of contaminated food and water. The European Food Safety Authority has identified the need to further characterize the toxicological profile of cyanotoxins and in this regard the genotoxicity is a key toxicological effect. The data available in the scientific literature show contradictory results. Thus, the aim of this study was to investigate the mutagenic and genotoxic effects of pure CYN using a battery of different *in vitro* assays including: the bacterial reverse-mutation assay in *Salmonella typhimurium* (Ames test) (0–10 μg/mL), the mammalian cell micronucleus (MN) test (0–1.35 μg/mL and 0–2 μg/mL in absence or presence of S9 fraction, respectively) and the mouse lymphoma thymidine-kinase assay (MLA) (0–0.675 μg/mL) on L5178YTk ± cells, and the standard and enzyme-modified comet assays (0–2.5 μg/mL) on Caco-2 cells. Positive results were obtained only when the metabolic fraction S9 was employed in the MN test, suggesting pro-genotoxic properties of CYN. Also, DNA damage was not mediated by oxidative stress as CYN did not induced changes in the modified comet assay. These data could contribute to a better risk assessment of this cyanotoxin.

1. Introduction

Cylindrospermopsin (CYN) is a cyanobacterial toxin considered as an emerging threat worldwide due to the progressive distribution of its main producer, *Cylindrospermopsis raciborskii* (Kinnear, 2010; Poniedziałek et al., 2012). CYN molecule consists of a tricyclic guanidine group combined with a hidroximethyl uracil (Ohtani et al., 1992). Its zwitterionic nature makes it highly water soluble and stable at extreme temperatures and pH (Falconer and Humpage, 2006). Humans are more susceptible to the exposure to CYN in comparison to other cyanotoxins because up to 90% of total CYN is found outside the cyanobacterial cells (Rücker et al., 2007). In this sense, CYN has been documented as being involved in at least two epidemical cases of human poisoning (Carmichael et al., 2001; Griffiths and Saker, 2003) and it has been identified in some water reservoirs supplying drinking water (Bittencourt-Oliveira et al., 2014; Lei et al., 2014). Moreover, human exposure to CYN is possible through other different pathways such as bathing, recreational water activities, and, mainly, by the consumption of contaminated food (fish, mollusks, vegetables, etc.) (Gutiérrez-Praena et al., 2013). In fact, the bioaccumulation of CYN on fish (up to 2.7 ng/g, Messineo et al., 2010), crayfish (up to 4.3 mg/g, Saker and Eaglesham, 1999), mussels (up to 2.52 mg/g, Saker et al., 2004), lettuce (up to 8.029 μg/kg, Cordeiro-Araújo et al., 2017) etc. has been reported. In this regard, the European Food Safety Authority (EFSA) has recommended to collect more data about the toxicological profile of cyanotoxins, including CYN (Testai et al., 2016), and Humpage and Falconer (2003) proposed a tolerable daily intake of 0.03 mg CYN/kg of body weight.

The main target of CYN activity is the liver (Bernard et al., 2003; Zegura et al., 2011a). However, it has been also characterized as cytotoxic due to its negative effects on different organs (Runnegar et al., 1994; Terao et al., 1994; Falconer and Humpage, 2006; Guzman-Guillen et al., 2013). One of CYN best-known modes of action is the irreversible inhibition of protein synthesis (Terao et al., 1994; Runnegar et al., 1995; Froscio et al., 2003) that leads to cytotoxic effects in a variety of cell cultures (hepatocellular, gastrointestinal tract and kidney cell lines) (Humpage et al., 2005; Froscio et al., 2009; Gutiérrez-Praena et al., 2012a). Moreover, some authors suggest that CYN cytotoxicity is also dependent on a cytochrome p-450 metabolism (Runnegar et al., 1995; Froscio et al., 2003) and the activation of CYN seems to enhance

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even more its toxicity (Norris et al., 2002; Humpage et al., 2005; Štraser et al., 2011; 2013a). It has been shown that CYN induces oxidative stress mediated by reactive oxygen species (ROS) in a variety of cell types including mouse hepatocytes (Humpage et al., 2005; López-Alonso et al., 2013); fish leucocytes (Sieroslawska and Rymuszka, 2013), fish hepatocytes (Liebel et al., 2011) and cell lines from different origin such as fish liver (PLHC-1) (Gutiérrez-Praena et al., 2011a); human colon (Caco-2) (Gutiérrez-Praena et al., 2012a), umbilical vein endothelium (HUVEC) (Gutiérrez-Praena et al., 2012b) and human hepatoma (Hep-G2) (Štraser et al., 2013b).

On the other hand, different authors considered CYN a genotoxic compound due to its effects on DNA (Zegura et al., 2011a; Moreira et al., 2012). Also, some structural properties, such as the presence of uracil (Shen et al., 2002) or potentially reactive guanidine and sulfate groups (Bain et al., 2007) have led researchers to suggest a possible interaction of CYN with nucleic acids. Two mechanisms have been suggested for its genotoxic activity: 1) loss of kinetochore/spindle functions; 2) induction of DNA strand breaks (SBs) at DNA level (Sieroslawska, 2010). However, these mechanisms potentially involved in its genotoxicity are still under investigation.

In the scientific literature there are different reports dealing with the *in vitro* mutagenic/genotoxic effects of CYN using different assays (for a review see Pichardo et al., 2017). Thus, with respect to the Micronucleus (MN) assay, one of the preferred methods for assessing chromosome damage (EFSA, 2011), positive results have been obtained in different experimental models. For example, Humpage et al. (2000) observed that CYN (6 and 10 μg/mL) increased the incidence of MN in the WIL2-NS lymphoblastic cell line after 48 h exposure. Similar results were obtained by Štraser et al. (2011) in Hep-G2 cells exposed to 0.05 and 0.5 μg/mL CYN and in peripheral blood lymphocytes (HPBLs) exposed to 0.1 and 0.5 μg/mL CYN as shown by Žegura et al. (2011b). In the common carp (*Cyprinus carpio* L.) leucocyte cell line 0.1 and 0.5 μg/mL CYN did not show genotoxic effects by the MN assay, but at 1 μg/mL, the frequency of MN was significantly higher (Sieroslawska and Rymuszka, 2015).

According to the studies performed with the comet assay, no DNA damage was detected on chinese hamster ovary (CHO-K1) cells after 24 h of treatment with CYN concentrations of 0.5 and 1 μg/mL (Fessard and Bernard, 2003) and on fish hepatocytes exposed to 0.1–10 μg/mL for 72 h (Liebel et al., 2011). However, significant increases of DNA SBs were observed in mouse hepatocytes (0.05–0.5 μg/mL) after 18 h exposure (Humpage et al., 2005), in HPBLs (0.05–0.5 μg/mL) after 4 h (Žegura et al., 2011b) as well as in Hep-G2 cells (0.01–0.5 μg/mL) after 12, 24 and 72 h (Štraser et al., 2011; Štraser et al., 2013b,c). Taking into account all these reports, it seems that the concentrations of CYN employed, the experimental model, and the type of assay used have a role on the results obtained. Moreover, there are additional data gaps regarding the genotoxic potential of CYN. Thus, although it is well known that CYN induces oxidative stress it has been not yet elucidated whether this mechanism contributes to CYN genotoxicity.

Studies concerning the influence of CYN on oxidative DNA damage *in vitro* are very scarce. Humpage et al. (2005) and Štraser et al. (2013b) concluded that ROS is not a mediator of CYN genotoxicity. However, 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-OHdG), a DNA oxidation product, was detected in a fish leucocyte cell line after 24 h treatment with CYN (Sieroslawska and Rymuszka, 2015). *In vivo*, DNA oxidation was also detected in tilapia sub-chronically exposed to environmental relevant concentrations (Guzman-Guillen et al., 2013). Another controversial point not yet fully elucidated is whether CYN is a genotoxic or a pro-genotoxic compound due to CYP-mediated metabolites (Zegura et al., 2011a; Štraser et al., 2013a).

All these contradictory results and uncertainties increase the concerns about CYN genotoxicity and make necessary to elucidate its genotoxic potential. In this sense, the scientific opinion of EFSA on genotoxicity testing strategies applicable to food and feed safety assessment recommends a step-wise approach beginning with a basic battery of *in vitro* tests, comprising 1) a bacterial reverse mutation test (OECD 471), which detects gene mutations and 2) an *in vitro* mammalian cell micronucleus test (OECD 487), that covers both structural and numerical chromosome aberrations (EFSA, 2011). Regarding to the Ames test, there is only one report which evaluated the capacity of CYN as mutagenic agent, showing negative results (Sieroslawska, 2013) whereas the MN assay has shown positive results (Bazin et al., 2010; Štraser et al., 2011; Hercog et al., 2017).

Taking into account these facts, the aim of this study was to investigate the potential mutagenicity and genotoxicity of CYN using a battery of different *in vitro* assays including: the bacterial reverse-mutation assay in *Salmonella typhimurium* (Ames test, OECD 471), the MN test (OECD 487), the standard comet assay on Caco-2 cells and the mouse lymphoma thymidine-kinase assay (MLA) on L5178Y$Tk^{+/-}$ cells, this one applied for the first time to study CYN genotoxicity. Moreover, to investigate the involvement of oxidative stress on CYN induced DNA damage, an enzyme-modified version of the comet assay was performed. In addition, the role of metabolism in CYN genotoxicity was studied by using the S9 fraction as metabolic activation system in the Ames, MN and mouse lymphoma assays in order to establish if CYN is a genotoxic or a pro-genotoxic cyanotoxin.

2. Materials and methods

2.1. Supplies and chemicals

Cylindrospermopsin standard (purity 95%) was supplied by Alexis Corporation (Lausen, Switzerland). All assay chemicals were purchased from Sigma–Aldrich (Madrid, Spain), Gibco (Biomol, Sevilla, Spain), Moltox (Trinova, Biochem, Germany) and C-Viral S.L. (Sevilla, Spain).

2.2. Cells and culture conditions

For the Ames test, five *Salmonella typhimurium* histidine-auxotrophic strains TA97A, TA98, TA100, TA102 and TA104 were used. For the MN and MLA tests, L5178Y $Tk^{+/-}$ mouse lymphoma cells were originally provided by Dr. Olivier Gillardeux (Safoni-Synthélabo, Paris, France). Caco-2 cells, used for standard and enzyme-modified comet assays are derived from a human colon carcinoma (ATCC© HTB-37). L5178Y$Tk^{+/-}$ cells and Caco-2 cell line were cultured and maintained according to Mellado-García et al. (2017).

2.3. Test solutions

A stock solution of CYN (1000 μg/mL) was prepared in milliQ sterile water and maintained at less than 4 °C. The exposure concentration solutions were made by dilution in sterile MilliQ water (Ames test), RPMI 1640 medium (MN and MLA assays) or MEM medium (standard and modified-comet assays).

2.4. Ames test

The mutagenicity test was performed following the principles of OECD guideline 471 (1997) with the modifications described in Mellado-García et al. (2015), as follows. Briefly, cultures of five *Salmonella typhimurium* histidine-auxotrophic strains (TA97A, TA98, TA100, TA102 and TA104) were prepared from their main strain plates and used in their late exponential growth phase. CYN mutagenic activity was assessed in three independent experiments using three technical replicates per concentration and per experiment, and in the absence and presence of S9 fraction as metabolic activation system from rat livers (S9 fraction induced by Aroclor-1254). The S9 fraction is an exogenous metabolising system that should be used when employing cells with inadequate endogenous metabolic capacity (OECD guideline 487). Each experiment included five increasing concentrations of CYN (0.625–10 μg/mL) selected according to Sieroslawska (2013). Higher

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