Genotoxicity of Cyanotoxins

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Abstract

According to the stability and propensity for biomagnification along food webs, the cyanotoxins produced by more than 40 species of cyanobacteria have a detrimental effect on the aquatic food web. A more plausible exposure scenario involves a combination of cyanotoxins, and interaction phenomena cannot be completely ruled out. Therefore, the combined effects of multiple cyanobacterial toxins can potentially be more dangerous than just one of them. In brackish and freshwater blooms, the cyclic heptapeptides microcystins (MCs) and nodularin (NODs) are the most ubiquitous and common cyanotoxins. The primary route through which humans are exposed to cyanotoxins is oral ingestion. Their hepatotoxic, dermatoxic, neurotoxic, cytotoxic, and genotoxic properties pose some risks to the general public's health with increasing attention. Well-known cyanotoxins with genotoxicity include microcystins (MCs), nodularin (NODs), and cylindrospermopsin (CYN), which are commonly found in freshwater environments. Several in vitro and in vivo assays may be used to examine the

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potential genotoxicity of cyanotoxins. Thus, the in vitro and in vivo assay protocols for determining the genotoxicity of cyanotoxins produced by cyanobacteria are covered in this chapter.

Keywords

Cyanobacteria · Cyanotoxins · Genotoxicity · In vitro and in vivo assays · Assay protocols

37.1 Introduction

Eutrophication of waterbodies and global warming is the long-term effect of environmental pollution, and they cause lots of damage to the flora and fauna diversity of the ecosystem. Consequently, the manifestation of cyanobacterial blooms in the aquatic environment has surprisingly increased in past decades. More than 40 cyanobacteria genera produce cyanotoxins that adversely affect the biome (Žegura et al. 2011). Toxin-producing cyanobacteria blooms have been recorded from terrestrial, freshwater bodies, viz., ponds, rivers, lakes, and brackish water throughout the world (Žegura et al. 2011; Yilmaz et al. 2022).

Cyanotoxins are precarious for humans, other animals, and aquatic organisms. In humans, intoxication with cyanobacteria-contaminated water causes symptoms such as nausea/vomiting, weakness, skin irritation, and illnesses ranging from gastroenteritis and pneumonia to hepatoenteritis (Zanchett and Oliveira-Filho 2013). Oral ingestion is the main method by which humans are exposed to cyanotoxins and carry some dangers for the public's health with increased exposure due to their hepatotoxic, dermatoxic, neurotoxic, cytotoxic, and genotoxic effects of them (Table 37.1). Cases of poisoning in humans and animals due to consuming contaminated water with cyanotoxins were reported for the first time in the late 1800s. The mechanisms behind the toxic effects of cyanotoxins are different because they are structurally diverse chemicals (Žegura et al. 2011; Zanchett and Oliveira-Filho 2013).

Microcystins, nodularin, and cylindrospermopsin are well-documented cyanotoxins with genotoxicity, and these cyanotoxins are commonly found in freshwater environments. The genotoxicity effect of the cyanotoxin can be analyzed using a set of in vitro and in vivo assays (Žegura et al. 2011). The in vitro assays can be performed using bacteria systems, viz., *Salmonella typhimurium* TA1537, *Escherichia coli* PQ37, *Bacillus subtilis* 168, animal cell lines, viz., rat hepatocytes, hamster ovary cell lines, and human cell lines such as human hepatoma cell line, human lymphocytes, and human glioblastoma cell lines (Žegura et al. 2011; Tejs 2008; Guo et al. 2020). Further, the genotoxicity of cyanobacteria can be assessed using animal models such as Swiss albino mice, Kunming mice, Fischer 344 rats, and Sprague–Dawley rats as test organisms and those studies have been categorized as in vivo assays. Thus, this chapter discusses the in vitro and in vivo assay protocols for detecting the genotoxicity of cyanotoxins produced by cyanobacteria.

Table 37.1 Types of cyanotoxins, toxin-producing cyanobacteria and effect on human health

Type of cyanotoxin	Toxin-producing cyanobacteria	Effect of human health	References
Microcystins (MCs)	*Microcystis aeruginosa, Cyanobium bacillare*	Neurotoxins, Genotoxin	Žegura et al. (2011), Yilmaz et al. (2022), Zanchett and Oliveira-Filho (2013)
Nodularin (NOD)	*Nodularia spumigena*	Hepatotoxins, Genotoxins	Žegura et al. (2011), Yilmaz et al. (2022)
Cylindrospermopsin (CYN)	*Anabaena lapponica, Cylindrospermopsis raciborskii, Lyngbia wollei*	Hepatotoxins, Genotoxins	Žegura et al. (2011), Yilmaz et al. (2022)
Anatoxin-a (Antx-a)	*Anabaena planctonica, Anabaena spiroides, Microcystis* sp.	Hepatotoxins, Neurotoxins	Žegura et al. (2011), Yilmaz et al. (2022), Zanchett and Oliveira-Filho (2013)
Anatoxin a-(s) (Antx-a(s))	*Anabaena flos-aquae and Anabaena lemmermannii*	Neurotoxins	Žegura et al. (2011), Zanchett and Oliveira-Filho (2013)
Saxitoxins (STX	*Anabaena circinalis, Cylindrospermopsis raciborskii*	Neurotoxins	Žegura et al. (2011), Zanchett and Oliveira-Filho (2013)
Microviridin J	*Microcystis* spp.		Žegura et al. (2011), Zanchett and Oliveira-Filho (2013)

37.2 Materials

- Cyanotoxin standards
- Bacteria culture media
- Bacteria strains
- Cell lines
- Cell culture media
- Cell culture treated culture plates
- Trypsin-EDTA
- Cell staining solutions

37.3 Methods

37.3.1 In Vitro Studies

37.3.1.1 Bacteria System

Bacterial Reverse Mutation Test (Ames test)

The Ames Salmonella/microsome mutagenicity assay (Salmonella test; Ames test) is a short-term bacterial reverse mutation assay specifically designed to detect a wide range of chemical substances that can produce genetic damage that leads to gene mutations (Tejs 2008).

1. Steps are taken prior to experimenting:
   (a) Inoculate Salmonella cultures 12 h prior to performing the experiment.
   (b) Label an appropriate number of minimal glucose (GM) agar plates and sterile test tubes for each test chemical.
   (c) Prepare metabolic activation system and keep on ice until use.
   (d) Prepare cyanotoxin dilutions.
   (e) Melt top agar supplemented with 0.05 mM histidine and biotin and maintained at 43 °C to 48 °C.
2. To the 100 mL sterile tubes maintained at 43 °C, add in the following order with mixing after each addition:
   (a) 0.01 mL of the test cyanotoxin dilution.
   (b) 2.5 mL of molten top agar with overnight culture of the *Salmonella* strain.
   (c) 0.5 mL of metabolic activation (S-9) mix.
3. The contents of the test tubes are then mixed and poured onto the surface of GM agar plates.
4. When the top agar has hardened (2-3 min), the plates are inverted and placed in a 37 °C incubator for 48 h.
5. The colonies are then counted, and the results are expressed as the number of revertant colonies per plate.

37.3.1.2 Cell Lines

Micronucleus (MN) Assay Using Human Hepatic Cell Line HepaRG

The micronucleus (MN) assay is a core test used to evaluate the genotoxic potential of xenobiotics. The traditional in vitro MN assay is usually conducted in cells lacking metabolic competency or by supplementing cultures with an exogenous rat S9 metabolic system. This creates a significant assay limitation for detecting genotoxic metabolites (Guo et al. 2020; Díez-Quijada et al. 2019).

1. Prepare HepaRG human hepatoma cell line.
   (a) Seed proliferative HepaRG cells at passages 13–19 at 1.3×10^4 cells cm^2 in a 100-mm tissue culture dish and culture in 10 mL William's E medium supplemented with 1% GlutaMax, 1% Pen-Strep solution, and growth

additives (Lonza, Walkersville, MD) at 37 °C for 14 days in a humidified atmosphere with 5% CO_2.

(b) Differentiate cells in William's E medium supplemented with differentiation additives (Lonza) for an additional 14 days.
(c) Detach the fully differentiated HepaRG cells by adding trypsin—EDTA and reseed in a 96-well plate at a density of 5×10^4 cells/well in William's E differentiation medium and cultured for 3 days prior to cyanotoxin treatment.
(d) Prepare toxin stock solutions by dissolving each cyanotoxin standard in DMSO.
(e) Serially dilute working solutions in 100 μL differentiation medium, and the final concentration of DMSO in the medium never exceed 1%.
(f) Freshly prepared Cisplatin, ENU, and hydroquinone (HQ) solutions before each experiment. Following 24-h treatment, remove media and maintain HepaRG cells in fresh differentiation medium supplemented with 100 $ngml^{-1}$hEGF for an additional 72 h prior to the MN assay.

2. Perform cytokinesis-block micronucleus (CBMN) assay.
(a) Detach the cells by trypsinization and adhere to Goldseal microscope slides (ThermoFisher) by centrifuging 200 to 300 μL cell suspension at 850 rpm for 4 min in a CytoFuge 2 (Statspin).
(b) Briefly air dry the slides and fix in absolute methanol for 10 min, and store at ambient temperature. Stain the HepaRG cell slides by applying 25 μL 250 $ng\mu l^{-1}$propidium iodide in 1× DPBS, covering with a 25 mm^2 cover glass and incubating for 5 min at ambient temperature.
(c) Wick away excess PI staining solution from the slides with a Kimwipe and mount the cells in 25 μL SlowFade Gold Antifade with DAPI.
(d) Examine slides with a DAPI-Texas Red-FITC triple band pass filter at 400× and MN verify using a DAPI single band pass filter at 1000 × magnification on an Eclipse 80i microscope.
(e) Evaluate the cytotoxic and cytostatic effect of each treatment using the cytokinesis-block proliferation index (CBPI), by counting 500 cells that were either single-nucleated, BN or multi-nucleated.

37.3.2 In Vivo Studies

Although the genotoxicity of cyanotoxin has been extensively studied in vitro, limited data are available on its in vivo genotoxicity effect of cyanotoxins (Díez-Quijada et al. 2019).

37.3.2.1 Comet Assay Using Wister Rats

1. Obtain ethical clearance for the animal study.
2. Purchase male Wistar rats and weigh the initial body mass of each animal.
3. Acclimates the animals to the environmental conditions.

(a) 12-h dark/light cycle, control temperature (23 ± 1 °C), relative humidity (55 ± 10%) for 1 week before the experiment.
(b) During this time, feed the animals with a standard laboratory diet and water.
(c) Use 5 animals per group and the 3 animals for positive controls.

4. Treat with different concentrations of cyanotoxins.
   (a) Weigh the body mass of each animal in order to ensure that weight variation did not exceed ±20% and randomly divide into groups.
   (b) Prepare the cyanotoxin concentration series in water in a final volume of 1 mL.
   (c) Feed the animals by gavage using an enteral feeding tube at 0; 24 and 45 h.
   (d) The animals were sacrificed 3 h after the final dose administration comet.
   (e) Record the clinical signs and body weight of each animal during the treatment period.
5. Sample collection.
   (a) Remove and dissect the elected tissues, then rinse with cold saline solution and weigh.
   (b) After weight, process stomach and liver quickly for the comet assay. Collect blood samples and maintain in Vacutainer® sodium heparin tubes.
   (c) For the histopathological study, collect a portion of stomach and liver samples from each individual separately and store.
6. Prepare samples for comet assay.
   (a) Prepare single cell suspensions from the stomach and liver.
   (b) Wash tissues with Merchant's buffer (MB) (0.14 M NaCl, 1.47 nM KH_2PO_4, 2.7 mMKCl, 8.1 mM Na_2HPO_4 , 10 mM Na_2 EDTA, pH 7.4) and discard.
   (c) Homogenize a portion of each tissue in cold.
   (d) Centrifuge the homogenates and mix with 5 mL buffer after filtration until slide preparation.
   (e) Mix heparinized blood samples v/v (1/1) with phosphate-buffered saline (PBS) solution and isolate the lymphocytes with Histopaque® (Sigma-Aldrich, Madrid, Spain) and centrifuge (400 G, 30 min).
   (f) Wash the cells twice with PBS and re-suspend in PBS at a concentration of 2×10^5 cells/mL.
7. Perform comet assay.
   (a) For blood samples, mix 30 μL of cells suspension with 140 μL of 0.5% low-melting point agarose.
   (b) Place 5 μL aliquots on a microscope slide.
   (c) For the stomach and liver, mix the cells suspensions with 1% low-melting point agarose and place the mixtures on a microscope slide similar to blood samples.
   (d) Perform standard and modified comet assays.
   (e) Briefly, after lysis, wash slides three times for 5 min with enzyme buffer (40 mM HEPES; 0.1 M KCl; 0.5 mM EDTA; 0.2 mg/mL bovine serum albumin; pH 8).

(f) Expose two gels in each slide to 30 μL lysis solution; enzyme buffer alone (buffer F); buffer F containing Fpg or Endo III for 30 min in a metal box at 37 °C.
(g) Denature the nuclei and perform electrophoresis for 20 min, 0.81 V/cmup to 400 mA.
(h) Neutralize the DNA in PBS, wash with water and fix with 70% and absolute ethanol before staining.
(i) Analyze at least 150 randomly selected nuclei per animal with the image analysis software Comet Assay IV.

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