Toxicon 86 (2014) 8–15

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

Saxitoxins induce cytotoxicity, genotoxicity and oxidative stress in teleost neurons *in vitro*

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ARTICLE INFO

Article history:
Received 11 January 2014
Received in revised form 25 April 2014
Accepted 29 April 2014
Available online 9 May 2014

Keywords:
Neuron primary culture
Environmental neuroscience
Neurotoxicology
Teleost

ABSTRACT

The aim of this study was establish a protocol for isolation and primary culture of neurons from tropical freshwater fish species *Hoplias malabaricus* for assessment of the effects of neurotoxic substances as saxitoxins (STXs). Cells from brain of *H. malabaricus* were treated with different concentrations of trypsin, dispase and papain for tissue dissociation. Cells type was separated by cellular gradient and basic fibroblast growth factor (bFGF) supplement nutrition media were added. The dissociated cells were plated with medium and different STXs concentrations and the toxic cellular effects such as oxidative stress, neurotoxicity, and genotoxicity and apoptosis process were evaluated. Cultures treated with bFGF showed the greatest adherence, survival and cellular development. STXs increased specific activity of glutathione peroxidase and lipoperoxidation levels, were cytotoxic and genotoxic indicated by the comet assay. Although the STXs effects due the blockage of sodium channels is reported to be reversible, the time exposure and concentration of STXs suggested cellular injuries which can lead to neuropathology. The establishment of primary neuronal culture protocol enables new applications for neurotoxicological assessments.

1. Introduction

Cyanobacterial blooms have become a serious environmental problem due changing the water quality and endanger the ecological balance due to the production of toxins strains such as saxitoxins (STXs) (Ibbelings and Chorus, 2007). Saxitoxins are also known as paralytic shellfish poisons (PSPs) and are water-soluble toxins that

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http://dx.doi.org/10.1016/j.toxicon.2014.04.016
0041-0101/

block sodium channels of neurons, leading in some cases death due the respiratory fault (Llewellyn, 2006). Many STXs analogs have been identified and are produced by several cyanobacteria such as *Cylindrospermopsis raciborskii*, a of highly tolerant species growing well at 20 °C and 35 °C, and is predominantly in tropical and subtropical areas, including freshwater ecosystems (Hamilton et al., 2005).

In southern Brazil, blooms of *C. raciborskii* in a water supply reservoir may pose significant risks to public health (Clemente et al., 2010). To protect the human population in this region, a maximum tolerance acceptable of 3 μg L^{-1} saxitoxin equivalents (STXeq) for drinking water was established (Brasil, 2012). *In vivo* studies showed the possible bioaccumulation of toxins in fish (Clemente et al., 2010; Silva et al., 2011), however, the neurotoxic effects of this toxin *in vitro* studies using fish neurons has not been reported yet.

Although mammalians species such as rats are widely used as models for studies *in vitro*, fish can also be used for assessing the effects of substances such as environmental pollutants (Bussolaro et al., 2010; Filipak Neto et al., 2007, 2008; Segner, 1998). In this context, the effects of neurotoxins have been studied in fish (Clemente et al., 2010; Silva et al., 2011; Silva de Assis et al., 2013); however, some mechanisms are still unknown and must be further examined.

In the present study, the freshwater fish *Hoplias malabaricus* was chosen as a model to establish neuronal primary cultures. This species occupies a high trophic level and is widely distributed in Brazilian rivers and reservoirs (Hensley and Moody, 1975) exhibiting blooms of *C. raciborskii*. Furthermore, it has been used by our group as an established model for *in vivo* (Miranda et al., 2008; Oliveira Ribeiro et al., 2006; Silva et al., 2011; Silva de Assis et al., 2013) and *in vitro* studies (Filipak Neto et al., 2007, 2008) with primary hepatocyte cell culture. To establish a robust protocol for studying neurotoxic effects in fish, a simple novel method is described here to obtain highly purified cultures of neuronal cells from *H. malabaricus* using biomarkers of oxidative stress and genotoxicity and cytotoxicity assays.

2. Materials and methods

2.1. Cells isolation and neuron primary culture: protocol

2.1.1. Isolation of brain cells

All procedures and protocols were submitted and approved by the Ethical Committee for Animal Experimentation at the Federal University of Paraná. For each experiment, a single adult fish (total experiment = 5 fish; 19.2 ± 1.6 cm; mean ± SD) was anesthetized and sacrificed by spinal transection. In a sterile culture hood the fish was rinsed with ethanol 70%, decapitated and the head was again rinsed with chlorhexidine 2%. The brain was removed, weighed and transferred to Hank's balanced salt solution (HBSS; Sigma) supplemented with 2 mM glucose (Sigma) and antibiotic penicillin/streptomycin (ATB; 50 U mL^{-1} Pen, 50 μg mL^{-1} Strept; Sigma). The meninges were removed and the brain was sliced at 0.5 mm using a scalpel. These were transferred to a 15 mL polystyrene centrifuge tube (Corning) containing 6 mL of Neurobasal A (Gibco) with B27 supplement (B27; 1:50 medium; Gibco) and dissociation enzymes (as described in Section 2.1.2). For dispase and papain, tissue was digested for 30 min at 30 °C with occasional gentle mixing. The tissue was triturated with a flame-polished Pasteur pipette for 1 min and incubated for 15 min at 30 °C (5 min for trypsin). This procedure was repeated twice. The total time of digestion was 1 h for dispase and papain and 15 min for trypsin. After digestion, the cellular suspension was transferred to a new 15 mL tube containing 4 mL density gradient prepared with Optiprep (Sigma) and Neurobasal/B27 medium according to Brewer and Torricelli (2007) and centrifuged at 8000 × g for 15 min at 22 °C. To remove the debris and other cell types, the top 6 mL, fraction 1 (oligodendrocytes) and fraction 2 (cellular fragments) were discarded. The volume 0.5 mL–2.0 mL (fraction 3) from the bottom of the tube containing the cellular gradient was transferred to a new 15 mL tube. Further, 5 mL of Dulbecco's Modified Eagle Medium (DMEM/F12; Gibco) or DMEM/F12 + 5% fetal bovine serum (FBS, Gibco) was added when trypsin was employed and cells were centrifuged at 800 × g for 5 min at 22 °C. The pelleted cells were washed in DMEM/F12 and collected by centrifugation at 800 × g for 5 min at 22 °C. The cells were re-suspended in culture medium and seeded in Petri dishes under different conditions.

2.1.2. Effect of different proteases on tissue dissociation

After the mechanic dissociation of the brain tissue, the slices were treated with three types of proteases in Neurobasal A medium supplemented with B27 and ATB: trypsin (1.33 mg mL^{-1}; Sigma), dispase (1 U mL^{-1}; 5 U mL^{-1}; 10 U mL^{-1}; Gibco), and papain (10 U mL^{-1}; 20 U mL^{-1}; 30 U mL^{-1}; Sigma) for a total duration of 1 h, except for the trypsin group that was treated for 15 min. The cellular suspension was transferred to a density gradient solution and centrifuged as described above. The total and viable cells were counted by Trypan Blue exclusion in a Neubauer chamber (Hu and Fakahany, 1994). This procedure was repeated at least five times for each protease.

2.1.3. Effect of coating substrate

The 96-well plates with flat bottom (Corning) were coated for 16 h at 24 °C with the following substrates: poly-L-lysine (15 μg mL^{-1}, Gibco), fibronectin from human plasma (15 μg mL^{-1}, Sigma) or matrigel (15 μg mL^{-1}, Kleinman et al., 1982). Prior to use, the plates were washed three times with phosphate buffered saline (PBS; Gibco). The viable cells from papain digestion (30 U mL^{-1}) were re-suspended in DMEM/F12 medium supplemented with B27 + 5% FBS + Glutamine (0.29 mg mL^{-1}, Dibco) + ATB + basic fibroblast growth factor (bFGF) (from bovine pituitary glands; 3 ng mL^{-1}; Sigma) and seeded at 2×10^5 cells for the experimental condition. The well volume was adjusted for 100 μL of medium and the cells were incubated for 1 day at 24 °C and 1.7% CO_2 in a humidified environment. The viability of the cultured cells was determined by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-bromide-diphenyl-2H tetrazolium (MTT method) (Sarmento et al., 2004).