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Effects of the cyanobacterial neurotoxin β-*N*-methylamino-L-alanine on the early-life stage development of zebrafish (*Danio rerio*)

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ABSTRACT

β-*N*-Methylamino-L-alanine (BMAA), a neurotoxic amino acid, is produced by members of all known groups of cyanobacteria. In the presence of added carbonate, BMAA generates an analogue of glutamate which has been associated with motor neuron (MN) diseases via a mechanism of motor neurone specific excitotoxicity. The toxicity of BMAA has been established in various mammalian test models, but the widespread aquatic production of BMAA raises questions of BMAA toxicity to aquatic organisms. Zebrafish (*Danio rerio*) embryos were exposed to varying concentrations of BMAA (5–50,000 $\mu g\,l^{-1}$) with and without added carbonate. BMAA exposure induced a range of neuro-muscular and developmental abnormalities in *D. rerio*, which can be directly related to disruptions to glutamatergic signalling pathways. When exposed to BMAA plus added carbonate, the incidence of pericardial oedema increased by up to 21% in test subjects, correlating with a reduction in heart rate. Increased incidence of abnormal spinal axis formation was seen in all *D. rerio* larvae exposed to BMAA concentrations of $\geq$50 $\mu g\,l^{-1}$, with a further 10% increase from $\geq$500 $\mu g\,l^{-1}$ BMAA when carbonate species were present. A dose-dependent increase in clonus-like convulsions was observable in embryos exposed to $\geq$5 $\mu g\,l^{-1}$ BMAA ± added carbonate. This is the first study on the neuro-muscular and developmental effects of BMAA exposure on aquatic vertebrates. The present findings, plus the potentially widespread production of BMAA in aquatic cyanobacteria, indicate a need for information of exposure levels, duration and toxic outcomes in aquatic biota.

1. Introduction

Cyanobacteria (blue-green algae) are receiving increased scientific and public attention, due in part to an apparent increasing incidence of cyanobacterial blooms in high resource waterbodies, and the production of a diverse range of potent cyanotoxins (Codd et al., 2005). β-*N*-Methylamino-L-alanine (BMAA) is a neurotoxic amino acid, first reported over 40 years ago in extracts of the cycad tree, *Cycas micronesica* (Vega and Bell, 1967). Evidence is emerging for the widespread production of BMAA by free-living freshwater, marine, brackish water and terrestrial cyanobacterial species, as well as by symbiotic cyanobacteria (Cox et al., 2003, 2005; Murch et al., 2004; Banack et al., 2007; Metcalf et al., 2008; Esterhuizen and Downing, 2008).

A causative association between human dietary exposure to BMAA and the increased incidence of amyotrophic lateral sclerosis–parkinsonism dementia complex (ALS–PDC), a motor neuron (MN) disease among native Chamorro people on the Island of Guam in Micronesia, has long been hypothesised (Spencer et al., 1987; Cox et al., 2003; Ince and Codd, 2005). Debate on this hypothesis (Papapetropoulos, 2007) and further research with environmental and human clinical material continue. In the presence of a carbonate species, BMAA forms the product L-BMAA-β-NCO_2, an analogue of glutamate. This analogue causes motor neuron selective toxicity via hyper-activation of MN glutamate (Glu) transporters at concentrations of 30 μM and potentiates MN injury induced by other insults from 10 μM (Nunn and O'Brien, 1989; Weiss et al., 1989; Rao et al., 2006; Lobner et al., 2007). During neurodegeneration, Glu transporters load excess Glu into extracellular spaces, inducing a flood of calcium (Ca^{2+}) into motor neurons via receptor channels causing mitochondrial damage, and Glu/Ca^{2+}-mediated increases in apoptotic transcription factors. Excitotoxicity due to excess Glu occurs as part of the ischemic cascade and is associated with stroke and diseases including amyotrophic lateral sclerosis, lathyrism, and Alzheimer's disease (Van Den Bosch et al., 2006; Hara and Snyder, 2007).

BMAA production by cyanobacteria suggests that this neurotoxin may be of toxicological significance to humans via aquatic exposure routes (Ince and Codd, 2005). Indeed, BMAA has been identified along with other cyanotoxins in environmental samples

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of cyanobacterial blooms from waterbodies used for abstraction for drinking water treatment, as fisheries and as recreational resources (Metcalf et al., 2008). Although the toxicity of BMAA has been investigated in various mammalian test models (Karamyan and Speth, 2008), the toxicity of BMAA to aquatic organisms has not been investigated. There is a need to develop effective models for motor neurone degeneration research, and the principles of humane experimental technique, as outlined by Russell and Burch (1959), place an emphasis on replacement, reduction, and refinement in animals used for toxicity testing, highlighting the need to further develop aquatic vertebrates as toxicity testing models. We have used the zebrafish (*Danio rerio*) as a model. This model has previously been used to identify and characterise other known cyanotoxins (Wiegand et al., 1999; Lefebvre et al., 2004; Wright et al., 2006; Berry et al., 2007).

Some of the main benefits of using zebrafish in toxicology are based on the early life-stage morphology, size and ease of husbandry of these fish. Optimal breeding and maintenance conditions are very well established and are also relatively easy to control. Their size allows standard microscopic analysis, whilst being small enough to be suitable for high-throughput screening, such as via multi-well plate analysis. The short gestational period and development of *D. rerio* make it ideal for both acute and chronic exposure analysis. Perhaps most importantly, the transparency of the embryo chorion allows straightforward identification of developmental stages and phenotype traits (Hill et al., 2005; Hinton et al., 2005; Berry et al., 2007). *D. rerio* is becoming increasingly popular as a standard vertebrate toxicity testing model and is particularly valuable for the study of MN degeneration due to conserved neurodevelopmental processes and signalling pathways. Glutamate is the key neurotransmitter in locomotor regions of the developing zebrafish, and glutamatergic synaptic drive systems in *D. rerio* show significant similarities with other vertebrates, with the universal motor pathway of synaptic drive underlying locomotion (Lein et al., 2005; Gabriel et al., 2008; Drapeau et al., 2002). *D. rerio* applications are being developed as standard Parkinson's disease and Huntington's disease models, among others (Bretaud et al., 2004; Linney et al., 2004; McKinley et al., 2005; Stehr et al., 2006; Schiffer et al., 2007).

This is to our knowledge the first investigation of the developmental effects of BMAA exposure on aquatic vertebrates. The potential for widespread BMAA production by cyanobacteria in aquatic environments means that both the toxicity to and fates of BMAA in aquatic organisms must be better understood. We report here on impacts of BMAA exposure on toxicological endpoints in *D. rerio*, including developmental, behavioural, physiological and in particular neurological and neuro-muscular responses (Fraysse et al., 2006).

2. Materials and methods

2.1. Fish

Adult zebrafish (*D. rerio*) were maintained in the University of Dundee aquarium in aerated de-chlorinated water at 26.5 ± 1.0 °C, with a light:dark cycle of 14:10 h. Spawning was induced by the onset of light and feeding. Eggs were collected from 2.25 h post-fertilisation (hpf) at the blastula (64-cell) developmental stage. Embryos were rinsed with aerated de-chlorinated water from the University of Dundee Aquarium, and placed in Petri dishes at 26.5 ± 1.0 °C where the unfertilised eggs could be distinguished and removed. Mean values for water quality in mmol l^{-1} were: Na^+ 0.19; K^+ 0.02; Ca^{2+} 0.24; Mg^{2+} 0.07; Cl^- 0.03; free CO_2 0.02; alkalinity as $CaCO_3$ 31.3 mg l^{-1}; non-bicarbonate hardness as $CaCO_3$ 10.6 mg l^{-1}; pH 8.2.

2.2. In vivo exposure of D. rerio embryos

After washing, embryos (n = 30) were placed in 25 well 12 mm cell macro-culture plates (Falcon 3503, Becton Dickinson and Co.) with 2 ml of Hank's egg hatching medium, with a composition of NaCl 80 g l^{-1}; KCl 4 g l^{-1}; Na_2HPO_4 3.58 g l^{-1}; KH_2PO_4 6 g l^{-1}; $CaCl_2$ 14.4 g l^{-1}; $MgSO_4{\cdot}7H_2O$ 24.6 g l^{-1}; $NaHCO_3$ 35 g l^{-1}. At 24 h post-fertilisation (hpf) embryos exposed to 0, 5, 50, 500, 5000 or 50,000 μg l^{-1} BMAA in 2 ml aerated de-chlorinated water from the University of Dundee Aquarium (composition outlined in section 2.1). Some embryos were exposed to a combination of BMAA and 0.1 mM Na_2CO_3. These were placed in 2 ml of aerated de-chlorinated water with 10.6 μg l^{-1} (0.1 mM) Na_2CO_3, ±0, ±5, ±500, ±5000 or ±50,000 μg l^{-1} BMAA. All treatments were performed in triplicate. Embryos were incubated at 26.5 ± 1.0 °C. The embryos were exposed to treatments for 5 days post-fertilisation (dpf). After hatching, the chorions were removed. After the 5 dpf exposure period, the larvae were placed in fresh aerated de-chlorinated water, referred to as the "recovery state" and observed until point of mortality (8–14 dpf).

2.3. Observations and data collection

Data for the morphological study and heart rate measurements were collected every 24 h. Physiological aspects monitored included heart rate, embryo area and yolk sac area, pericardial area, head and eye length and width, larval spinal axis angle, fin and tail length and width, and occurrence of clonus-like convulsions. Every individual (n = 30) from each treatment was observed. Any observable abnormalities or deformities were noted and photographed. At least 5 apparently normal individuals per group were randomly selected, along with all affected individuals, for photography every 24 h using phase contrast microscopy and a graticule. Morphological features were measured using Image Tool software (UTHSCSA ImageTool Version 3.0). Every day, the numbers of hatched and damaged embryos, abnormal and dead larvae were recorded. All damaged embryos and dead larvae were removed from the wells.

Heart rate (beats per minute, bpm) was observed via light microscopy, due to the transparency of the embryo/larvae. Heart rate monitored until the development of pigmentation occludes observation (96–120 hpf). Heart rate calculated by time taken for 20 heart beats, converted to bpm. Clonus-convulsions were observed as extended periods of rapidly alternating muscular contraction and relaxation, apparently affecting the entire body (dorsal and caudal regions observed). Each well monitored for 3 min and the number of convulsing individuals within that period recorded. Distinguished from reflex tail flicks as must involve full body, and only recorded when convulsion duration over 3 s.

2.4. Chemicals

Synthetic L-BMAA hydrochloride was purchased from Sigma–Aldrich. A stock solution of 10 mg ml^{-1} in deionised water was diluted to 5, 50, 500, 5000 and 50,000 μg l^{-1} of BMAA with de-chlorinated water for fish-exposure studies. All other chemicals, including anhydrous sodium carbonate, were of analytical grade quality and were purchased from BDH Laboratory Supplies (Poole, UK).

2.5. Statistical analysis

For endpoints described by 'normal' law or Gaussian distribution, statistical analysis was performed in the form of single ANOVA, assuming equal variances between exposure groups ($p^* < 0.05$; $p^{**} < 0.01$; $p^{***} < 0.001$). For all data expressed the statistical dif-