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In vitro and in vivo safety assessment of edible blue-green algae, *Nostoc commune* var. *sphaeroides* Kützinger and *Spirulina plantensis*

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Abstract

Blue-green algae (BGA) have been consumed as food and herbal medicine for centuries. However, safety for their consumption has not been well investigated. This study was undertaken to evaluate in vitro and in vivo toxicity of cultivated *Nostoc commune* var. *sphaeroides* Kützinger (NO) and *Spirulina platensis* (SP). Neither NO nor SP contained detectable levels of microcystin (MC)-LA, MC-RR, MC-LW and MC-LR by LC/MS/MS. Cell viability remained ~70-80% when HepG2 cells were incubated with 0-500 µg/ml of hexane, chloroform, methanol and water-extractable fractions of NO and SP. Four-week-old male and female C57BL/6J mice were fed an AIN-93G/M diet supplemented with 0, 2.5% or 5% of NO and SP (wt/wt) for 6 months. For both genders, BGA-rich diets did not induce noticeable abnormality in weight gain and plasma alanine aminotransferase (ALT) and aspartate aminotransferase concentrations except a significant increase in plasma ALT levels by 2.5% NO supplementation in male mice at 6 month. Histopathological analysis of livers, however, indicated that BGA did not cause significant liver damage compared with controls. In conclusion, our results suggest that NO and SP are free of MC and the long-term dietary supplementation of up to 5% of the BGA may be consumed without evident toxic side-effects.

Keywords

blue-green algae; safety; mouse; *Nostoc commune* var. *sphaeroides* Kützinger; *Spirulina platensis*

1. Introduction

Blue-green algae (BGA), also known as cyanobacteria, are a phylum of bacteria that utilize photosynthesis to obtain energy. BGA have been used as food or medicine by humans in Asian, African and South American countries for centuries (Johnson et al., 2008; Qiu et al.,

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2005). Studies have reported various health benefits of BGA, including immune functions, anti-inflammatory, anti-bacterial, anti-viral, anti-cancer, hypocholesterolemic and hypotriglyceridemic properties (Esser et al., 1999; Hori et al., 1994; Knubel et al., 1990; Rodriguez-Hernandez et al., 2001; Samuels et al., 2002; Smith et al., 1994). *Spirulina platensis* (SP), the most common BGA species used for human consumption, reportedly has antioxidant, anti-inflammatory and hypolipidemic properties (Tiniakos et al., 2010). Another BGA species, *Nostoc commune* var. *sphaeroides* Kützinger (NO), also has a long history of human consumption for a medicinal purpose such as inflammation, night blindness, digestion, and chronic fatigue (Qiu et al., 2002). In particular, we previously reported that lipid extract from NO can inhibit cholesterol biosynthesis as well as lipogenesis *in vitro* (Rasmussen et al. 2008). Mice fed a diet supplemented with 5% NO for 4 wk showed significantly lowered plasma total cholesterol and triglyceride concentrations (Rasmussen et al. 2009). Moreover, in RAW 264.7 macrophages, NO lipid extract repressed the production of pro-inflammatory cytokines. These studies suggest that NO may have an athero-protective potential and can be a candidate for alternative treatment of hypercholesterolemia.

Despite the purported health benefits of BGA, naturally harvested BGA products may be contaminated with algal toxins, such as microcystins (MC), nodularins, anatoxins, saxitoxins and β -Methylamino-L-alanine (BMAA), from toxin-producing algae species (Carmichael, 1994; Cox et al., 2005). MC and nodularins, produced by *Microcystis* and *Nodularia*, respectively, are hepatotoxins that disrupt the cytoskeleton and inhibit protein phosphatases (Carmichael, 1994). Anatoxin, saxitoxins and BMAA are neurotoxins that interfere with the nervous system (Carmichael, 1994; Cox et al., 2005; Johnson et al., 2008). In 1996, dietary supplements with naturally harvested *Aphanizomenon flos-aquae* (AFA) were found being contaminated with MC-producing *Microcystis aeruginosa* (Gilroy et al., 2000). This incidence urged the State of Oregon Department of Agriculture to estimate a regulatory limit of MC and a safe level of MC in BGA products was determined to be 1 $\mu\text{g/g}$ (Gilroy et al., 2000).

SP is recognized as a MC-free BGA and considered to be a “safe food with no adverse side-effects” by the United Nations Industrial Development Organization program (Samuels et al., 2002). However, no comprehensive examination of the safety of BGA supplementation, particularly on the long-term effects of BGA consumption, has been conducted. Therefore, the objective of this study was to examine the *in vitro* and *in vivo* toxicity of NO and SP cultivated in a controlled environment.

2. Materials and Methods

2.1. MC content measurement

MC contents in NO and SP were measured by liquid chromatography tandem mass spectrometry (LC/MS/MS) using a Thermo LCQ ion trap coupled to a Waters 2695 HPLC. Reference standards for MC-LR, LA, and LW were obtained from Alexis Biochemicals (Plymouth, PA), Calbiochem (Gibbstown, NJ), and Sigma-Aldrich (St Louis, MO), respectively. Powdered NO and SP were generously provided by Algaen Corp (Winston-Salem, NC) and Earthrise Nutritionals (Irvine, CA), respectively. Three different batches of NO and SP samples were extracted using hot water extraction (Metcalf and Codd, 2000). Briefly, 0.1 g of sample was weighed into a Teflon centrifuge tube and mixed with 50 ml of purified reagent water heated to 100°C. The contents were vortexed, cooled to room temperature and centrifuged. The supernatant was filtered through a glass fiber filter and then fortified with nodularin as a surrogate compound. The aqueous extract was passed through a 200mg Oasis HLB solid-phase extraction cartridge (Waters Corporation, Milford, MA). The cartridge was eluted with 10 ml of methanol into an evaporation tube and 100 μl of Leu-Enkephalin in water was then added as an internal standard. A small amount (300 μl)

of reagent water was added and the extract was evaporated to a final volume of 500 μ l using a stream of N₂. The concentrated extract was then transferred to an HPLC autosampler vial for analysis by LC/MS/MS (Cong et al., 2006). Compounds were separated using a small-bore reversed-phase column (Thermo Scientific HyPurity C18, 250 mm $\times$ 2.1 mm, 5 μ m particle size, temperature 50°C). Detection was in electrospray negative ion mode and quantification used multiple reaction monitoring. Based on the precision of a low-level fortified blank, the detection limits were close to 50 ng/g.

2.2. Fractionation of BGA and cytotoxicity measurement

Powdered NO and SP were sequentially extracted into hexane, chloroform, methanol and water. For each extraction, 50 ml of solvent was added to 15 g of BGA and the mixture was stirred for 2 h at room temperature. The supernatant was collected and the extraction was repeated twice for 3 h. Hexane, chloroform and methanol extracts were dried under a stream of N₂ and the water extract was lyophilized. All the extracts were stored at -80°C until use.

HepG2 cells, a human hepatocellular carcinoma cell line, from ATCC (Manassas, VA) were cultured in DMEM with 4.5g/l glucose at 37 $^{\circ}\text{C}$ in an incubator with 5% CO₂. Cell culture medium was supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μ g/ml streptomycin, and 2 mmol/l L-glutamine. To test the cytotoxicity of BGA, 0–500 μ g/ml of the hexane, chloroform, methanol and water-extractable fractions from NO and SP were solubilized in the FBS-free medium by sonication and the cells were incubated with each fraction for 24 h. Cell viability was measured by Cell Counting Kit-8 (Dojindo Inc., Rockville, MD) according to the manufacturer's protocol.

2.3. Diet and animal study

Male and female C57BL/6J mice at 4 wk of age were purchased from Jackson Laboratory (Bar Harbor, ME) and randomly assigned to one of the following five groups, i.e., control, 2.5% NO, 5% NO, 2.5% SP and 5% SP (n=8). Mice were housed in a polycarbonate cage under a 12-hr light/dark cycle. Powdered NO or SP (2.5 and 5% by wt) was incorporated into AIN-93G and AIN-93M diets at the expense of cornstarch, casein, oil and fiber based on the composition of BGA (Table 1). Mice were fed an AIN-93G diet with BGA supplementation for 4 wk and subsequently an AIN-93M diet for the rest of the feeding period. During the 6 month feeding period, bodyweights were recorded biweekly. Blood samples were obtained at 2, 4 and 6 month by tail bleeding after 4 hr-fasting. At the end of the 6 month feeding period, mice were fasted for 4 hr and anesthetized subcutaneously with ketamine HCl (50 mg/Kg)/xylazine (10 mg/Kg). Blood samples were collected into tubes containing EDTA (BD Vacutainer) by cardiac puncture and the animals were then euthanized by cervical dislocation. Liver samples were weighed and then immersed in 10% buffered formalin. All animal experiment protocols were approved by the Institutional Animal Care and Use Committee at the University of Nebraska-Lincoln.

2.4. Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) measurement

Blood samples were centrifuged for 10 min at 1,500 $\times$ g at 4 $^{\circ}\text{C}$ to remove red blood cells and plasma samples were analyzed for ALT and AST concentrations using Cholestech LDX ALT/AST test cassettes and the Cholestech LDX System (Cholestech Corporation, Hayward, CA).

2.5. Histopathologic assessment of mouse liver samples

Paraffin-embedded liver specimens were sectioned at 4 μ m, stained with hematoxylin and eosin. The staining was examined by light microscopy.

2.6. Statistical Analysis

ANOVA and Tukey's pairwise comparison were used to identify statistically significant differences between treatments with $P < 0.05$ being considered significant by GraphPad InStat 5 (GraphPad Software, La Jolla, CA). Data were expressed as mean $\pm$ SEM.

3. Results

3.1. MC content in BGA

Three batches of NO and SP samples were analyzed for four major MC isoforms known to be highly present in certain species of BGA, i.e., MC-LA, MC-RR, MC-LW and MC-LR, by LC/MS/MS. There was no detectable levels of the MC isoforms in the 3 different batches of NO and SP. The selective negative ionization mode ESI multiple reaction monitoring fragmented the deprotonated molecular $[M-H]^-$ ion for each MC though loss of water to a product $[M-H-H_2O]^-$ ion. None of the MC isoforms were detected above the detection limits (20-50 ng/g). Extracts of all three NO samples contained other peaks, with one co-eluting with MC-LR MRM transition (993 $\rightarrow$ 975). Reanalysis in positive ion mode produced different product ion spectra, confirming that the co-eluting compound was not consistent with the structures of MC. The positive MS/MS spectrum for the co-eluting compound in the NO extract exhibited 4 major ions at $m/z=906$, 793, 583, and 413 while major ions at $m/z=995$, 977, 967 and 599 were observed in the spectrum for MC:LR.

3.2. BGA cytotoxicity

Cytotoxicity of NO and SP fractions including hexane, chloroform, methanol and water-extractable fractions was assessed in HepG2 cells. We chose this cell line as hepatotoxins are major algal toxins in certain BGA species. Cell viability slightly decreased with increasing concentrations of all the fractions (Figure 1). However, cell viability remained at ~70-80% of the control when cells were treated with 500 μ g/ml of each fraction. We conducted the assay with three different batches of NO and SP and found similar results from all the batches.

3.3. In vivo assessment of BGAtoxicity

For in vivo assessment of BGA toxicity, 4 wk-old male and female C57BL/6J mice were fed an AIN-93G/M diet supplemented with 0, 2.5 or 5% of NO or SP for 6 months to monitor growth. During the course of 6 month feeding, there were no signs of illness and behavioral changes in any of the mice. Initial body weights of mice were not significantly different between treatment groups for males ($P = 0.15$) and females ($P=0.69$), and mice on any BGA diets did not show aberrant growth (Figure 2A). There was no significant difference in body weight gain between treatment groups for each gender of mice at the end of the 6 month feeding period (Figure 2B).

Plasma concentrations of ALT and AST were measured with samples from mice on the experimental diets for 2, 4 and 6 months to assess liver and tissue damages, respectively (Giboney, 2005). Mice fed diets supplemented with SP showed no increase in plasma ALT levels when compared with control animals. However, NO supplementation for 6 months showed significantly higher plasma ALT concentrations at 2.5%, but not at 5%, level of supplementation in male than those of the other groups (Figure 3A). Plasma AST concentrations were not different between both genders of control and NO-fed mice at 2, 4 or 6 month of supplementation (Figure 3B). However, SP supplementation significantly lowered plasma AST levels in male mice after 6 month feeding compared with the control.

Histopathological examination of liver samples from mice fed experimental diets for 6 months was performed to evaluate a potential liver toxicity of BGA supplementation. We

observed that regardless of a gender or an experimental diet, most of mice developed mild to moderate lipidosis after 6 months of control or experimental diets (Figure 4). However, no other signs of liver damages were observed in all the animals.

4. Discussion

Because of increasing public demand for natural products with health-promoting properties, the development of safe and reliable natural sources is needed. Although various BGA species have been consumed as food and natural medicines for hundreds of years, evaluation of their safety for consumption has been lacking. The safety of BGA supplements has been a health issue due to toxin contamination in naturally-harvested BGA products. This has hindered the use of non-toxin producing edible BGA grown in a controlled environment to obtain potential health benefits. In the present study, we evaluated toxicity of two cultivated BGA species, i.e., NO and SP, in vitro as well as in vivo. We found that both BGA do not contain MC, the major toxins in BGA, and have minimal cytotoxicity. Additionally, the BGA species were safely consumed at the supplemental level of 5% (by wt) in mice.

Cyanotoxins produced by certain BGA species have become a major health concern. The most common cyanotoxins are MC (Namikoshi et al., 1993). With an algal bloom of toxin-producing *Microcystis aeruginosa* in Upper Klamath Lake where AFA had been harvested for human consumption (Gilroy et al., 2000), algal toxins drew lots of public's attention. In responding to this concern, Health Canada performed broad sampling of BGA products available on the Canadian market in May 1999 for testing their MC contents. The survey revealed that only SP has no detection of MC and many non-SP BGA products harvested from natural lakes contain the toxins above that considered acceptable by Health Canada and the World Health Organization. In consistent with this report, we did not detect any of four major MC, i.e., MC-LA, MC-RR, MC-LW and MC-LR, in SP. Although a few NO species have been reported to produce MC (Dixon et al., 2001), the particular NO species used in our study did not contain detectable MC. Furthermore, in our in vitro assessment of BGA cytotoxicity, hexane, chloroform, methanol and water extracts from NO and SP showed little cytotoxicity with ~80% cell viability at 500 µg/ml concentrations.

To evaluate long-term in vivo toxicity of BGA in mice, we fed male and female mice for 6 months a diet supplemented with BGA according to the recommendation by International Conference on Harmonisation (ICH); Guidance on the duration of chronic toxicity testing in animals, which is endorsed by Food and Drug Administration (S4A Duration of Chronic Toxicity Testing in Animals (Rodent and Nonrodent Toxicity Testing)) (Administration, 1999). Mice were fed an experimental diet from 4 wk of age to monitor their growth for 6 months. Both genders of mice fed 2.5% or 5% BGA supplemented diets showed normal growth with no noticeable adverse side effects throughout the dietary period. Long-term consumption of two BGA species did not significantly elevated plasma ALT and AST concentrations. One exception was observed with male mice fed 2.5% NO. The group of mice showed significantly higher plasma ALT levels than other groups at 6 months despite 5% supplementation did not increase the levels. However, histological analysis of liver samples demonstrated that livers of male mice fed 2.5% NO did not differ from those of controls. Almost all mice in our study developed mild lipidosis regardless of a gender or a diet with no obvious tissue damage or the development of hepatitis. Interestingly, plasma AST levels were increased ~2-fold in control groups at 6 months both in male and female mice. Supplementation with 2.5 and 5% SP showed significantly lower AST concentrations in males and a trend toward a reduction in female mice. The reason for this observation is not known. However, considering mice were 7 month old, SP may prevent aging-related tissue damages. Further studies are necessary to evaluate this possibility.

5. Conclusions

Our results indicate that cultivated NO and SP do not contain MC and have minimal cytotoxicity. Furthermore, long-term BGA supplementation in mice did not induce any evident adverse side-effects. Therefore, NO and SP may be developed as a safe natural food for health benefits. Clinical trials are warranted to assess the safety of the BGA supplements in humans.

Acknowledgments

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Abbreviations

AFA	Aphanizomenon flos-aquae
ALT	alanine aminotransferase
AST	aspartate aminotransferase
BGA	blue-green algae
BMAA	β -Methylamino-L-alanine
FBS	fetal bovine serum
LC/MS/MS	liquid chromatography tandem mass spectrometry
MC	microcystins
NO	Nostoc commune var. sphaeroides Kützinger
SP	Spirulina platensis

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Figure 1.

Cytotoxicity of SP and NO fractions. HepG2 cells were incubated with increasing concentrations (0-500 $\mu\text{g/ml}$) of hexane, chloroform, methanol and water extract from NO and SP for 24 hr and cell viability was measured. Cell viability of NO (A) and SP (B) fractions is shown relative to control (0 $\mu\text{g/ml}$).

Figure 2.

Body weights of male and female C57BL/6J mice fed an AIN-93G/M diet with 0, 2.5 or 5% BGA supplementation by wt. (A) Body weights during 6 months of dietary period. (B) Body weight changes after 6 months on an experimental diet. n = 7-8. Mean $\pm$ SEM.

Figure 3. Plasma ALT and AST concentrations of male and female C57BL/6J mice fed an AIN-93G/M diet with 0, 2.5 or 5% BGA supplementation for 2, 4 and 6 months. (A) Plasma ALT and (B) AST concentrations. $n = 7-8$. Bars with different letters are significantly different ($P < 0.05$). Mean $\pm$ SEM.

Figure 4. Histopathological analysis of liver samples from male and female C57BL/6J mice fed a control AIN-93G/M or 2.5% or 5% BGA supplemented diet for 6 months. Formalin fixed liver samples were stained with hematoxylin and eosin. Representative liver sections from each dietary group are shown.

Table 1

Composition of AIN-93G/M diet supplement with 0, 2.5 or 5% BGA (wt/wt)

Ingredient (g)	AIN-93G			AIN-93M		
	Control	2.5% BGA	5% BGA	Control	2.5% BGA	5% BGA
Cornstarch	397.5	390	382	465.7	458.2	450.7
Casein	200	185	170	140	126.2	112.4
Dextrinized cornstarch	132	132	132	155	155	155
Sucrose	100	100	100	100	100	100
Soybean oil	70	70	70	40	38.4	36.85
Fiber	50	47.5	45.5	50	47.9	45.75
Mineral mix	35	35	35	35	35	35
Vitamin mix	10	10	10	10	10	10
L-Cystin	3	3	3	1.8	1.8	1.8
Choline bitartrate	2.5	2.5	2.5	2.5	2.5	2.5
Algae	0	25	50	0	25	50
Total	1000	1000	1000	1000	1000	1000