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The multidisciplinary approach to safety and toxicity assessment of microalgae-based food supplements following clinical cases of poisoning

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ABSTRACT

Commercially available food supplements based on microalgae such as Spirulina (Cyanobacteria) or Chlorella (Chlorophyta) are becoming increasingly popular. Both are considered as non-toxic *per se* but the quality and safety of the final product depends on culturing and manufacturing conditions. This study presents two cases of human poisoning following the simultaneous use of Spirulina and Chlorella food products and a multidisciplinary approach to their evaluation: cytotoxic tests using human whole-blood *in vitro* and a suite of analytical screenings of over 30 elements, arsenic species and cyanotoxins: cylindrospermopsin (CYN), anatoxin-a (ANA) and three microcystin (MC) analogues. To compare metal content the Food Supplement Metal Index and Toxic Elements Contamination Index were also introduced. In all performed analyses two other commercial products were also investigated. Reported clinical symptoms of poisoning included the spreading of atopic dermatitis, nausea, dizziness, headache and fatigue. Extracts of supplements obtained from affected subjects were found to act pro-necrotically in human neutrophils, while tablets contained higher levels of several metals including Cd, Pb and Hg. All analyzed food supplements contained a significant content of Al. Neither CYN, ANA nor MC were present in any examined product. The quality of both Spirulina-based and Chlorella-based food supplements was very doubtful. The contamination problem of some commercially available microalgae-based supplements appears to be pleiotropic. The present study clearly indicates that such products should be subject to strict and routine monitoring before being registered and distributed as some of them may pose a distinct threat to human health.

1. Introduction

The global interest, consumption and market for dietary supplements based on natural compounds has greatly increased over recent decades (Kennedy, 2005). An important group of such products is represented by microalgae biomass obtained from species such as *Spirulina* sp. (Cyanobacteria) and *Chlorella* sp. (Chlorophyta). As a rich source of proteins, chlorophyll, minerals, digestive and restriction enzymes, antioxidants and various vitamins, they possess the potential to improve nutritional status in the diet of humans and prevent deficiency disorders (Nakano et al., 2010; De et al., 2011). Furthermore, their immunomodulatory potencies (Selmi et al., 2011; Kwak et al., 2012), oxidative stress protection (Lu et al., 2006; Kalafati et al., 2010; Lee et al., 2010), anti-tumour, anti-bacterial and anti-viral activities (Yamani et al., 2009), as well as a positive effect on lipid profile (Torres-Durán et al., 2012; Mazokopakis et al., 2014) have been demonstrated using *in vitro* and *in vivo* models and also, to some extent in clinical trials. Cells of *Spirulina* sp. and *Chlorella* sp. are not known to produce any known toxic metabolites and are generally considered *per se* as safe for human consumption (Yang et al., 2011). Serious concerns have been raised as to the quality of the products manufactured from these microalgae due to recent reports of their contamination with toxic metals (Al-Dhabi, 2013), inorganic arsenic (Hedegaard et al., 2013) or cyanotoxins (Draisci et al., 2001).

Serious contamination of microalgae-based food supplements can result from the unsuitable location of the cultivation ponds

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(*e.g.* near rural, industrial or agricultural areas). The environmental quality of these ponds can be affected through inflows of effluents containing some of the most widespread pollutants such as toxic metals and it has been widely demonstrated that these elements possess a high affinity to bioaccumulate in microalgae through sorption processes (Rzymski et al., 2014a, 2014b). Therefore, final products manufactured from such biomass can be potentially hazardous to human health. As reported in many studies, exposures to heavy metals such as Cd, Pb, Hg and others can adversely affect human health in a variety of ways, including, *inter alia*, the disruption of neurological function (Jang and Hoffman, 2011), immunosuppresion (Poniedziałek et al., 2012a) and alterations in the reproductive system (Chojnacka, 2010; Rzymski et al., 2014c). Another ubiquitous and potentially health-threatening element that can be introduced to the environment at elevated levels from anthropogenic sources is arsenic (As). Its toxicity depends primarily on the form in which it occurs. In general, inorganic species, As(III) and As(V), are considered to be the most toxic while organic forms are known to be less or even non-toxic to humans (Hughes, 2002). The need for As speciation analyses in the evaluation of the safety of natural products has been strongly urged by international authorities (EFSA, 2009; WHO, 2011).

A further cause of serious contamination in microalgal products may arise from the improper culture purity and the co-occurrence of potentially toxic cyanobacteria species such as *Microcystis aeruginosa* (Vichi et al., 2012). Moreover it has been shown that the presence of *M. aeruginosa* and microcystins (MCs) in cultures of Spirulina and Chlorella do not only fail to retard their growth but can even stimulate it (Costa et al., 2006; Campos et al., 2013). Under such conditions the final Spirulina and Chlorella products can be potentially contaminated with the MCs. This group of compounds, comprising over 80 congeners, is known to induce irreversible inhibition of protein phosphatases, alteration of the hepatic structure and in extreme cases, liver failure. One of the most common MC analogues, MC-LR was shown to act as a potent promoter of tumours (Rzymski et al., 2011) and on the basis of these results it was classified as possibly carcinogenic to humans (group B2) by the International Agency for Research on Cancer (IARC, 2006). Commercial products using Spirulina from certain origins (*e.g.* Chinese) have already been reported to contain traceable levels of this cyanotoxin (Jiang et al., 2008).

Altogether, the aforementioned findings indicate the need for systematic quality monitoring and assessment of commercially available microalgae-based food supplements. This can, however, be a serious challenge if one considers the large number of different manufacturers and the various routes of their distribution, including online sales which are less subject to control. Due to the present lack of proper regulations the safety of dietary supplements remains more the subject of scientific investigation than part of a thorough monitoring programme (Heussner et al., 2012; Vichi et al., 2012).

The present study reports two cases of intoxication following the simultaneous oral use of commercially-available food supplements based on Spirulina and Chlorella. The *in vitro* cytotoxicity of these products in human white cells was also assessed using whole-blood assay. Finally, a suite of analytical screening was performed which included a determination of the total content of over 25 elements (including obligatory toxic metals), As species and cyanobacterial toxins: CYN, ANA-A and three MCs analogues. For the first time X-ray spectrometry was introduced as a convenient method for analysing the elementary content of microalgae-based supplements. This study reveals that these products should be subject to strict quality monitoring to protect humans from exposure to health threatening impurities, it also describes and demonstrates the application of a range of tools that can be potentially useful in such a procedure.

2. Material and methods

2.1. Studied individuals

Two cases of side effects manifested following oral administration of the microalgae-based food supplements Spirulina and Chlorella (both of Chinese origin), were examined retrospectively. Both cases occurred in Poland, Europe. Information on the clinical image, background and treatment was collected by interviewing the affected individuals and staff at the medical unit in which they were treated.

2.2. Dietary supplement samples

The Spirulina and Chlorella supplements (hereafter SPIR-TOX and CHLOR-TOX) were provided by the affected individuals for further analyses. Both products were of Chinese origin and were purchased online from a non-registered distributor. For comparison, all analyses were also performed using commercially available Spirulina and Chlorella products (hereafter SPIR-C and CHLOR-C) purchased from a registered merchandizer. Performed analyses included *in vitro* cytotoxicity tests using cells derived from healthy human subjects, determination of trace and rare earth element content, As speciation and quantification of cyanotoxins.

2.3. Cytotoxicity studies

To evaluate the potential cytotoxicity of algal supplements, cell-free extracts were obtained from each product. Ten randomly selected tablets of each Spirulina and Chlorella supplement were homogenized in a mortar and 2.5 g of powdered material was flushed with UV-sterilized and filtered MilliPore water, vigorously shaken for 10 min and subjected to 3×10 min cycles of freezing and thawing performed at $-80\,^{\circ}C$ and $37\,^{\circ}C$, respectively. To ensure total cell lyses, samples were additionally ultrasonicated and examined under a light microscope. The broken cell suspensions were centrifuged. The supernatants were sterilized with UV light and filtrated through an injection filter with a nominal pore size of 0.4 μm (Sartorius, Germany) to protect extracts from microbial contamination. The obtained solutions were then diluted to reach a final concentration of 250 $\mu g\ mL^{-1}$.

Whole-blood assays were used to evaluate the toxicity of the obtained extracts. Heparinized blood samples (6.0 mL) were collected in lithium heparin tubes from 5 healthy (screened by physical examination, medical history and initial blood tests), non-smoking and normal weighted (BMI 18.5–24.9) donors (aged 21–51 years old; 1 female, 4 male) at the Regional Centre of Blood and Blood Treatment in Poznan, Poland, according to accepted safeguard standards and legal requirements.

Samples of human blood (45 μL) were exposed for 1.5 h to each extract solution (5 μL). A control was constituted of blood and phosphate buffered saline. Incubation was carried out in a CO_2 incubator under controlled conditions (5% CO_2, $37\,^{\circ}C$, 95% humidity). Following the exposure, samples were labelled fluorescently for detection of live, apoptotic and necrotic cells (lymphocytes and neutrophils) by adding 20 μl of binding buffer, 5 μL of Annexin V-FITC and 5 μl of propidium iodine (Pharmingen, San Diego, CA). Samples were mixed gently and incubated at $25\,^{\circ}C$ in darkness for 15 min. Next, 1 mL of red blood cell lysis solution was added and after 10 min, all samples were analyzed by flow cytometry (CyFlow Space flow cytometer with 488 nm excitation, Partec GmbH, Germany) using FL1 (Annexin V-FITC) and FL3 (propidium iodine) channels. Using a log FL-1 *versus* log FL-3 quadrant dot-plot, three cell subpopulations were identified: live,