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Safety evaluation of Algal Oil from *Schizochytrium* sp.

I. Fedorova-Dahms [a,*], P.A. Marone [b], E. Bailey-Hall [a], A.S. Ryan [a]

[a] Martek Biosciences Corporation, 6480 Dobbin Road, Columbia, MD 21045, United States
[b] Eurofins Product Safety Laboratories, US Highway 130, Dayton, NJ 08810, United States

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ABSTRACT

The safety of Algal Oil from *Schizochytrium* sp. was evaluated by testing for gene mutations, clastogenicity and aneugenicity, and in a subchronic 90-day Sprague–Dawley rat dietary study. The results of all genotoxicity tests were negative. The 90-day study involved dietary exposure to 0.5, 1.5, and 5 wt.% of Algal Oil and two control diets: a standard low-fat basal diet and a basal diet supplemented with 5 wt.% menhaden oil (the fish oil control). There were no treatment-related effects of Algal Oil on clinical observations, body weight, food consumption, behavior, hematology, clinical chemistry, coagulation, or urinalysis parameters. Increased mean liver weights and alveolar histiocytosis were observed in both the fish oil control and the high-dose Algal Oil-treated animals and were not considered to be adverse. Algal Oil was bioavailable as demonstrated by the dose-related increase of DHA and EPA levels in tissues and plasma. The no observable adverse effect level (NOAEL) for Algal Oil under the conditions of this study was 5 wt.% in the diet, equivalent to an overall average Algal Oil intake of 3250 mg/kg bw/day for male and female rats. Based on the body surface area, the human equivalent dose is about 30 g Algal Oil/day for a 60 kg adult.

1. Introduction

The long-chain polyunsaturated n-3 fatty acids (LC-PUFA), docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3) are obtained primarily from fatty fish, such as tuna, salmon and mackerel. However, fish do not synthesize these fatty acids *de novo*, lacking the required key enzymatic activities (Sargent and Tacon, 1999). Instead, fish obtain DHA and EPA primarily from their diet in the form of plankton. DHA is the most abundant LC-PUFA in the gray matter of the brain and in the retina of the eye (Salem, 1989). DHA occurs naturally in breast milk and is essential for normal infant brain and eye development (Hoffmann et al., 2009). In adults both EPA and DHA support heart health (Mori and Woodman, 2006). The Food and Drug Administration has concluded that DHA and/or EPA up to 3 g/day are generally recognized as safe (GRAS) (FDA, 1997).

Large-scale fermentation technology has made it possible to bypass the marine food chain and produce DHA and EPA oils directly from microalgae. Martek Biosciences Corporation (Martek) manufactures commercially available DHA oil from microalgae *Schizochytrium* sp., a thraustochytrid and a member of the Stramenoplia kingdom (Chromista) (Ryan et al., 2010). The kingdom includes the golden algae, diatoms, yellow-green algae, haptophyte and cryptophyte algae oomycetes. Thraustochytrids are found throughout the world in estuarine and marine habitats. Consumption by humans of thraustochytrids, especially those of the genus *Schizochytrium* sp. is primarily indirect, through consumption of fish and shellfish. There are no reports of this organism producing toxic chemicals nor is it pathogenic (Ryan et al., 2010). A number of studies have confirmed the safety of DHA-rich dried *Schizochytrium* sp. These studies considered subchronic feeding to rodents (Hammond et al., 2001a) and non-rodents (Abril et al., 2003), developmental toxicity in rodent and non-rodent species (Hammond et al., 2001b), reproduction (Hammond et al., 2001c), and *in vitro* mutagenicity and genotoxicity (Hammond et al., 2002). Presently, DHA Algal Oil derived from *Schizochytrium* sp. is generally recognized as safe (GRAS) and available for food use and for dietary supplements (FDA, 2004).

Recently, Martek produced an omega-3 oil obtained from a different strain of *Schizochytrium* sp., herein named Algal Oil

Abbreviations: ALB, albumin; ALKP, alkaline phosphatase activity; ALP, alanine aminotransferase activity; ARA, arachidonic acid; AST, aspartate aminotransferase activity; A/G, ratio albumin to globulin; bw, body weight; BUN, blood urea nitrogen; DHA, docosahexaenoic acid; CHOL, cholesterol; CPA, cyclophosphamide; EMS, ethylmethanesulfonate; EPA, eicosapentaenoic acid; GLP, good laboratory practices; GRAS, generally recognized as safe; LC-PUFA, long-chain polyunsaturated fatty acids; Martek, Martek Biosciences Corporation; NOAEL, no observable adverse effect level; OECD, Organization for Economic Co-operation and Development; SDH, sorbitol dehydrogenase; TG, triglycerides.

* Corresponding author. Tel.: +1 443 542 2388; fax: +1 410 997 7789.
E-mail address: idahms@martek.com (I. Fedorova-Dahms).

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from *Schizochytrium* sp. Algal Oil contains approximately 37% DHA and 16% EPA (wt/wt). Algal Oil is intended for use as a food ingredient and dietary supplement as a source of DHA and EPA.

In this report, we describe the results from comprehensive genetic toxicity testing and a repeat-dose toxicological study that evaluated the safety of Algal Oil from *Schizochytrium* sp. Potential genotoxicity of Algal Oil was investigated using a bacterial reverse mutation assay (Ames test), a chromosome aberration test in human lymphocytes, and an *in vivo* micronucleus test in mouse immature erythrocytes. The results from the 90-day subchronic toxicity evaluation in Sprague–Dawley rats are also described. For the purpose of regulatory filings, Martek considers the data herein to be proprietary.

2. Materials and methods

The battery of genetic toxicity tests was performed at BSL Bioservice Scientific Laboratories GmbH (Planegg, Germany) under good laboratory practice (GLP) conditions and followed the Organization for Economic Co-operation and Development (OECD) Guidelines for the Testing of Chemicals and Food Ingredients, Section 4, Parts 471, 473, and 474.

The 90-day subchronic toxicity study was conducted by Eurofins Product Safety Laboratories (Dayton, NJ) in accordance with the GLP Regulations issued by the U.S. FDA (Title 21 of the CFR, Part 58; effective 1987). The study followed OECD Guidelines for the Testing of Chemicals and Food Ingredients, Section 4 (Part 408): Health Effects, Repeated Dose 90-Day Oral Toxicity Study in Rodents (1987) and US FDA Toxicological Principles for the Safety Assessment of Food Ingredients, Redbook 2000, IV.C. 4. a. Subchronic Toxicity Studies with Rodents (2003).

2.1. Test Substances

The material tested was Algal Oil from *Schizochytrium* sp. Algal Oil (Lot No. 98–5814E) contained 37% DHA and 16% EPA of total fatty acids. The oil was assayed for elemental composition and chemical characteristics and met previously established specifications. Analysis of the oil has confirmed the absence of the common algal toxins, domoic acid and prymnesin. The antioxidants ascorbyl palmitate and tocopherols were added to the final product to enhance stability. The oil was analyzed for DHA and EPA content using gas chromatography at the beginning and completion of the 90-day subchronic toxicity study. The concentration of DHA and EPA remained constant throughout the duration of the study (data not shown). The control article in the 90-day subchronic toxicity study was OmegaPure menhaden oil (lot number 8042TE) manufactured by Omega Protein Inc. (Houston, TX). Corn oil from Welch, Holme & Clarke Co., Inc. (Lot Nos. 12–493 & 12–498) was added to the diet to balance the total fat content; it was also used as a control in the genotoxicity tests.

2.2. Genotoxicity studies

In the Ames test (Ames et al., 1973), Algal Oil was investigated for its potential to induce gene mutations according to the plate incorporation test using *Salmonella typhimurium* strains TA 98, TA 100, TA 1535, TA 1537 and tester strain *Escherichia coli* WP2 *uvr*A. In two independent experiments several concentrations up to 5000 μg/plate of the test item were used. Each assay was conducted with and without metabolic activation (S9 mix).

In the chromosome aberration test (Ishidate and Sofuni, 1985) in human lymphocytes, chromosomes were prepared 24 h after the start of treatment with the test item. The treatment interval was 4 h with and without metabolic activation (experiment I) and 4 h with and 24 h without metabolic activation (experiment II). Two parallel cultures were used. One hundred (100) metaphases per culture were scored for structural chromosomal changes. Concentrations up to 5 μL/mL were used. Ethylmethanesulfonate (EMS) at 400 μg/mL and cyclophosphamide (CPA) at 5 μg/mL were used as positive controls.

The micronucleus test (Heddle, 1973) was performed to investigate the potential of Algal Oil to induce micronuclei in polychromatic erythrocytes (PCE) in murine peripheral blood. The test item was diluted with corn oil to achieve an orally administered volume of 10 mL/kg. A dose of 2000 mg/kg of Algal Oil was selected as the maximum tolerable dose. Corn oil was administered at the same dose. Peripheral blood samples were collected for micronuclei analysis 44 h and 68 h after a single application of the test item. CPA (40 mg/kg i.p.) was used as a positive control. For all experimental groups, including positive and negative controls, 10,000 polychromatic erythrocytes per animal were scored by flow cytometry analysis (FACScan, BD Biosciences, San Jose, CA) for incidence of micronucleated immature erythrocytes.

2.3. 90- Day subchronic toxicity study

2.3.1. Animals

Seven-week-old male and female Sprague–Dawley rats were obtained from Harlan (Frederick, MD) and acclimated for one week before assigning to treatment groups. Animals were examined for overall health and ophthalmology prior to study initiation and those considered unsuitable for the study based on physical examination, ophthalmologic evaluation, or body weight were eliminated. The remaining animals were randomly distributed into five groups of 20 animals each (10 per sex). The body weight means for each group were comparable. Ophthalmologic examination included a focal illumination and indirect ophthalmoscopy and was performed prior to study initiation and before terminal sacrifice (Day 91). During the study, animals were housed individually in suspended stainless steel cages and kept in an environment maintained at 22 ± 3 °C, 30–70% humidity, using a 12-h light/dark cycle. Certified rodent diet 2016CM Harlan Teklad was used as a basal diet with a fat content of 4%.

2.3.2. Test material administration

The test material was added to the basal diet and administered at dietary levels of 0.5% (5000 ppm), 1.5% (15,000 ppm) and 5% (50,000 ppm). The low- and mid-dose diets were adjusted with corn oil to obtain 5wt.% added fat. There were two control groups: basal diet control containing 4wt.% of total fat and fish oil control supplemented with 5wt.% of menhaden oil in addition to the basal diet fat (9wt.% of total fat). All three Algal Oil diets contained 9wt.% of total fat. Diets were provided *ad libitum* and prepared weekly at the testing facility. The stability, homogeneity and concentration of Algal Oil and fish oil in the diet were confirmed by analysis performed at Eurofins Central Analytical Laboratories, Metairie, LA.

2.3.3. Animal observations

Animals were checked for mortality, moribundity, and clinical signs twice daily. Detailed observation, body weights and food consumption measurements were conducted weekly. During the last week of exposure period, a functional observational battery and motor activity measurements were performed. Motor activity was monitored for 60 min using an automated Photobeam Activity System (San Diego Instruments, Inc., San Diego, CA). Each rat was evaluated during handling and while in an open field for excitability, autonomic function, gait and sensorimotor coordination (open field and manipulative evaluations), reactivity and sensitivity (elicited behavior) and other abnormal clinical signs including convulsions, tremors, unusual or bizarre behavior, emaciation, dehydration, and general appearance. The rats were observed in random order and without the observer having knowledge of the treatment group. In addition to the above observations, forelimb and hindlimb grip strength and foot splay measurements were obtained and recorded. The grip strength was measured with a digital force gauge (Wagner Force Five, Model #FDV-5, Greenwich, CT). Triplicate measurements of grip strength and duplicate measurements for foot splay were taken for each animal and a mean was calculated for each measurement.

2.3.4. Hematology and clinical chemistry

Blood samples were collected via sublingual bleeding under isoflurane anesthesia after fasting overnight at the end of the study. Erythrocyte count, hemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin concentration, red cell distribution width, total leukocyte and neutrophil counts, platelet count and lymphocyte and reticulocyte counts were measured in samples collected in EDTA containing tubes. Sodium citrate-treated blood was used to measure prothrombin time and activated partial thromboplastin time. Clinical chemistry measurements included alkaline phosphatase activity (ALKP), aspartate aminotransferase activity (AST), alanine aminotransferase activity (ALT), sorbitol dehydrogenase (SDH), total protein, albumin (ALB), ratio albumin to globulin (A/G), blood urea nitrogen (BUN), creatinine, total bilirubin, glucose, cholesterol (CHOL), and triglycerides (TG).

2.3.5. Urinalysis

The day before collection of samples for the clinical pathology evaluation, the animals were placed in metabolic cages. Animals were fasted for at least 15 h prior to urine collection from each animal. Analysis included evaluation of quality, color, clarity, volume, pH, glucose concentration, specific gravity, protein, ketone, bilirubin, and blood content, and urobilinogen.

2.3.6. Histopathology

A complete gross necropsy was conducted at terminal sacrifice performed by exsanguination from the abdominal aorta under isoflurane anesthesia. Organ weights were obtained for the brain, heart, liver, spleen, thymus, adrenal gland (paired), kidney (paired), epididymides, ovary (paired), uterus, fallopian tubes, and testis (paired). Relative organ weights: organ-to-body (organ weight/body weight × 100) and organ-to-brain (organ weight/brain weight) ratios were calculated based on body weights measured at study termination. In addition to the above-mentioned organs, the following organs and tissues were preserved in 10% neutral buffered formalin: lung, aorta, stomach, intestine, trachea, bone (femur, sternum, marrow), eye, pancreas, skeletal muscle, skin, pituitary gland, thyroid