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Studies on *meso*-zeaxanthin for potential toxicity and mutagenicity

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ABSTRACT

The purpose of these studies was to examine the potential toxicity and genotoxicity of *meso*-zeaxanthin (MZ). Toxicity was assessed by administering MZ daily to rats for 13 weeks followed by a 4-week recovery period. Potential genotoxicity was assessed in separate experiments using the Ames test method. Rats were randomly assigned to four groups to receive corn oil (control) or MZ at dose levels of 2, 20 and 200 mg/kg/day by oral gavage (10/sex/group). Additional rats (five of each sex) in the control and the 200 mg/kg/day groups were retained for the recovery period. No compound-related clinical, biochemical or pathological signs or symptoms were noted and the no-observed-adverse-effect-level (NOAEL) of MZ was >200 mg/kg/day. To investigate genotoxicity, MZ was tested for its ability to induce reverse mutations (±microsomal enzymes) at 2 genomic loci; the histidine locus of 4 strains of *Salmonella typhimurium* and the tryptophan locus of *Escherichia coli* strain WP2*uvr*A. Six doses of MZ ranging from 10 to 5000 μg/plate were tested twice with vehicle and positive controls using 3 plates/dose. MZ did not cause any increase in the mean number of revertants/plate with any bacterial strain, with or without microsomal enzymes, and was therefore unlikely to be mutagenic.

1. Introduction

The carotenoid *meso*-zeaxanthin (MZ; (3R,3′S)-dihydroxy-β,β-carotene-3,3′-diol) first rose to importance when it was discovered to be one of the three main carotenoids in the macula of the eye (Bone et al., 1993). The other two carotenoids in the macula are stereoisomers; lutein ((3R,3′R,6′R)-β-ε-carotene-3,3′-diol) and zeaxanthin ((3R,3′R)-dihydroxy-β,β-carotene-3,3′-diol). Lutein and zeaxanthin are widely distributed in the human diet in a ratio of approximately 5:1, respectively. The daily intake of lutein and zeaxanthin in the USA has been estimated at between 1 and 3 mg per day; white individuals tend to be nearer the bottom and blacks nearer the top (Mares-Perlman et al., 2001). In contrast there are fewer reports of MZ in foods.

The first report was of MZ in human foods was in shrimp carapace, depot fat deposits in turtles and in the integument of 20 species of fish (Maoka et al., 1986). More recently it has been reported in the yolk of chicken eggs that were obtained from Mexico (Thurnham, 2007). However, the MZ was found in Mexican eggs because it has been added to the pigment supplied to chicken industry in Mexico since the mid 1990s. The main pigment used for layers in Mexico was Yemix® (Industrial Orgánica SA, Monterray, Mexico) which comprised 70% xanthophyll concentrate of which 50% was MZ.

The source of the MZ in the macula of the eye is believed to be dietary lutein. Monkeys that were deprived of all dietary xanthophyll were later fed either lutein or zeaxanthin. Only those given lutein were found to have MZ in their maculae while those given only zeaxanthin had none (Johnson et al., 2005). Persons with age-related macular disease (ARMD) have low concentrations of the macular pigments in the fovea. MZ may be of specific importance as it has also been found to be concentrated centrally in the macula (Bone et al., 1997) and the pigment profile of persons where macular pigment concentration was low at the centre, was found to benefit from supplements containing MZ (Nolan et al., 2012).

The cause of ARMD is currently not known but supplements containing MZ, other xanthophyll carotenoids and anti-oxidants may be of benefit especially since the diet contains very little if any MZ. The purpose of this study was to determine whether MZ when given by oral gavage at high daily doses had any toxic effects in male or female rats during a period of 13 weeks or the following 4 weeks on the control diet. The rat was selected for these studies

Abbreviations: MZ, *Meso*-zeaxanthin (3R,3′S)-dihydroxy-β,β-carotene-3,3′-diol; ARMD, age-related macular disease; NOAEL, no-observed-adverse-effect-level; DMSO, di-methyl sulphoxide; TA98, TA100, TA1535 and TA1537, tester strains of *Salmonella typhimurium*; WP2*uvr*A, tester strain of *Escherichia coli*; S9 homogenate, liver microsomal enzyme preparation.

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as it is the standard species for use in toxicology studies as recommended by the Food and Drug Administration and the International Committee on Harmonization guidelines (Food and Drug Administration, 2012).

We also examined MZ concentrate for possible genotoxic effects using the Ames test (Ames et al., 1975; Office for Economic Co-operation and Development, 1997). The objective of this study was to evaluate whether MZ induced reverse mutations either in the presence or absence of mammalian microsomal enzymes at (1) the histidine locus in the genome of several strains of *Salmonella typhimurium* and at (2) the tryptophan locus of *Escherichia coli* tester strain WP2*uvr*A. Further details of the animal and genotoxicity studies can be found on the Howard Foundation web site (The Howard Foundation, 2006).

2. Methods

2.1. Rat-feeding study

2.1.1. Husbandry

The study was done at Gene Logic Laboratories Inc., 610 Professional Drive, Gaithersburg, MD 20879, USA (Gene Logic) between 2005 and 2006. Gene Logic's Institutional Animal Care and Use Committee approved the protocol and found it to be in accordance with the provisions of the USDA Animal Welfare Act, the Public Health Service Policy on Humane Care and use of laboratory animals and the US Interagency Research Animal Committee Principles for the Utilization and Care of Research Animals.

The Han Wistar rats were obtained from Charles River Laboratories and were acclimatised to the laboratory conditions for 10 days prior to the first dose and release from quarantine by the staff veterinarian. Rats were caged individually at 64–79°F, 30–70% humidity, a 12-h dark and 12-h light cycle with a minimum of 10 air changes per hour controlled by a computerised system. Water was provided by an automatic watering system and water-bottles. Feed (Teklad Global 2018 18% protein diet, Harlan Laboratories) and water were provided *ad libitum* except on day 90–91 (13 week sacrifice) or day 118–9 (following recovery sacrifice). On those two occasions, food fasting was implemented for 19–23 h before termination. No contaminants were known to be present in the water, diet or bedding that levels that might have interfered with the objectives of the study.

2.1.2. Test and control treatment solutions

The stock test article used for the first 12 weeks contained MZ in corn oil (~210 g/kg) was supplied by Industrial Orgánica SA, (Monterray, Mexico) and stored refrigerated (5 ± 3 °C) and protected from light on receipt. Total carotenoids in the product were 344 g/kg; the principle impurities being lutein (76 g/kg), and zeaxanthin (53 g/kg). For week 13 dosing, a second batch of MZ concentrate was obtained and contained MZ 207 g/kg in a total carotenoid mixture of 324 g/kg. Three batches of corn oil used for animal dosing and in the preparation of the test dilutions (Spectrum Chemical Company, New Brunswick, NJ (2 batches); ACH Food Company, Memphis, TN (1 batch)).

The stock test article in both cases was assumed to contain 200 mg/mL and to be 100% pure for formulation purposes. It was however further diluted for dosing purposes on a stated density of 0.9189 g/mL. Dose formulations were prepared weekly and used within 8 days. Prior to use, the stock MZ was warmed overnight in a circulating water bath at 50 °C (protected from light). The corn oil was also warmed at 50 °C for 20 min prior to use. Dose formulations were prepared by adding an appropriate amount of the MZ stock (200 mg/mL) into a mortar, adding a small amount of corn oil and mixing into a paste and then transferring the paste to a pre-calibrated beaker. A sufficient quantity of corn oil was added to achieve the desired final volume which was then placed in a circulating water bath for 15 min to raise to 50 °C and stirred for 10 min with a magnetic stirring bar or until a suspension was achieved. Following preparation, the total volume of the 3 formulations (0.2, 2 and 20 mg/mL) was dispensed in 7 amber glass vials (one for each day of dosing) and stored between 2 and 8 °C. When the refrigerated formulations were used, they were first warmed in a water batch at 40 °C for at least 15 min followed by mixing on a stir-plate for at least 5 min and during the dosing period.

2.1.3. Quality assurance of dosing solutions

Triplicate 5 mL samples were taken from the top, middle and bottom portions of each dose formulation in week 1 for homogeneity analysis and dose verification. In addition 5 mL samples of each dosing formulation prepared for weeks 5, 9 and 13 were also collected for dose verification. The samples were protected from light and stored refrigerated (5 ± 3 °C) prior to shipping on ice to Industrial Orgánica SA (Monterray, Mexico) for analysis.

2.1.4. Experimental design

Fifty animals of each sex were assigned to four study groups using computer-generated random numbers (Table 1). Males and females were randomised separately. At randomisation the mean body weight of each group was not significantly different from the control mean ($P > 0.05$). After randomization each study animal was given a unique number based on cage and ear tag. Rats were 7–8 weeks of age at the time of the first dose. Animals were observed at least twice daily for any mortality, moribundity, general health and signs if toxicity. Clinical observations and body weight were made once weekly prior to oral gavage and at terminal sacrifice. Clinical observations included an evaluation of skin and fur characteristics, eye and mucous membranes, respiratory, circulatory, autonomic and central nervous systems and somatomotor and behaviour patterns. Ophthalmological examinations were made using indirect ophthalmoscopy prior to terminal sacrifice and following 1% Tropicamide dilation of the pupil (mydriasis). The first 10 rats/sex/group were sacrificed after 13 weeks and the remaining rats were sacrificed after a 4 week recovery period.

2.1.5. Clinical pathology

On termination days prior to necropsy, blood was obtained through the retro-orbital plexus, abdominal aorta or cardiac puncture when rats were under anaesthesia (70% CO_2/3%O_2). Blood was collected into 3 tubes; at least 1 mL serum for clinical chemistry using a serum separator, 0.5 mL plasma using potassium EDTA for haematology and 1.8 mL plasma using a sodium citrate tube for coagulation studies. Haematology and coagulation samples were stored refrigerated and the clinical chemistry samples were stored frozen before analysis. Blood for clinical chemistry was transported on ice packs to Gene Logics Clinical Pathology Laboratory for analysis. The clinical variables measured and methods of analysis are described in Table 2.

2.1.6. Haematology and coagulation

The following haematological variables were measured or calculated by the Bayer Advia 120 Haematology Analyser; white blood cell count, erythrocyte count, haemoglobin, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, mean platelet volume, platelet count, absolute counts of neutrophils, lymphocytes, monocytes, oesinophils, basophils, reticulocytes. Blood smears for cellular morphology were prepared and stained using a quick Romanowsky type of stain. Cellular morphology was determined by visual examination of the stained smear. Coagulation variables were measured on a Beckman Coulter ACL 1000 Coagulation Analyzer. Beckman control samples were analysed each day of testing. Coagulation variables measured included the activated partial thromboplastin time and prothrombin time using a laser-nephelometric centrifugation.

2.1.7. Post mortem examination

On day 91 following MZ feeding and day 119 following recovery, all designated animals were killed by carbon dioxide inhalation followed by exsanguination. Animals were autopsied as soon as possible after the time of death. A full gross autopsy, which included examination of the external surface of the body, all orifices, the cranial, thoracic, and abdominal cavities, and contents within each body cavity was performed. Protocol-specified organs were weighed as soon as possible after dissection; paired organs were weighed together. Bone marrow smears were prepared from the sternum; bone marrow slides were air dried, fixed in methanol, and stored

Table 1
Design of the rat study.

Group	Treatment	MZ concentrate		Numbers of Han Wistar rats	
		Target dose mg/kg/day	Concentration administered[a] mg/mL corn oil	Males	Females
1	Corn oil	0	0	15	15
2	MZ	2	0.2	10	10
3	MZ	20	2.0	10	10
4	MZ	200	20.0	15	15

[a] Animals were administered the solutions shown daily at a dose volume of 10 mL/kg based on the most recent weight. Oral gavage was achieved using 3 mL or 5 mL syringes with 16 gauge, 10 cm needles at approximately the same time late morning each day.