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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.

Table 2
Methods used for clinical chemistry analysis of rat serum[a].

Variable (units)	Method	
	Name	Procedure
Albumin/globulin ratio	A/G ratio (g/L)	Calculated albumin/glubulin
Albumin (g/L)	Bromcresol Green	Colorimetric
Alkaline phosphatase (Units/L)	p-Nitrophenyl-phosphate	Rate reaction
Alanine aminotransferase (Units/L)	L-alanine + α-ketoglutarate	Rate reaction
Aspartate aminotransferase (Units/L)	Aspartate + α-ketoglutarate oxaloacetate leuko dye	Rate reaction
Blood urea nitrogen (g/L)	Urease	Colorimetric
Calcium (g/L)	Arsenazo III dye	Colorimetric
Cholesterol (g/L)	Cholesterol oxidase peroxidase	Colorimetric
Chloride (mmol/L)	Io-selective electrode	Potentiometric
Creatinine (g/L)	Creatinine aminohydrolase	Two point rate
Globulin (g/L)	Total protein minus albumin	Calculation
Glucose (g/L)	Glucose oxidase peroxidase	Colorimetric
Potassium (mmol/L)	Ion-selective electrode	Potentiometric
Sodium (mmol/L)	Ion-selective electrode	Potentiometric
Phosphorus (g/L)	Ammonium molybdate	Colorimetric
Total bilirubin (g/L)	Diazo	Colorimetric
Total protein (g/L)	Biuret with lithium hydroxide	Colorimetric
Triglycerides (g/L)	Glycerophosphate	Colorimetric

[a] An Ortho-Clinical Diagnostics Vitros chemistry analyser was used to measure the clinical variable above. All reagents were obtained from Ortho-Clinical Diagnostics. Commercially available controls were assayed each day of testing.

for possible future evaluation. The eyes, together with optic nerves, Harderian and lacrimal glands, testes and epididymides, were fixed in modified Davidson's fixative and transferred to 70% ethanol within 24–48 h of collection. All other tissue samples and the animal identification (ear tag) were preserved in 10% neutral buffered formalin.

2.1.8. Histopathology

All tissue samples from the control and 200 mg MZ/kg/day animals sacrificed following the treatment phase (week 13) and the liver, kidneys, spleen, and stomach from the 2 and 20 mg MZ/kg/day animals were processed and evaluated. The liver, kidneys, spleen, and stomach from the recovery sacrifice animals were also processed and evaluated. Those tissue samples were embedded in paraffin, sectioned, stained with haematoxylin and eosin and examined microscopically by a board-certified veterinary pathologist.

2.2. Genotoxicity testing

2.2.1. Test material

The test material 'Aztec Marigold Carotenoid Concentrate' was received from Industrial Orgánica SA (Monterray, Mexico) on 24 August 2004. Carotenoid activity was 745,200 ppm. It was an orange powder comprising free xanthophylls with a composition of MZ, zeaxanthin and lutein (51.15%, 17.05%, 30.4%, respectively). Suspensions of the xanthophyll preparation were prepared using di-methyl sulphoxide (DMSO). The experimental materials, methods and procedures followed previously described methods (Office for Economic Co-operation and Development, 1997; Ames et al., 1975; Green and Muriel, 1976; Maron and Ames, 1983). Initial tests using *S. typhimurium* TA100 with or without the presence of microsomal enzymes found no evidence for cytotoxicity up to the maximum concentration of 5000 μg/plate so this was the maximum dose used for the mutagenicity assay.

2.2.2. Mutagenicity assay

2.2.2.1. The tester strains. The tester strains used were the *S. typhimurium* histidine auxotrophs TA98, TA100, TA1535 and TA1537 (Ames et al., 1975) and *E. coli* tryptophan auxotroph WP2*uvr*A (Green and Muriel 1976). In addition to mutations in the histidine or tryptophan operons, the tester strains also contained additional repair (uvrB or uvrA) and cell wall (rfa) mutations to enhance their sensitivity to some mutagenic compounds. Tester strains TA98 and TA1537 are reverted from histidine dependence to independence by frameshift mutations. Tester strains TA100 and TA1530 are reverted by base substitution mutagens. The *S. typhimurium* tester strains were obtained from Dr. B Ames (Department of Biochemistry, University of California) and the *E. coli* was obtained from the National Collection of Industrial Bacteria (Torrey Research Station, UK).

2.2.2.2. Preparation and storage of bacterial cultures. Frozen permanent stock cultures of the tester bacteria were prepared by growing fresh overnight cultures, adding DMSO (0.09 mL/mL of culture) and freezing appropriate aliquots in vials at −60 to −80 °C. Master plates of test strains were prepared by streaking each strain from a frozen permanent stock onto minimal agar appropriately supplemented with histidine and biotin or tryptophan, and for strains containing the pKM101 plasmid (TA98 and TA100), ampicillin. Tester strain master plates were stored as >0–10 °C. Overnight cultures for use in all testing procedures were inoculated by transferring a colony from the appropriate master plate to a flask containing culture medium. The broth used to grow overnight cultures of the tester strains was Vogel-Bonner salt solution (Vogel and Bonner, 1956) supplemented with 2.5% (w/v) Oxoid nutrient broth no 2 (dry powder). Inoculated flasks in a shaker/incubator (125 ± 25 rpm, 37 ± 2 °C) were programmed to begin operation so that overnight cultures were in late log phase when density monitoring began. Once a density of a least 0.5×10^9 cells/mL was achieved the cultures were held at >0–10 °C until use.

2.2.2.3. Confirmation of bacterial genotype. Tester strain mutants were checked for the following genetic markers; *rfa* wall mutation was confirmed by the sensitivity of the culture to crystal violet, pKM101 plasmid was confirmed by resistance of the strains to ampicillin and all strains were checked for the characteristic number of spontaneous revertants when grown on selective media.

2.2.2.4. Top agar for selection of revertants. The top agar was prepared using 0.7% (w/v) agar and 0.5% (w/v) sodium chloride supplemented with 10 mL of 0.5 mmol/L histidine/biotin solution or 10 mL 0.5 mmol/L tryptophan solution per 100 mL. Two mL of top agar were overlayed onto 25 mL bottom agar (Vogel-Bonner minimal medium E supplemented with 1.5% (w/v) agar and 0.2% (w/v) glucose in plates (15 × 100 mm Petri dishes)). To the top layer was also added 0.5 ml of water or a liver microsomal enzyme preparation (S9 homogenate; Molecular Toxicology Inc. Boone, NC; Lot numbers 1698 and 1718). The S9 homogenate was prepared for immediately before use by diluting 0.1 mL with water (0.7 mL), 1.0 M sodium phosphate buffer (pH 7.4, 0.1 mL), 0.25 M glucose-6-phospate (0.2 mL), 0.1 M NADP (0.04 mL) and 0.04 mL of a mixture of KCl (0.825 M) and Mg_2Cl_2 (0.2 M).

2.2.2.5. Positive controls. Specific positive control were used with the different tester strains as follows; TA 98 (2.5 μg/plate, benzo[a]pyrene), TA98 plus S9 (1.0 μg, 2-nitrofluorene), TA100 (2.5 μg, 2-aminoanthracene), TA100 plus S9 (2.0 μg, sodium azide), TA1535 (2.5 μg 2-aminoanthracene), TA1535 plus S9 (2.0 μg sodium azide), TA1537 (2.5 μg 2-aminoanthracene), TA1537 (2.0 μg ICR-191), WP2uvrA (25 μg 2-aminoanthracene) and WP2uvrA plus S9 (1.0 μg 4-nitroquinoline-N-oxide).

2.2.2.6. Test procedure. The tester strain (100 μl) and 100 μl of the test article were added to 2.0 mL of the molten selective top agar with or without 500 μl of water or S9 mix. The mixture was vortexed, overlaid onto the bottom agar contained in the petri dish and allowed to solidify. Plates were then inverted and incubated for 52 ± 4 h at 37 ± 2 °C. Positive control substances were plated using a 50 μl aliquot and incubated with and without the S9 mix. Revertant colonies were counted by an automated colony counter or by hand. For tester strains TA98, TA100 and WP2*uvr*A, a positive count had to produce a 2-fold increase in the mean revertants per plate of the appropriate vehicle controls. For strains TA1535 and TA1537, a positive count had to be at least 3-fold higher the corresponding vehicle control. In both cases positivity had also to show a dose response to increasing concentrations of the test article and vehicle controls had to fall within the range of historical controls.

2.3. Data analyses

2.3.1. Animal experiments

Quantitative data were check for normality using Kolmogorov–Smirnov tests, the Leven Median test of equal variance and by one-way analysis of variance (ANOVA) tests. Kruskal–Wallis ANOVA was used on non-normal data to distinguish differences between groups. For parametric data, if the ANOVA indicated a significant difference between groups then the Dunnett '*t*' test was used to determine which groups if any differed from the controls. The probability value of less than 0.05 (two tailed) was used as the critical level of significance for all tests.

2.3.2. Genotoxicity data

Genotoxicity data were obtained as replicate analyses for each concentration of the test material. Differences between the groups were assessed using ANOVA. There were no group differences between concentrations (LSD test).

3. Results

3.1. Animal toxicity experiment

3.1.1. Stability of MZ formulations

Stability data indicated that the 200 mg/mL stock formulation and the dilutions in corn oil (0.2, 2.0 and 20.0 mg/mL) were stable for up to 14 days after storage at 3–5 °C or at 25 °C. Analysis of the dose formulations prepared in weeks 1, 5, 9 and 13 indicated the test material was properly prepared and stable. Mean test article concentrations ranged from 92.22% to 110.4% of target values. Analysis of week 1 samples for homogeneity gave a coefficient of variation <6% of the target values.

3.1.2. Animal behaviour and clinical observations

The only noticeable features during the feeding and recovery phases were alopecia ($n = 7$) abrasions ($n = 4$) and hyperactivity ($n = 3$). Alopecia occurred in both control and test groups as did hyperactivity. Abrasions only occurred in the MZ-treated animals but numbers were small and self-correcting. The clinical features were found in both sexes and were probably unrelated to the treatments given (Table 3).

3.1.3. Ophthalmology

A few ophthalmological findings were noted in the controls, the 20 mg/kg/day and 200 mg/kg/day groups (Table 4). As the changes also occurred in the controls, they were unrelated to the *MZ* treatment. They were therefore incidental as they were infrequent, sporadic, not dose related and without histopathological correlations.

3.1.4. Clinical pathology, haematology and coagulation

There were some significant differences between groups in a small number of variables (Table 5). In the data obtained on clinical pathology, serum alkaline phosphatase activity in male rats in the 20 and 200 mg/kg/day groups was higher than in the controls but only the 20 mg/kg/day group was significant. The distribution of alkaline phosphatase activities were skewed in all groups including the controls, indicating the high activities in the control and the 20 and 200 mg/kg/day groups were most likely the result of individual animal variability rather than a compound effect. Serum total bilirubin concentrations for the 20 mg/kg/day female rats was also significantly lower when compared with female controls. The difference was minimal, inconsistent with a dose response and neither biologically or toxicologically significant.

In the recovery data, there were significantly higher serum sodium, total protein and globulin concentrations in the 200 mg/kg/day group than in the male control rats. In addition, prothrombin time was significantly higher for the 200 mg/kg/day females rats than the respective controls. These differences were minor and not considered pathologically important. Otherwise, no compound-related changes in haematology, clinical chemistry or coagulation were noted.

3.1.5. Gross pathology

The changes in weights of the 100 animals in the four groups over the first 12 weeks are shown in Figs. 1 (males) and 2 (females). Data for week 13 is not shown as animals were fasted overnight prior to sacrifice. No compound-related body weight changes were noted. However, there were two significant differences in total body weight change over the course of the study. In the males

Table 3
Observations of animal behaviour and clinical conditions.

Observations	Treatment groups											
	Corn oil			MZ – 2 mg/kg/day			MZ – 20 mg/kg/day			MZ – 200 mg/kg/day		
	N	Duration (weeks)	Site	N	Duration (weeks)	Site	N	Duration (weeks)	Site	N	Duration (weeks)	Site
Alopecia – males	0			1	7–9	Dorsal thorax and left scrotum	1	2–9	Abdomen Dorsal thorax	0		
								6–9				
				1	7–9	Dorsal thorax						
Alopecia – females	1	3–9	Forelimbs	1	2–6	Ventral neck	1	4–9	Right inguinal & ventral thorax	1	8–13	Forelimbs
	1	2–9	Ventral thorax									
	1	2–9 4–9	Abdomen Dorsal thorax									
Abrasions – male	0			0			0			1	12–13	Right hind paw
Abrasions – females	0			1	1–8	Ventral neck	1	2	Cranium	1	11–12	Right ear
					3–4							
Hyperactivity – males	0			0			0			0		
Hyperactivity – females	1	7–8		0			1	6–8		0		

Ten male (m) and female (f) animals of both sexes were fed corn oil (10 mL/kg) or *meso*-zeaxanthin (MZ) by oral gavage at the doses shown for 13 weeks (91 days). An additional 5 animals of both sexes in the corn oil and 200 mg/kg/day groups only were kept for another 4 weeks for observation only with no further treatment.

Table 4
Ophthalmological findings at terminal sacrifice and at sacrifice following recovery period.

			Observations
Terminal sacrifice			
Group	Sex	Number	
Control	M	3	Crystalline corneal opacities (2 right eye, one both)
Control	F	2	Crystalline corneal opacities (1 right, 1 both)
Control	F	1	Crystalline corneal opacity, anterior synechiation (adhesion) of cornea with irregular pupil and retinal degeneration
20 mg MZ/kg/day	M	1	Pinpoint corneal opacity (left eye)
200 mg MZ/kg/day	M	1	Crystalline corneal deposits (right eye)
Recovery sacrifice			
Control	F	3	Crystalline lens opacities (1 right eye; 2 both eyes)
Control	F	1	Crystalline lens opacities, irititis, focal lens cataract and focal retinal degeneration (both eyes)
200 mg MZ/kg/day	M	3	Crystalline lens opacities (2 right 1 left eye)
200 mg MZ/kg/day	M	1	Lens opacity (left eye)
200 mg MZ/kg/day	F	1	Crystalline lens opacities (both eyes)

Ophthalmological examinations were conducted using an indirect ophthalmoscope following 1% Tropicamide mydriasis.

Table 5
Clinically abnormal findings at termination of *meso*-zeaxanthin feeding and after 4-weeks recovery[a].

Clinical measurement	Sex	Control group	2 mg MZ/kg/day	20 mg MZ/kg/day	200 mg MZ/kg/day	P=[b,c]
At termination of MZ; $N = 10$/group						
Alkaline phosphatase activity U/L	Male	131 (83–195)	136 (80–237)	172 (109–283)*	154 (109–445)	0.038[a]
	Female	58 (43–114)	63 (49–102)	69 (54–91)	71 (58–138)	0.34[a]
Total bilirubin mg/L	Male	1.29 (0.49)	1.59 (0.53)	2.09 (1.46)	1.46 (0.57)	0.21[b]
	Female	2.30 (0.48)	1.30 (0.67)*	1.69 (0.5)*	1.80 (0.42)	0.002[b]
Following 4 week recovery; control $n = 5$; 200 mg/kg/day $n = 5$						P=[d]
Sodium mmol/L	Male	150.6 (0.89)	–**	–	154 (2.7)	0.032
	Female	153.2 (3.19)	–	–	154 (2.0)	0.65
Total protein g/L	Male	67.8 (3.1)	–	–	74.0 (4.2)	0.03
	Female	79.6 (7.8)	–	–	76.0 (2.7)	0.36
Globulin g/L	Male	30.2 (2.17)	–	–	33.4 (1.52)	0.027
	Female	32.6 (2.88)	–	–	31.6 (1.82)	0.53
Prothrombin time seconds, mean (SD)	Male	15.0 (0.39)	–	–	15.1 (0.48)##	0.74
	Female	14.8 (0.42)	–	–	15.5 (0.43)##	0.034

[a] Blood samples were taken at termination of feeding *meso*-zeaxanthin (MZ) for 13 weeks at doses shown or after 4 weeks recovery on a normal diet. Data are means (SD) except alkaline phosphatase activity for which medians (ranges) are given.
[b] Differences analysed using Kruskal–Wallis ANOVA for non-parametric data. * Differences between medians of control and specific feeding groups $P < 0.05$ (Kruskal–Wallis test).
[c] Differences analysed using ANOVA for normal data. # Differences between control and specific feeding groups at $P < 0.05$ (Dunnett's two-sided test).
[d] Differences analysed by independent '*t*' tests. ##$N = 4$. ** '–' indicates no animals retained in these groups.

the weight gain was greater in the 200 mg MZ/kg/day than the control group ($P = 0.049$) and in the females, weight gain in the 200 mg MZ/kg/day was greater than in the 2 mg MZ/kg/day group ($P = 0.026$, repeated measure ANOVA, LSD test). In addition some significant increases in weekly body weight changes were noted as follows: 20 mg/kg/day males on study days 22–29 and 43–50; 200 mg/kg/day males on study days 36–43; 2 mg/kg/day females on study days 43–50; and 200 mg/kg/day females on study days 22–29 and 71–78. All the significant changes were considered incidental and unrelated to treatment because the changes were infrequent, sporadic and/or not dose related.

Weight changes over the entire 17 weeks in the control and 200 mg MZ/kg/day animals that were retained for recovery, are shown in Fig. 3. In both males and females there were no differences between the two groups.

3.1.6. Organ weight

At the 13 weeks sacrifice, the following significant differences in absolute and relative organ weight data were noted: lower adrenal and/or adrenal/body weight ratios in all treated females; lower brain/body weight ratios in the 20 and 200 mg/kg/day females; and higher liver/brain weight ratio in the 20 mg/kg/day females.

At recovery sacrifice, the following significant differences in absolute and relative organ weight data were noted: lower thymus weight, heart/body weight ratio, thymus/body weight ratio, and thymus/brain weight ratio in the 200 mg/kg/day males. No significant differences were noted in the female data.

All organ weight changes noted above were considered incidental and unrelated to treatment, due to lack of dose responses and/or microscopic correlations.

3.1.7. Histopathology

No compound-related histopathology findings were noted.

Lesions considered to be spontaneous and incidental were observed in treated and control rats. These lesions consisted of early lesions of nephropathy (tubular regeneration; cortical, medullary and mucosal mononuclear cell infiltrates; and mineralisation within the kidney); vacuolation within the adrenal gland; mononuclear cell infiltration within the Harderian gland; hepatocellular vacuolation and mononuclear cell infiltration within the liver; acute haemorrhage within the lung, mandibular lymph node, and thymus; dilation of uterus; and mononuclear cell infiltration within the prostate. These lesions were noted sporadically, in low frequency, and/or were not dose-proportional, and are recognised as background findings of rats.

Some microscopic observations seen only in compound-treated animals were also considered to be spontaneous due to incidence and severity. At week 13, focal, minimal, granulomatous inflammation within the liver in a female animal (200 mg MZ); unilateral, pelvic dilation within the kidney in a male animal (20 mg MZ);

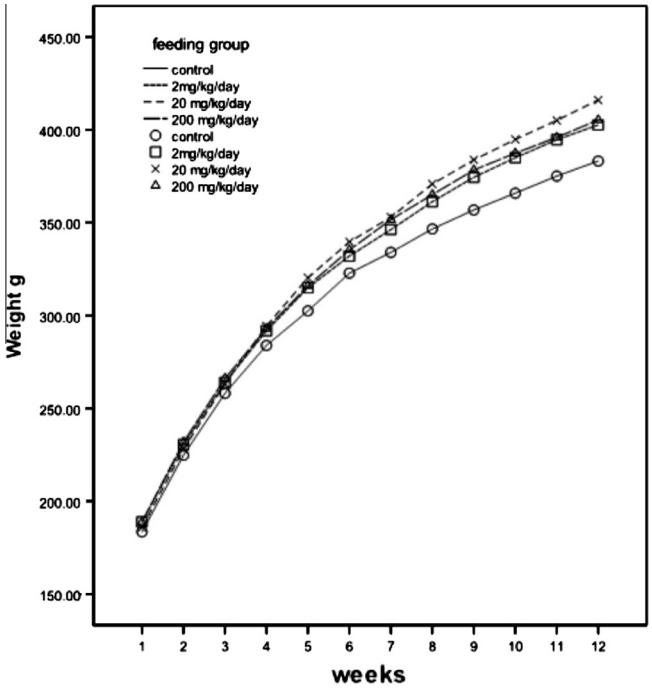

Fig. 1. Effects of feeding *meso*-zeaxanthin in corn oil at 2, 20 or 200 mg/kg/day on male rats for 12 weeks. There were 10 animals per group and food and water were supplied *ad libitum*. Control rats (-o-) received corn oil 10 mL/kg based on the most recent weight. MZ was fed at the same rate from solutions containing 0.2 (-□-), 2.0 (-x-) and 20 mg (-Δ-) MZ/mL corn oil. Rats were 7–8 weeks of age at the time of the first dose. Body weights at initiation of treatment were 150–170 g (males) and 105–125 (females). Growth rates were not significantly different between groups (repeated measures ANOVA). For clarity, error bars are not shown. Standard deviations of the weights of male rats (expressed as percentages of the means) increased over the 12 weeks from 3.4% to 8.3%.

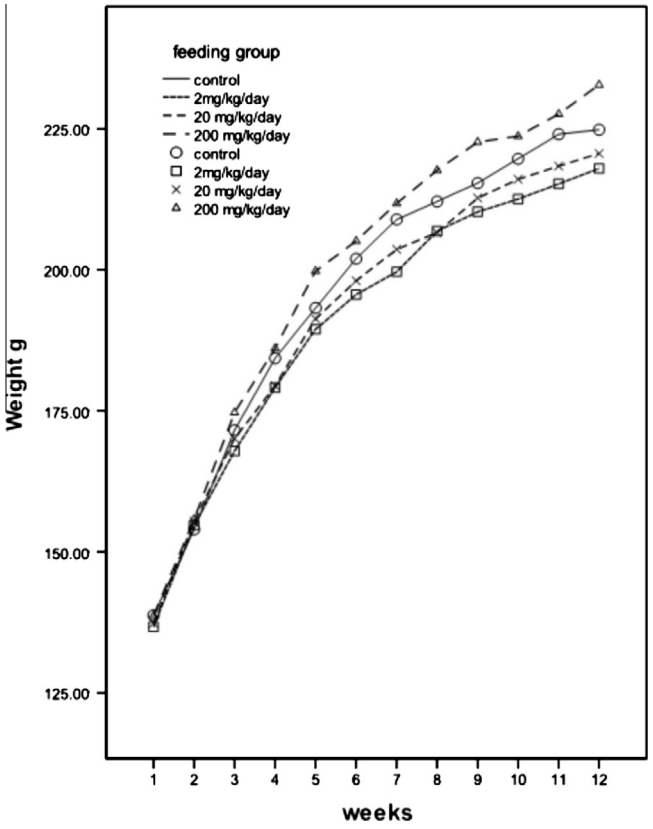

Fig. 2. Effects of feeding *meso*-zeaxanthin in corn oil at 2, 20 or 200 mg/kg/day on female rats for 12 weeks. See Fig. 1 for details. For clarity, error bars are not shown. Standard deviations of the weights of male rats (expressed as percentages of the means) increased over the 12 weeks from 3.4% to 7.1%.

multifocal, minimal, histiocytosis within the lung in one female animal (200 mg MZ); focal, minimal, perivascular mononuclear cell infiltrate within the pancreas and focal, minimal, luminal neutrophilic infiltrate within the prostate in a male animal (200 mg MZ); multifocal, minimal, sub-acute inflammation within the stomach in another male animal (2 mg MZ); multifocal, minimal, sub-acute haemorrhage within the thymus in a female animal (200 mg MZ) and multifocal, minimal, mononuclear cell infiltrate with the lachrymal gland in a male animal (200 mg MZ) were considered incidental and/or spontaneous. Multifocal, unilateral, sub-acute, mucosal inflammation within the kidney in male animals (200 mg MZ) and (control), at 13 and 17 weeks respectively, were also considered incidental and unrelated to the test article administration.

3.2. Genotoxicity studies

3.2.1. Cytotoxicity

The cytotoxicity study served to determine the dose range for the mutagenicity study and determine any cytotoxic properties of the test material. The test material was suspended in DSMO and the amounts tested ranged from 6.67 to 5000 μg/plate in the presence and absence of the S9 mix. There was no evidence of cytotoxicity; the numbers of revertants per plate for the vehicle controls in the presence of absence of the S9 mix for the TA100 strain were 122 and 88 and for WP2*uvr*A strains 18 and 16 respectively. The range of values for the test material were 90–118 and 71–106 for TA100 and 9–21 and 11–16 for WP2*uvr*A respectively. Amounts of the test article >333 μg tended to precipitate in the plates and >1000 μg to obscure the background 'lawn'.

3.2.2. Mutagenicity

The amounts of the MZ material tested covered a range from 10 to 5000 μg/plate. Mean (SD) of the number of revertants per plate for triplicate assays is shown in Table 6. All bacterial tester strains used reacted with the expected sensitivity to the positive control substances. However, the number of revertants obtained at any concentration of MZ, with or without microsomal stimulation, was no different from the rat-liver vehicle-control responses obtained for each bacterial test strain. The experiment was repeated once more and confirmed the above findings.

All data not displayed can be viewed at (The Howard Foundation, 2006).

4. Discussion

4.1. Animal toxicology study

The studies reported in this paper provide no evidence to suggest that MZ demonstrates, or suggest any grounds to suspect, a hazard to public health when provided as a dietary supplement. The product tested was a mixture of mainly MZ (~61%) with smaller amounts of lutein (~22%) and zeaxanthin (~15%). It has been argued for the mixture of lutein and zeaxanthin, that safety testing of substances that are intended to supplement the diet, should be done on the whole product (Kruger et al., 2002) since the safety of the product is determined by evaluating the source of the product, the production process, nature and quantify of impurities and product specifications. Like the lutein and zeaxanthin mixtures, the original source of the MZ was from marigold flowers (*Targetes erecta*) but the lutein extracted was converted to MZ using alkaline

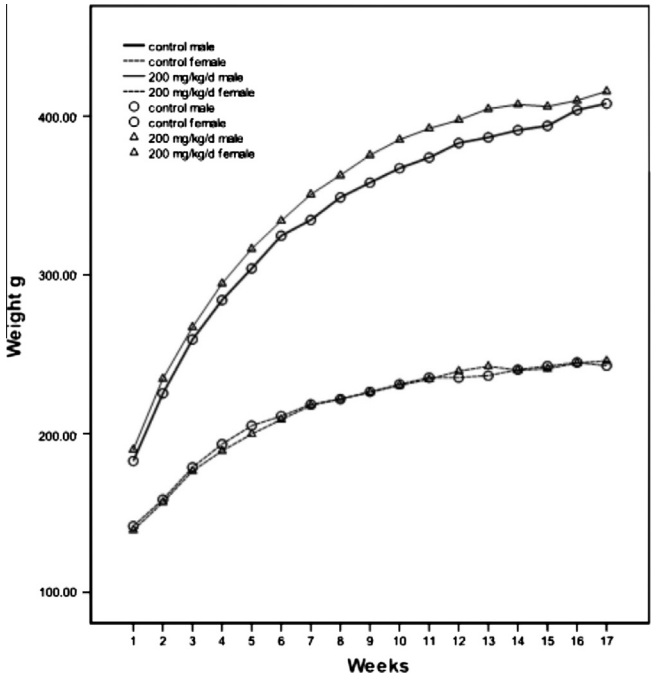

Fig. 3. Effects of feeding *meso*-zeaxanthin in corn oil at 200 mg/kg/day for 13 weeks followed by 4 weeks recovery. *Meso*-zeaxanthin (MZ) treated rats were fed 200 mg/kg/day diet for 13 weeks before placement on the control diet for the last 4 weeks. See Fig. 1 for fuller details of feeding. Growth was significantly different between the sexes but there was no difference between control and MZ-treated rats of either sex (repeated measures ANOVA). Symbols used in growth curves; male control (––o––), 200 mg/mL (––Δ––), female control (–o–) and 200 mg/mL (–Δ–).

hydrolysis and the non-esterified carotenoids were purified by a patented procedure (Montoya-Olvera et al., 2003). Using the whole product, corroboration of safety has been obtained by animal toxicological studies which indicated a no-observed-adverse-effect-level (NOAEL) for MZ of >200 mg/kg body weight or 344 mg/kg whole product. Supplements of MZ currently being assessed for effectiveness for eye health provide approximately 10 mg or 20 mg whole product. Ten mg MZ for 70 kg person is ~0.143 mg/kg, that is the NOAEL for MZ is >1400 times higher than the proposed intake of MZ or >700 times higher than the NOAEL of the whole product.

These results have also been confirmed very recently by Chinese workers who carried out acute toxicity testing in male and female rats for 90 days at a top dose of 300 mg MZ/kg/day (Xu et al., 2013). They also found no acute toxicity and no genotoxicity and, after applying a 100 fold safety factor, arrived at an acceptable dietary intake of 3 mg/kg/day.

The procedure we used to test the MZ concentrate for potential toxicity was that recommended by the FDA in which rats are given the substance under examination daily for 13 weeks (Food and Drug Administration, 2012). Even the lowest rat dose of 2 mg/kg/day is 4–5 times higher than those of the xanthophyll carotenoids typically given to man (Sabour-Pickett et al., 2012; Nolan et al., 2012; Olmedilla et al., 2001). Human studies have shown for lutein (Thurmann et al. 2005) and zeaxanthin (Hartmann et al., 2004) that plateau concentrations in plasma proportional to dose are achieved after 3 weeks daily dosing. MZ appears to be less well absorbed than either lutein or zeaxanthin (Schiedt et al. 1985; Thurnham et al., 2008) nevertheless macular pigment optical density (MPOD) was significantly increased at 0.25, 0.5 and 1.0 degrees at 2 weeks following oral MZ treatment of 5 normal adult subjects and 5 subjects with ARMD with 7.3 mg MZ, 1.7 mg lutein and 0.8 mg zeaxanthin (Connolly et al., 2010). The Connolly study was only an 8 week intervention but longer studies of 6 months (10 mg MZ, 10 mg lutein and 2 mg zeaxanthin) (Loughman et al., 2012) have also been reported and no evidence of any harmful effects of treatment with MZ containing preparations has resulted.

The rats we used for the toxicity studies were from the Wistar Han strain and are recommended for general research applications including toxicology. Among the characteristics of the rats are alopecia, typically sides and neck, and spontaneous pathology which

Table 6
Mutagenicity assay results[a].

Test article	Dose/plate[b] μg	Revertants per plate					Background Lawn[f]
		TA98[e]	TA100	TA1535	TA1537	WP2*uvr*A	
Microsomal test system							
Vehicle control[c]		17 (4)	96 (9)	10 (4)	8 (4)	15 (1)	N
Meso-Zeaxanthin; plating aliquot 100 μL	10.0	20 (2)	100 (14)	10 (3)	6 (1)	9 (4)	N
	33.3	16 (5)	96 (23)	13 (4)	6 (4)	17 (1)	N
	100	24 (4)	94 (12)	12 (3)	6 (3)	15 (3)	N
	333	18 (8)	97 (12)	11 (2)	6 (2)	13 (2)	NP/OP
	1000	21 (4)	90 (12)	13 (3)	7 (2)	17 (4)	OP
	5000	23 (3)	105 (5)	10 (2)	11 (7)	13 (3)	OP
Positive control[d]		364 (48)	727 (90)	138 (10)	157 (9)	512 (33)	N
Microsome-free test system							
Vehicle control		9 (3)	68 (8)	!3 (2)	6 (3)	12 (3)	N
Meso-zeaxanthin; plating aliquot 100 μL	10.0	10 (1)	81 (10)	16 (3)	5 (1)	14 (3)	N
	33.3	9 (3)	65 (15)	14 (4)	7 (1)	19 (3)	N
	100	12 (3)	78 (11)	13 (4)	6 (4)	17 (6)	N
	333	9 (3)	78 (10)	10 (4)	6 (4)	17 (3)	NP
	1000	8 (3)	79 (14)	10 (4)	3 (4)	13 (3)	OP
	5000	8 (3)	81 (11)	11 (4)	4 (3)	12 (3)	OP
Positive control[d]		357 (33)	1182 (76)	690 (93)	959 (127)	267 (50)	N

[a] Numbers of revertants following incubation for 52 h at 37 °C with the test articles shown are shown as means (±SD).
[b] Test substances were suspended in dimethyl sulphoxide (DMSO) and all tests were analysed in triplicate.
[c] Vehicle controls were DMSO in place of MZ or positive control substances all analysed in the presence or absence of microsomal enzyme preparation.
[d] Positive controls were known mutagens for the specific bacterial test systems.
[e] Tester strains were *Salmonella typhimurium* (TA) auxotrophs for histidine dependence and additional sensitivities and Escherichia coli (WP2*uvr*A) which was tryptophan dependent. See methods for additional information on all aspects of the above experiment.
[f] Background lawn evaluation codes: N, normal; O, obscured; P, precipitate. Where there are two entries, the first is the lawn evaluation for the TA strains and the second for WP2*uvr*A.

makes them useful as models for long-term studies (Charles River, 2013). We found no consistent, compound-related abnormalities occurred during the feeding or recovery studies. Alopecia was noted in both controls and MZ-treated groups and the abrasions noted spontaneously recovered (Table 3). The incidental ophthalmological, clinical and haematological findings (Tables 4 and 5) were not treatment or dose-related and were more probably spontaneous pathologies. Growth characteristics in an actively-growing animal are a good indication of potential pathology. In this study the animals were fed MZ from 7 to 8 weeks of age. Published data for these rats indicates rapid growth for a further 6 weeks (Charles River, 2013). The growth data shown in Figs. 1–3 indicate the most rapid growth over the first 6 weeks of the study in both sexes after which the rate declined. There were no significant differences in the rates of growth between the groups during treatment or recovery phases; if anything both male and female rats receiving 200 mg MZ/kg/day tended to grow slightly more rapidly than the controls, but all rats grew within the reference range reported by the supplier (Charles River, 2013).

4.2. Genotoxicity and mutagenicity of MZ

The results of the *S typhimurium–E. coli*, mammalian-microsome reverse mutation assay indicated that MZ did not increase the mutation rate by either insertion or substitution of nucleotides to cause a positive increase in the mean number of revertants per plate with any of the tester strains either in the presence or absence of mammalian microsomes. The doses tested ranged from 10 to 5000 μg/plate. Supplements would typically contain 10 mg MZ but it is difficult equating dose/plate to a physiological effect in man. Nevertheless it has recently been reported that amounts of MZ of as little as 5 μg/plate had anti-mutagenic activities when added to positive controls in the genotoxicity assay (Firdous et al., 2010). That is the number of revertants/plate was reduced when MZ was co-incubated with a relevant mutagen in the presence of *S. typhimurium* strains TA98, TA100, TA102, TA1535 in a dose-dependent fashion. Furthermore, the authors showed that MZ inhibited microsomal CYP450 enzymes and thus was able to reduce the risk of liver cancer and liver damage in a dose-dependent fashion when rats were exposed to nitroso-diethylamine – a potent cancer-forming compound (Magee et al., 1976). However the lowest dose of MZ with anti-cancer properties was 50 mg MZ/kg/day which is far higher than is likely to be used in humans. The study of Firdous and colleagues however do indicate why no genotoxic effects were found when MZ was incubated with the tester strains; the data suggest that MZ itself may potentially react with environmental carcinogens to lower the risk of genetic mutations.

5. Conclusions

Rat toxicity studies showed that amounts of MZ of 2, 20 and 200 mg/kg/day for 13 weeks had no adverse effects on animal health. That is the NOAEL is >200 mg MZ/kg body weight and this is at least 1400 times higher than the typical supplement dose. Genotoxicity testing indicated that amounts of MZ from 10 to 5000 μg/plate with or without microsomal enzymes did not increase mutation rates in five bacterial tester strains.

Disclosure of interests

DIT is a consultant to the Howard Foundation and receives a fee for services. AHN is a trustee of the Howard Foundation.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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