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 (smicrosomai enzymes) at 2 genonomic loci; the histidine locus of 4 strains of Salmonella typhimurium and the tryptophan locus of Escherichia coli strain WP2uvrA. 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.

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1. Introduction

The carotenoid meso-zeaxanthin (MZ; (3R,3S)-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′S)-dihydroxy-β, β-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 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 with age-related macular disease (ARMID) 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...
as it is the standard species for use in toxicology studies as recom-
med by the Food and Drug Administration and the Interna-
tional 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 Salmo-
nella typhimurium and at (2) the tryptophan locus of Escherichia coli
tester strain WP2uvRA. Further details of the animal and genotox-
icty 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, Gai-
thersburg, MD 20879, USA (Gene Logic) between 2005 and 2006. Gene Logic’s Insti-
tutional 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 Inter-
agency 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 re-
lease 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% pro-
tein 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 hr 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, (Monte-
ray, 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 ob-
tained and contained MZ 207 g/kg in a total carotenoid mixture of 324 g/kg. Three
batches of corn oil were used for animal dosing and in the preparation of the test dilu-
tions (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
warned at 50 °C for 20 min prior to use. Dose formulations were prepared by add-
ing 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 formu-
lations (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 bath at 40 °C for at least 5 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 (Monterrey, 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 sepa-
ately. 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 observa-
tions and body weight were made once weekly prior to oral gavage and at terminal
sacrifice. Clinical observations included body weight, clinical chemistry, blood
angle and mucus membranes, respiratory, circulatory, autonomic and central nerv-
sous systems and somatomotor and behaviour patterns. Ophthalmological examin-
ations were made using indirect ophthalmoscopy prior to terminal sacrifice and
following 1% Tropicamide dilution 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 anaes-
thesia (70% CO2/30% O2). 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 Labora-
tory for analysis. The clinical variables measured and methods of analysis are de-
scribed 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 haemoglo-
bin, mean corpuscular haemoglobin concentration, mean platelet volume, platelet
count, absolute counts of neutrophils, lymphocytes, monocytes, eosinophils, baso-
phil, reticulocytes. Blood smears for cellular morphology were prepared and
stained using a quick Romanowsky type of stain. Cellular morphology was deter-
mimed by visual examination of the stained smear. Coagulation variables were mea-
sured on a Beckman Coulter ACL 1000 Coagulation Analyzer. Beckman control
samples were analysed each day of testing. Coagulation variables measured in-
cluded the activated partial thromboplastin time and prothrombin time using a lar-
s-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. Ani-
mals 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 cra-
nial, thoracic, and abdominal cavities, and contents within each body cavity was
performed. Protocol—specified organs were weighed as soon as possible after dissec-
tion; 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

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>MZ concentrate</th>
<th>Numbers of Han Wistar rats</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Target dose mg/kg/day</td>
<td>Concentration administereda mg/mL corn oil</td>
</tr>
<tr>
<td>1</td>
<td>Corn oil</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>MZ</td>
<td>20</td>
<td>2.0</td>
</tr>
<tr>
<td>3</td>
<td>MZ</td>
<td>200</td>
<td>20.0</td>
</tr>
<tr>
<td>4</td>
<td>MZ</td>
<td>200</td>
<td>20.0</td>
</tr>
</tbody>
</table>

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.

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

*AnOrtho-Clinical Diagnostics Vitro 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 Margold 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). Suspend- tions 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/ml so this was the maximum dose used for the mutagenicity assay.

2.2.2. Mutagenic 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 trypto- phan auxotroph WP2uvrA (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 (raf) 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 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 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 at least 0.5 x 10^8 cells/ml was achieved the cultures were held at >0-10 °C until use.

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 (0.05 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 at least 0.5 x 10^8 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. 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-nitroguanin-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 59 mlx. 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 59 mlx. Revertant colonies were counted by an automated colony counter or by hand. For tester strains TA98, TA100 and WP2uvrA, 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 po- sitive 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.
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 20 mg/kg/day female 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

<table>
<thead>
<tr>
<th>Observations</th>
<th>Treatment groups</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Corn oil MZ – 2 mg/kg/day MZ – 20 mg/kg/day MZ – 200 mg/kg/day</td>
</tr>
<tr>
<td></td>
<td>N Duration Site</td>
</tr>
<tr>
<td>Alopecia – males</td>
<td>0 1 1</td>
</tr>
<tr>
<td>Alopecia – females</td>
<td>1 1 1</td>
</tr>
<tr>
<td></td>
<td>1 1 1</td>
</tr>
<tr>
<td></td>
<td>1 1 1</td>
</tr>
<tr>
<td>Abrasions – males</td>
<td>0 0 0</td>
</tr>
<tr>
<td>Abrasions – females</td>
<td>0 0 0</td>
</tr>
<tr>
<td>Hyperactivity – male</td>
<td>0 0 0</td>
</tr>
<tr>
<td>Hyperactivity – females</td>
<td>1 1 1</td>
</tr>
</tbody>
</table>

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.
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 in the 20 mg MZ/kg/day females on study days 36–43; 2 mg/kg/day females on study days 43–50; and 200 mg MZ/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 MZ/kg/day females; and higher liver/body weight ratio in the 20 mg MZ/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);
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 inflammation 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 DMSO and the amounts tested ranged from 6.67 to 5000 \( \mu g/\)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 WP2uvrA 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 WP2uvrA respectively. Amounts of the test article >333 \( \mu g/\)tended to precipitate in the plates and >1000 \( \mu g/\)to obscure the background ‘lawn’.

3.2.2. Mutagenicity

The amounts of the MZ material tested covered a range from 10 to 5000 \( \mu g/\)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 (\( \sim 61\% \)) with smaller amounts of lutein (\( \sim 22\% \)) and zeaxanthin (\( \sim 15\% \)). 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 quantity of impurities and product specifications. Like the lutein and zeaxanthin mixtures, the original source of the MZ was from marigold flowers (Tagetes erecta) but the lutein extracted was converted to MZ using alkaline
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-Picket 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; Thurham 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

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**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 (– –), female control (–o–) and 200 mg/mL (– –).
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 reverse mutation assay indicated that MZ did not increase the

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