Food Control 32 (2013) 678–686

Contents lists available at SciVerse ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Safety evaluation of *meso*-zeaxanthin

Xinde Xu [a,*], Lihua Zhang [a], Bin Shao [a], Xiaoxia Sun [a], Chi-Tang Ho [b], Shiming Li [b,**]

[a] Zhejiang Medicine Co., Ltd., Xinchang Pharmaceutical Factory, Xinchang, Zhejiang Province 312500, PR China
[b] Department of Food Science, Rutgers University, The State University of New Jersey, New Brunswick, NJ 08901, USA

ARTICLE INFO

Article history:
Received 8 August 2012
Received in revised form
5 February 2013
Accepted 6 February 2013

keywords:
Meso-zeaxanthin
Safety evaluation
Xanthophylls
In vivo toxicity study
Carotenoids
Food safety

ABSTRACT

Meso-zeaxanthin is a xanthophyll family member of carotenoids. Having closely related structures, *meso*-zeaxanthin, zeaxanthin and lutein have high concentration in macula and are believed to play a major role in protecting retinal constituents from free radicals. Due to the current technical difficulties in measurement, the presence of *meso*-zeaxanthin in human blood or tissues has not been reported except in human eyes, which has promoted scientists' interest in exploring potential health benefits of *meso*-zeaxanthin. Herein, we report a complete toxicological safety assessment of *meso*-zeaxanthin for use as an ingredient in food, dietary and nutritional supplements, as well as medical food. Assays of acute toxicity, genetic toxicity (Ames test, mice bone marrow erythrocyte micronucleus and mice sperm abnormality) and 90-day sub-chronic toxicity were performed. In the acute oral toxicity tests, maximum tolerable dose was more than 10.0 g/kg bw in SD rats and ICR mice, and showed no toxicological signs during the period of the study. The testing results for three terms of hereditary toxicity (Ames test, mice bone marrow erythrocyte micronucleus and mice sperm abnormality) were all negative. For 90-day feeding of *meso*-zeaxanthin at the dosage of 300 mg/kg/d in both male and female SD rats, there is no noticeable toxicological effects observed. Therefore, *meso*-zeaxanthin has no acute toxicity and no genotoxicity and the use of *meso*-zeaxanthin is safe at dose of 300 mg/kg bw/day in rats from a 90-day feeding study. After the application of a 100-fold safety factor, we obtained the ADI (acceptable daily intake) value of 3 mg/kg body weight per day.

1. Introduction

The carotenoid pigments of the macula are collectively known as macular pigments, composed of oxygenated carotenoids lutein and zeaxanthin. The human body cannot synthesize the macular pigments and completely relies on diet intake (Landrum & Bone, 2001).

Meso-zeaxanthin is a unique member of the xanthophyll family of carotenoids. Unlike other carotenoids, *meso*-zeaxanthin and its stereoisomer, zeaxanthin, and its structural isomer, lutein, have particular high concentrations in the macula (Landrum & Bone, 2001). *Meso*-zeaxanthin, zeaxanthin and lutein are believed to play major roles in protecting retinal constituents from free radicals (Li, Ahmed, & Bernstein, 2010; Wooten & Hammond, 2002). Scientists became interested in exploring the potential health benefits of *meso*-zeaxanthin because *meso*-zeaxanthin is not usually found in the human blood or other organ tissues, but it can be always found in human eyes, especially in the fovea. *Meso*-zeaxanthin represents approximately one third of the total macular pigment in fovea of retina, whereas 15% in the whole retina (Bone, Landrum, Hime, Cains, & Zamor, 1993; Chang, 2006; Landrum & Bone, 2001), suggesting that *meso*-zeaxanthin might come into being in the eyes rather than other organ tissues (Bone et al., 1997; Loane, Kelliher, Beatty, & Nolan, 2008; Neuringer, Sandstrom, Johnson, & Snodderly, 2004).

It has been reported that the mechanism of *meso*-zeaxanthin protecting eyes' health includes at least two aspects: absorbing harmful high energy blue light and possessing strong anti-oxidative properties. As natural colorants and also for their role in human health, xanthophylls like lutein, (*R,R*)-zeaxanthin and (*R,S*)-zeaxanthin have attracted much attention of scientists and researchers in the biomedical, chemical and nutritional fields in recent years (Bone, Landrum, Alvarez-Correa, Eeienne, & Ruiz, 2003; Bone, Landrum, Cao, Howard, & Alvarez-Calderon, 2007; Connolly et al., 2011; Connolly, Beatty, Loughman, & Nolan, 2010; Firdous, Preethi, & Kuttan, 2010). A few years ago, Chang (2006) examined the potential toxicity of *meso*-zeaxanthin in a 13

* Corresponding author. Tel.: +86 (0)575 8613 2603.
** Corresponding author. Tel.: +1 973 919 3702.
E-mail addresses: xuxinde@xcpharma.com (X. Xu), shiming3702@yahoo.com, shiming3702@gmail.com (S. Li).

0956-7135/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.foodcont.2013.02.007

consecutive week study with gavage feeding of dosages upto 200 mg/kg in Han Wistar rats and found no signs of toxicity. Herein we report our thorough evaluation and toxicological assessment of *meso*-zeaxanthin in rodent model, such as genotoxicity, acute oral toxicity, and sub-chronotoxicity, to serve as scientifically defensible safe data and no observed adverse effect level (NOAEL) on *meso*-zeaxanthin as well.

2. Materials and methods

2.1. Preparation of meso-zeaxanthin

Meso-zeaxanthin (lot# 100303) was provided by Zhejiang Medicine CO., Ltd., Xinchang Pharmaceutical Factory, Xinchang, Zhejiang, China. The content of *meso*-zeaxanthin in total xanthophylls is over 85%, which has been quantified by HPLC (high performance liquid chromatography) using a chiral column (Chiral PAK AD-H 5 μm, 250 × 4.6 mm). The HPLC conditions are as follows: isocratic mobile phase, n-Hexane:ethanol:isopropyl = 80:10:10; column temperature, 35 °C; flow rate, 0.5 ml/min; wavelength, 453 nm; injection volume, 20 μL. The substance was stored in an air tight and light-resistant container in a cold place at 4 °C no more than six months prior to its consumption.

2.2. Animals and animal housing

Male and female ICR mice and SD rats were purchased from Experimental Animal Center of Zhejiang Province, China (laboratory animal reproduction license # SCXK (Z) 2008-0033). Animal feed was purchased from the same company (operative standard# GB14924.1-2001). The studies were conducted in compliance with Good Laboratory Practices (GLP) at the Center for Disease Control and Prevention of Zhejiang (ZJCDC) (laboratory animal use permit# SYXK [Z] 2008-0106). Temperature was controlled at 22 °C–24 °C; relative humidity was controlled at 50%–70% and light control was maintained as a 12 h dark–light cycle throughout the test period. All animals were examined for their general physical conditions upon adoption and acclimatized for 3 days before any test. Prior to *meso*-zeaxanthin feeding, the animals were fasted overnight, with unlimited water supply.

2.3. Dose formulation

In this study, each dosing suspension was prepared individually by mixing *meso*-zeaxanthin with distilled water in a homogenizer. Each dosage is standardized to pure *meso*-zeaxanthin based on its purity. Dosing formulations were stored at 4 °C, homogenized daily for at least 2 min and allowed to warm up to room temperature prior to administration.

2.4. Experiment designs and methods

2.4.1. Acute oral toxicity test in SD rats

Maximum tolerable dose (MTD) test was conducted because *meso*-zeaxanthin had not been reported for its toxicity or adverse effects in human being. Twenty of SD rats, ten males and ten females, weighed 180–220 g, were used in this MTD test. *Meso*-zeaxanthin (50.0 g) was mixed with distilled water to a total volume of 200 ml, and administered twice at 4 h intervals, 20 ml/kg bw for each oral administration, the accumulated *Meso*-Zeaxanthin dose was equal to 10.0 g/kg bw. Toxicological signs and morbidity were monitored and recorded daily for two week period after intragastric gavage (Ig) administration.

2.4.2. Acute oral toxicity test in ICR mice

Maximum tolerable dose (MTD) test was conducted. Twenty of ICR mice (ten males and ten females), weighed 18–22 g, were used. *Meso*-zeaxanthin (50.0 g) was mixed with distilled water to a total volume of 200 ml and administered twice at 4 h intervals, 20 ml/kg bw for each oral administration, the accumulated *meso*-zeaxanthin dosage was 10.0 g/kg bw. Toxicological signs and morbidity were monitored and recorded daily for two weeks after Ig administration.

2.4.3. Ames test

Ames test was performed with and without metabolic activation. Supernatant *S*9 was obtained from PCB-induced SD rat liver homogenate, and tested by 2-AF and 1,8-dihydroxyanthraquinone to confirm its bioactivity. Four certificated histidine-deficient strains of *Salmonella typhimurium* TA97, TA98, TA100, and TA102 were provided by toxicology department of the CDC of Shanghai, China. The plate incorporation method was used in Ames test. The maximum dose was 5000 μg/dish. 1 g of *meso*-zeaxanthin samples and distilled water were sterilized and mixed to make a 20 ml of sample solution with a concentration of 50,000 μg/ml. The resulted sample solution (0.1 ml) was added to each Petri dish, which is equivalent to 5000 μg/dish. The four testing dosages of *meso*-zeaxanthin were 5000, 1000, 200 and 40 μg/dish, respectively. Blanket control group, negative solvent control (distilled water) and strain-specific positive controls were included in each test. All strains were tested using three plates per dosage. The tests were repeated under the same conditions to confirm the results.

The number of reverted colonies were counted with or without metabolic activation (*S*9) and compared to the number of spontaneous reverting colonies of blank control group and negative solvent control group, respectively. The test substance will be considered to be mutagenic if the reverted colonies are two folds or more than that of the number of the spontaneous reverting colonies on negative solvent control plates. A dose response relationship was observed in at least two concentrations.

2.4.4. Mice bone marrow erythrocyte micronucleus assay

Fifty of ICR mice, weighed 25–30 g, twenty-five males and twenty-five females, were divided into five groups randomly. Cyclophosphamide (60 mg/kg bw) was administered as a positive control and distilled water was used as a negative control. *The meso*-zeaxanthin dose levels of testing groups were 1.25, 2.5 and 5.0 g/kg bw, respectively. The testing samples were prepared by mixing *meso*-zeaxanthin (2.5, 5.0, and 10.0 g) with distilled water to a total volume of 40 ml, to obtain the concentrations of 0.0625, 0.125, 0.25 g/ml, respectively. Testing materials were Ig administered twice at 20 ml/kg bw at 24 h intervals. Execution *via* cervical vertebra dislocation was conducted 6 h after the last dose administration. Sternum bone was removed and the bone marrow cells were pulled out and mixed with fetal bovine serum immediately following the sacrifice. One drop of the mixture was smeared onto a clean slide and air-dried. The slides were briefly flamed, then fixed with immersion in 95% methanol for 10 min, and stained in ordinary staining jars with Giemsa Working Solution for 15 min. The stained slides were washed gently with ddH_2O (double distilled water), air-dried, and cover-slipped for microscope examination. All slides were coded to ensure that the evaluation was blinded. Micronucleus frequencies were determined for each animal by counting 1000 of polychromatic erythrocytes (PCE) and the micronucleus occurrence rate per one thousand PCE was recorded. The proportion of immature erythrocytes (i.e. PCE) to total erythrocytes (RBC) was determined for each animal by counting a total of 200 erythrocytes. Mean ± SD of micronucleus occurrence rate and PCE/RBC ratio of each group were compared using SPSS11.0 software.

2.4.5. 5. Mice sperm abnormality test

Thirty-five male ICR mice, weighed 25–30 g, were divided into five groups randomly. The testing samples were prepared by mixing *meso*-zeaxanthin (2.5, 5.0, and 10.0 g) with distilled water to a total volume of 40 ml, to obtain the concentrations of 0.0625, 0.125, 0.25 g/ml, respectively. Mitomycin C (MMC) (2.0 mg/kg bw) was used as a positive control and distilled water was used as a negative control. *Meso*-zeaxanthin dose levels of testing groups were 1.25, 2.5, 5.0 g/kg bw, respectively. Intubations occurred daily at 20 ml/kg bw for 5 days. Five mice were selected randomly in each group. Execution was conducted *via* cervical vertebra dislocation 35 days after the first dose administration. Epididymis was isolated and placed on a flat dish containing 2 ml of 0.9% NaCl solution. The epididymis was cut by using ophthalmological scissors longitudinally once or twice, allowed to settle for 3–5 min, vibrated gently, and filtered with four layers of microscopy cleaning paper. One drop of the filtrate was smeared onto a clean slide and air-dried. Slides were then fixed with immersion into 95% methanol for 5 min, stained with 1% Eosin dye for 1 h, washed gently with ddH_2O, and air-dried. Sperm morphology was examined at high magnification. A total of 1000 sperm was counted for each animal in an optical microscope. The percentage of abnormalities was calculated, first as a total, then further classified in relation to the specific location of each abnormality in the sperm.

2.4.6. 90-Day feeding study

Eighty of SD rats were randomly divided into four groups, 20 rats (10 males and 10 females) were used in each group. Rats were caged individually in stainless steel open-mesh cages, freely eating and drinking during the study period. The experiment was performed at three dosage groups and one negative control (distilled water) group. The dose levels of *meso*-zeaxanthin was 300 (low dose group), 600 (intermediate dose group), 1200 mg/kg bw/day (high dose group), respectively. Samples of 7.5 g, 15 g and 30 g were measured and diluted with distilled water to a total volume of 250 ml to yield the concentrations of 0.03, 0.06, 0.12 g/ml, respectively. The testing sample solutions were Ig administered at the level of 10 ml/kg bw/day for 13 consecutive weeks. The volume administrated was calculated based on the most recently measured body weight. The control groups were treated with the same procedure.

Routine cage-side observations were conducted on all animals once a day throughout the study period for general behavior and toxicological signs. Body weight, feed consumption (feed added/feed left), as well as physical examinations were conducted weekly. In the middle of the study (day 42), animals were fast for 16 h, blood samples were collected from the caudal vein and analyzed using MEK-6318K automatic blood cell counter for hematology measurement, parameters including hemoglobin(HB), red blood cell count(RBC) and white blood cell count (WBC). Blood samples were collected from the orbital sinus and the serum were analyzed using TBA-40FR automatic biochemistry analyzer for clinical biochemistry measurement, parameters including alanine amino-transaminase (ALT), aspartate amino-transferase (AST), blood urea nitrogen (BUN), creatinine (Cr), lipoprotein (CHOL), triglyceride (TG), blood glucose, total protein, albumin and globulin (GLU). In the later period of the experiment (one day after the last dose on day 90), animals were fasted for 16–18 h, then blood samples were collected and the hematology and clinical biochemistry analysis were conducted as described above. Necropsy after taking blood samples was performed thoroughly and systematically by dissection of the viscera and carcass. Liver, kidney, spleen, testes/ovaries were weighed and calculated in organ-to-body weight ratios, and

Table 1
Effects of *Meso*-Zeaxanthin on Ames test.

Groups		Dose (μg/dish)	TA97	TA98	TA100	TA102
			$-S_9$ $+S_9$	$-S_9$ $+S_9$	$-S_9$ $+S_9$	$-S_9$ $+S_9$
Group I	*Meso*-zeaxanthin	40	119 ± 4.6 132 ± 3.1	31 ± 1.5 32 ± 1.2	130 ± 3.8 131 ± 2.5	255 ± 8.3 256 ± 10.0
		200	126 ± 3.5 133 ± 3.2	32 ± 1.0 33 ± 1.0	135 ± 3.6 136 ± 3.1	266 ± 8.0 265 ± 5.1
		1000	134 ± 3.5 145 ± 5.6	32 ± 0.6 36 ± 1.0	146 ± 2.5 146 ± 2.5	273 ± 6.7 279 ± 9.6
		5000	139 ± 8.7 147 ± 9.1	32 ± 2.0 39 ± 1.0	149 ± 7.0 151 ± 2.1	272 ± 14.2 297 ± 8.9
	Blank control	–	128 ± 6.6 138 ± 8.1	36 ± 1.5 38 ± 1.5	131 ± 9.7 143 ± 2.5	269 ± 8.1 278 ± 7.0
	Solvent(H_2O)	–	124 ± 2.0 136 ± 3.6	35 ± 1.0 40 ± 2.1	127 ± 2.1 143 ± 1.7	275 ± 8.7 278 ± 5.7
	Positive	–	3012 ± 99.0 1923 ± 99.0	2196 ± 162.1 2351 ± 173.9	2115 ± 119.2 1822 ± 125.2	1848 ± 108.6 1849 ± 79.1
Group II	*Meso*-zeaxanthin	40	118 ± 4.7 129 ± 5.0	31 ± 1.2 31 ± 1.2	127 ± 4.6 128 ± 3.2	253 ± 5.9 257 ± 5.1
		200	123 ± 3.2 134 ± 4.2	32 ± 1.5 32 ± 2.3	134 ± 4.5 133 ± 2.1	258 ± 4.7 274 ± 4.7
		1000	131 ± 3.6 143 ± 6.5	33 ± 2.5 35 ± 1.2	143 ± 2.6 140 ± 4.5	272 ± 6.0 284 ± 6.5
		5000	137 ± 5.7 149 ± 5.7	34 ± 1.5 40 ± 1.0	150 ± 4.2 152 ± 3.8	272 ± 7.5 298 ± 8.1
	Blank control	–	121 ± 6.7 146 ± 8.7	33 ± 2.6 38 ± 1.2	127 ± 7.0 141 ± 7.0	268 ± 6.4 281 ± 10.2
	Solvent(H_2O)	–	126 ± 5.1 141 ± 2.5	34 ± 2.1 38 ± 1.0	129 ± 4.7 142 ± 2.8	279 ± 7.1 278 ± 2.6
	Positive	–	2880 ± 166.2 1857 ± 194.7	1951 ± 128.5 1940 ± 72.3	1965 ± 217.8 1912 ± 187.1	1819 ± 97.7 1819 ± 151.0

Note: The result was mean ± SD of 3 plates. Positive control: TA97 + S_9, TA98 + S_9, TA100 + S_9 used 2-AF at 10.0 μg/dish; TA97 − S_9 used ICR-191 at 1.0 μg/plate; TA98 − S_9 used daunomycin at 6.0 μg/plate; TA100 − S_9 used NaN_3 at 1.5 μg/plate; TA102 + S_9 used 1,8-dihydroxyanthraquinone at 50.0 μg/plate; TA102 − S_9 used MMC (Mitomycin C) at 0.5 μg/plate.

Table 2
Effects of *Meso*-zeaxanthin on mice bone marrow micronucleus and PCE/RBC ratio ($n = 5$).

Sex	Dose (mg/kg)	Total PCE counted	Total micronucleus contain PCE	Micronucleus ratio($\bar{x} \pm s$) (‰)	*P*	PCE/RBC ($\bar{x} \pm s$)
Female	0	5000	6	1.2 ± 0.8	/	1.30 ± 0.28
	1250	5000	6	1.2 ± 0.8	0.554	1.16 ± 0.11
	2500	5000	7	1.4 ± 0.6	0.394	1.17 ± 0.10
	5000	5000	7	1.4 ± 0.9	0.394	1.23 ± 0.22
	60(CP)	5000	123	24.6 ± 10.2$^{\#}$	<0.001	1.03 ± 0.29
Male	0	5000	7	1.4 ± 1.1	/	1.35 ± 0.33
	1250	5000	7	1.4 ± 0.9	0.550	1.34 ± 0.34
	2500	5000	8	1.6 ± 0.6	0.401	1.32 ± 0.26
	5000	5000	8	1.6 ± 0.9	0.401	1.31 ± 0.27
	60(CP)	5000	150	30.0 ± 7.65$^{\#}$	<0.001	1.15 ± 0.25

Note: $^{\#}P < 0.01$ *vs.* negative group.

liver, kidney, spleen, stomach, intestine and testes/ovaries were conducted histopathological examination (paraffin section, H-E staining, microscopic examination).

2.5. Statistical analysis

The SPSS Statistical System was used to analyze the data for variance homogeneity. Homogenous data were analyzed using a One-Way Aanlysis of Variance (ANOVA), heterogeneous data were analyzed using the Rank-Sum test, and the significance of inter-group differences between the control and treatment groups was assessed using *t*-test for pair-wise comparisons to the control group. All statistical tests were performed at the $P < 0.05$ and $P < 0.01$ levels of significance.

3. Results

3.1. Acute oral toxicity test in SD rats

The exploration of maximum tolerable dose (MTD) test was performed to examine the acute toxicity in animals. The acute oral toxicity study was conducted in 10 males and 10 females of SD rats with a dose of 10.0 mg/kg body weight. The animals' body weight at the beginning of the test was 192.6 ± 5.5 g for females and 193.8 ± 5.4 g for male rats. At the end of the test, the body weight for female rats was 277.9 ± 11.8 g and for males 320.8 ± 8.3 g. There was no dead rats observed, means no mortality in this test and in the specified time frame of 14 days and applied dosage range. Hence, the results of acute oral toxicity test in SD rats regardless of gender profile showed that rats fed with 10.0 g/kg bw of *meso*-zeaxanthin by Ig administration expressed no toxicological effects and no morbidity observed in the 14 days monitoring period. Therefore, the acute oral toxicity MTD of *meso*-zeaxanthin in both male and female SD rats is over 10.0 g/kg bw.

3.2. Acute oral toxicity test in ICR mice

Besides SD rats, the maximum tolerable dose (MTD) test was also performed in ICR mice. The acute oral toxicity test in ICR mice, 10 males and 10 females, have also resulted in no findings of toxicity. Body weight at the beginning of the test was 19.5 ± 1.0 g for females and 18.6 ± 0.5 g for males, and body weight at the end of test was 30.1 ± 1.9 g for females and 37.9 ± 1.4 g for male mice. For both male and female ICR mice fed with 10.0 g/kg bw of *meso*-zeaxanthin by Ig administration, no toxicological effects and no morbidity were observed during the observation period. Therefore, the acute oral toxicity MTD of *meso*-zeaxanthin in both male and female ICR mice is more than 10.0 g/kg bw.

3.3. Ames test

Ames test is the most often used traditional method to examine the genotoxicity of a compound or agents. In our study, the Ames Test was performed on supernatant *S*9 obtained from PCB-induced SD rat liver homogenate. The results of Ames test illustrated in Table 1 are two groups of data from two completely different sets of experiments. The two sets of data in Table 1 explicitly showed that there were no findings of cytotoxicity (reduced rate of spontaneously occurring colonies and visible thinning of the bacterial lawn) at all testing dosages of *meso*-zeaxanthin. The mean number of revertants per plate of *meso*-zeaxanthin treatment groups at all dose levels of four strains,TA97, TA98, TA100 and TA102, with or without *S*9 were negative. None of the treated groups has two folds or more revertant counts than the blanket control and solvent control, and no dose-relationship observed. The positive control mutagens induced the increases in revertant colonies, confirming the validity of the assay. The results of Ames test indicate that *meso*-zeaxanthin has no genetic toxicity.

3.4. Mice bone marrow erythrocyte micronucleus assay

The results of mice bone marrow erythrocyte micronucleus assay are shown in Table 2. The micronucleus of the *meso*-zeaxanthin treatment groups at all dose levels and the negative control group were significantly lower than those in positive control group treated with cyclophosphamide ($P < 0.01$), confirming the validity of the trial. There were no significant differences of micronucleus between the negative control group and the *meso*-zeaxanthin treatment groups ($P > 0.05$). The PCE/RBC ratio of each group was within normal range. The result proves that *meso*-zeaxanthin is not mutagenic at the tested dosage range.

3.5. Mice sperm abnormality trial

The results of mice sperm abnormality trial were shown in Table 3. The sperm abnormality of the *meso*-zeaxanthin treatment groups at all dose levels had no significant differences compared

Table 3
Effects of *Meso*-zeaxanthin on sperm counts and sperm morphology in mice ($n = 5$).

Dose (mg/kg)	Total sperms counted	Total abnormal sperms	Abnormal ratio ($\bar{x} \pm s$)/%	Abnormal sperms counted					
				No hook	Large round head	Banna shape	Two tail/head	Kinks tail	Amorphous
0	5000	104	2.08 ± 0.41	40	8	2	0	0	54
1250	5000	106	2.12 ± 0.13	46	3	5	0	0	52
2500	5000	96	1.92 ± 0.41	32	5	3	2	0	54
5000	5000	108	2.16 ± 0.34	45	3	4	0	0	56
2.0 (MCC)	5000	246	4.92 ± 1.69$^{\#}$	127	12	2	3	10	92
H				1.350					
P				0.717					

$^{\#}P < 0.05$ *vs.* negative group.

with the negative control group ($P > 0.05$). The sperm abnormality ratio in all treatment groups and the negative control group was significantly lower than those in positive control group treated with Mitomycin C (MMC) at 2.0 mg/kg ($P < 0.01$).

3.6. 90-Day subchronic oral toxicity study

3.6.1. Meso-zeaxanthin on clinical observations

No decease, no abnormal behaviors, nor physical signs of toxicity was observed for the SD rats throughout the experimental period.

3.6.2. Effects of meso-zeaxanthin on body weight

In SD rats, the long term effects of *meso*-zeaxanthin on body weight are listed in Table 4, with group summary and individual body weight data. Raw data were consistent with the requirements of homogeneity of variance ($P > 0.05$). Body weights of the *meso*-zeaxanthin treated groups at all dose levels had no significant differences ($P > 0.05$) compared with the negative control group, showing that *meso*-zeaxanthin has no effects on body weight, a biomarker of no harmfulness.

3.6.3. Effects of meso-zeaxanthin on feed consumption and feed efficiency

Feed consumption and feed efficiency data of SD rats (Table 5 and supplemental data) in 90 day study were shown to be consistent with the requirements of homogeneity of variance ($P > 0.05$). Feed consumption and feed efficiency of the *meso*-zeaxanthin treatment groups at all dose levels had no significant differences ($P > 0.05$) compared with those of the negative control group (Table 5 and more data in supplemental materials). There has been no noticed abnormal data in feed consumption and feed efficiency of SD rats.

3.6.4. Effects of meso-zeaxanthin on the total feed efficiency

The total feed efficiency data of SD rats and mice in thirteen weeks study are shown in Table 5 (and more in supplemental material). From Table 5 we noticed that all raw data except those of female rats on 1–6 weeks were consistent with the requirements of homogeneity of variance ($P > 0.05$). Although raw data of female rats on 1–6 weeks were inconsistent with the requirements of homogeneity of variance after conversion, there are no significant differences (Rank-Sum test, $P > 0.05$) compared with the negative control group after they were analyzed using Rank-Sum test. Data of body weight gain, feed consumption and feed efficiency of the *meso*-zeaxanthin treatment groups at all dose levels had no significant differences ($P > 0.05$) compared with the negative control group in the period of 1–6 weeks, 7–13 weeks and 1–13 weeks (ANOVA, $P > 0.05$).

3.6.5. Effects of meso-zeaxanthin on the hematology

The effects of *meso*-zeaxanthin on the hematology were examined in SD rats (10 female and 10 male rats). We found that all hematological data were in the normal range (Table 6 and supplemental materials). Raw data were consistent with the requirements of homogeneity of variance ($P > 0.05$). HB, RBC and WBC of the *meso*-zeaxanthin treatment groups at all dose levels had no significant differences (ANOVA, $P > 0.05$) compared with the negative control group in the middle and end periods of the thirteen weeks study, indicating no adverse effects of *meso*-zeaxanthin on the hematology of SD rats.

3.6.6. Effects of meso-zeaxanthin on WBC

In the WBC category, lymphocytes, monocytes and granulocytes were examined for their potential abnormality caused by *meso*-

Table 4
Effects of *Meso*-zeaxanthin on body weight in SD rats ($\bar{x} \pm SD$).

Sex	Dose (mg/kg bw/day)	Number	Body weight (g)									
			0 Week	1 Week	2 Week	3 Week	4 Week	6 Week	8 Week	10 Week	12 Week	13 Week
Female	Control	10	65.5 ± 4.0	105.6 ± 8.4	133.5 ± 10.4	166.2 ± 9.9	193.9 ± 10.8	227.2 ± 13.8	257.6 ± 12.7	280.5 ± 14.3	294.8 ± 13.6	305.6 ± 14.2
	300	10	65.0 ± 4.2	106.1 ± 8.1	136.9 ± 12.2	164.1 ± 12.2	195.5 ± 13.0	231.6 ± 14.0	260.0 ± 20.9	278.2 ± 23.9	292.3 ± 25.8	301.7 ± 26.0
	600	10	66.4 ± 5.8	106.8 ± 8.1	137.1 ± 9.1	164.2 ± 10.5	191.4 ± 12.9	224.9 ± 11.0	261.7 ± 24.1	281.4 ± 21.7	295.6 ± 24.7	305.7 ± 25.1
	1200	10	66.3 ± 4.8	107.3 ± 7.2	138.2 ± 9.4	168.6 ± 12.2	196.3 ± 12.4	230.4 ± 9.9	257.2 ± 17.9	275.5 ± 18.2	285.7 ± 19.0	296.2 ± 19.1
	F		0.185	0.091	0.379	0.348	0.309	0.608	0.121	0.178	0.355	0.427
	P		0.906	0.964	0.769	0.791	0.819	0.614	0.947	0.911	0.786	0.735
Male	Control	10	71.1 ± 4.3	120.8 ± 6.5	171.4 ± 8.8	218.9 ± 13.9	270.9 ± 14.8	345.5 ± 26.8	409.0 ± 32.3	452.5 ± 35.2	491.0 ± 45.7	516.2 ± 50.5
	300	10	70.7 ± 6.0	117.0 ± 5.9	167.1 ± 13.1	219.8 ± 15.4	267.7 ± 15.1	331.1 ± 28.9	390.2 ± 35.8	433.1 ± 32.7	468.0 ± 37.1	489.6 ± 38.2
	600	10	71.8 ± 6.4	118.3 ± 10.1	172.5 ± 14.1	222.4 ± 15.6	271.3 ± 15.7	329.3 ± 27.9	395.7 ± 32.9	447.9 ± 34.7	488.7 ± 35.7	515.3 ± 39.2
	1200	10	70.3 ± 7.0	121.3 ± 11.0	176.3 ± 14.2	232.5 ± 16.2	285.1 ± 14.1	356.5 ± 22.7	425.1 ± 18.6	470.5 ± 17.4	501.4 ± 21.3	523.8 ± 23.0
	F		0.115	0.552	0.867	1.662	2.691	2.322	2.588	2.488	1.505	1.461
	P		0.951	0.650	0.467	0.192	0.061	0.092	0.068	0.076	0.230	0.241

Table 5
Effects of *Meso*-Zeaxanthin on the total feed efficiency in SD rats ($\bar{x} \pm SD$).

Sex	Dose (mg/kg bw/day)	Number	1–6 weeks			7–13 weeks		
			Body weight gain (g)	Feed consumption (g)	Feed efficiency (%)	Body weight gain (g)	Feed consumption (g)	Feed efficiency (%)
Female	Control	10	161.7 ± 12.5	935.6 ± 45.5	17.3 ± 1.1	78.4 ± 17.1	1201.4 ± 58.0	6.6 ± 1.7
	300	10	166.5 ± 12.4	947.4 ± 36.5	17.6 ± 1.2	70.2 ± 17.9	1190.0 ± 64.8	5.9 ± 1.4
	600	10	158.5 ± 15.5	929.0 ± 36.8	17.1 ± 1.6	80.8 ± 23.0	1232.1 ± 40.0	6.6 ± 1.8
	1200	10	164.1 ± 9.0	928.9 ± 40.4	17.7 ± 1.0	65.9 ± 11.8	1180.9 ± 71.3	5.7 ± 1.4
	F		0.739	0.473	0.992	1.526	1.400	0.885
	P		0.536	0.703	0.803(*H*)	0.224	0.259	0.458
Male	Control	10	274.5 ± 26.6	956.2 ± 28.4	28.7 ± 2.5	170.6 ± 26.1	1520.7 ± 39.1	11.2 ± 1.5
	300	10	260.4 ± 28.2	943.6 ± 42.6	27.6 ± 3.0	158.6 ± 16.2	1485.4 ± 43.9	10.7 ± 1.0
	600	10	257.5 ± 26.1	954.5 ± 29.5	26.9 ± 2.2	186.0 ± 33.9	1534.6 ± 59.2	12.1 ± 1.9
	1200	10	286.2 ± 20.9	981.2 ± 50.0	29.2 ± 2.2	167.3 ± 19.2	1482.8 ± 54.4	11.3 ± 1.5
	F		2.693	1.689	1.694	2.132	2.684	1.543
	P		0.061	0.187	0.186	0.113	0.061	0.220

zeaxanthin. All the WBC data parameters examined in SD rats were in the normal range (data see data in supplemental materials). Raw data were consistent with the requirements of homogeneity of variance ($P > 0.05$). Lymphocytes, monocytes and granulocytes of the *meso*-zeaxanthin treated groups at all dose levels had no significant differences (ANOVA, $P > 0.05$) compared with the negative control group in the middle and end periods of the thirteen weeks study.

3.6.7. Effects of meso-zeaxanthin on clinical blood biochemistry

Both midway and termination clinical blood biochemistry parameters were measured and values are shown in Table 7, all data were in the normal range and no adverse effects were observed. Raw data were consistent with the requirements of homogeneity of variance (ANOVA, $P > 0.05$). Ten parameters, namely, ALT, BUN, Cr, CHOL, TG, blood glucose, total protein, albumin, GLU and albumin/GLU, of the *meso*-zeaxanthin treated groups at all dose levels had no significant differences (ANOVA, $P > 0.05$) compared with the negative control group in the middle period of the thirteen week study. We observed that AST levels at all dose groups of female rats and at low and intermediate dose groups of male rats had no significant differences (NOVA, $P > 0.05$) compared with the negative control group, whereas AST levels only at high dose (1200 mg/kg bw/day) groups of male rats had significant differences (ANOVA, $P < 0.05$) compared with the negative control group.

Termination clinical blood biochemistry parameters were also measured and all data were in the normal range (data shown in supplemental materials). Raw data were consistent with the requirements of homogeneity of variance ($P > 0.05$). Again, the measured ten parameters, i.e. ALT, AST, BUN, Cr, CHOL, TG, blood glucose, total protein, albumin, GLU and albumin/GLU of the *meso*-zeaxanthin treated groups at all dose levels, including AST level at high dosed male rat group, had no significant differences (ANOVA, $P > 0.05$), compared with the negative control group in the end period of the thirteen weeks study, further indicated the no adverse and non-detrimental effects of *meso*-zeaxanthin, even at such high dosage levels as 1200 mg/kg bw/day per rat.

3.6.8. Effects of meso-zeaxanthin on the organ-to-body weight ratios

Table 8 illustrates the results of the effects of *meso*-zeaxanthin on the organ-to-body weight ratio. Raw data were consistent with the requirements of homogeneity of variance ($P > 0.05$). There was no statistically significant difference found (ANOVA, $P > 0.05$) in the weights of liver, kidney, spleen, and testes/ovaries, and the ratio of organ-to-body weight, compared with the negative control group in the end period of the thirteen weeks study, proved no effects of *meso*-zeaxanthin on the organ weight examined and the ratio of organ-to-body weight.

3.6.9. Effects of meso-zeaxanthin on the histopathology

There were no obvious abnormality in the examination of gross anatomy as evaluated by eye observation. Liver pathological examination of 90-day feeding study were as followed: liver velamen was intact; hepatic lobule structure was clear; liver plate arrangement was not in disorder; nuclear morphology rule, portal

Table 6
Effects of *Meso*-Zeaxanthin on HB, RBC and WBC in SD rats ($\bar{x} \pm SD$).

Sex	Dose (mg/kg bw/day)	Number	HB (g/L)		RBC ($\times 10^{12}$/L)		WBC ($\times 10^{9}$/L)	
			Midway	Termination	Midway	Termination	Midway	Termination
Female	Control	10	145.6 ± 18.7	155.8 ± 13.0	8.17 ± 1.24	9.12 ± 1.16	16.59 ± 4.92	20.30 ± 5.44
	300	10	154.2 ± 17.4	151.0 ± 23.7	8.33 ± 0.98	8.30 ± 1.25	17.38 ± 5.89	18.82 ± 4.74
	600	10	154.1 ± 14.4	167.6 ± 22.4	8.21 ± 0.99	8.46 ± 1.19	14.52 ± 2.99	19.06 ± 6.75
	1200	10	141.3 ± 21.1	147.5 ± 12.4	7.45 ± 1.12	7.71 ± 0.80	15.53 ± 3.77	22.95 ± 7.17
	F		1.262	2.219	1.364	2.702	0.758	0.962
	P		0.302	0.103	0.269	0.060	0.525	0.421
Male	Control	10	165.0 ± 15.0	161.8 ± 25.0	8.87 ± 1.52	9.46 ± 0.91	18.05 ± 1.89	22.56 ± 3.70
	300	10	165.4 ± 20.2	152.2 ± 27.3	9.20 ± 1.04	9.22 ± 0.98	19.36 ± 5.20	20.11 ± 2.66
	600	10	166.1 ± 13.3	147.5 ± 15.4	8.97 ± 0.97	8.56 ± 0.75	20.86 ± 10.44	22.35 ± 4.01
	1200	10	172.6 ± 13.2	161.9 ± 36.7	8.90 ± 0.62	8.97 ± 0.96	19.31 ± 2.89	23.13 ± 3.93
	F		0.521	0.700	0.188	1.803	0.357	1.347
	P		0.671	0.558	0.904	0.164	0.784	0.274

Table 7
(I) Midway clinical biochemistry results of thirteen weeks in SD rats ($\bar{x} \pm SD$).

Sex	Dose (mg/kg bw/day)	Number	ALT (U/L)	AST (U/L)	BUN (mmol/L)	Cr (μmol/L)	CHOL (mmol/L)	TG (mmol/L)	Blood glucose (mmol/L)	Total protein (g/L)	Albumin (g/L)	GLU (g/L)	Albumin/GLU
Female	Control	10	46.7 ± 6.7	147.5 ± 25.0	5.6 ± 0.9	56.4 ± 1.4	1.97 ± 0.41	0.48 ± 0.17	7.05 ± 0.74	66.6 ± 3.0	33.4 ± 0.9	33.2 ± 2.5	1.01 ± 0.07
	300	10	47.7 ± 4.1	138.2 ± 24.4	5.3 ± 0.9	55.5 ± 3.0	1.70 ± 0.40	0.39 ± 0.09	7.00 ± 0.66	67.0 ± 3.6	33.9 ± 1.2	33.1 ± 2.5	1.03 ± 0.06
	600	10	53.1 ± 6.6	139.8 ± 23.9	5.0 ± 1.3	54.4 ± 4.2	1.60 ± 0.25	0.50 ± 0.15	6.17 ± 0.84	64.7 ± 2.4	32.9 ± 1.1	31.8 ± 1.7	1.04 ± 0.05
	1200	10	52.8 ± 8.0	153.8 ± 15.4	4.6 ± 0.6	53.4 ± 3.5	1.62 ± 0.22	0.55 ± 0.11	6.51 ± 1.02	67.0 ± 3.1	34.0 ± 1.5	33.0 ± 1.9	1.03 ± 0.04
	F		2.663	1.008	1.946	1.640	2.665	2.458	2.572	1.272	1.637	0.904	0.378
	P		0.063	0.400	0.140	0.197	0.062	0.079	0.069	0.298	0.198	0.449	0.770
Male	Control	10	50.7 ± 7.9	150.5 ± 30.9	5.2 ± 0.4	57.4 ± 3.7	1.41 ± 0.21	0.56 ± 0.16	6.97 ± 0.67	67.5 ± 3.1	34.5 ± 1.2	33.0 ± 2.2	1.05 ± 0.05
	300	10	46.6 ± 8.6	135.0 ± 35.4	4.7 ± 0.6	56.3 ± 4.3	1.69 ± 0.23	0.55 ± 0.22	6.36 ± 0.88	65.8 ± 4.8	33.3 ± 1.9	32.5 ± 3.4	1.03 ± 0.08
	600	10	45.4 ± 5.0	142.3 ± 17.1	5.3 ± 0.8	57.0 ± 11.3	1.65 ± 0.40	0.57 ± 0.23	6.35 ± 1.05	65.7 ± 3.4	33.4 ± 1.5	32.2 ± 2.3	1.04 ± 0.05
	1200	10	53.2 ± 6.2	247.4 ± 62.5*	5.3 ± 0.9	59.9 ± 2.7	1.69 ± 0.23	0.78 ± 0.27	7.18 ± 0.64	64.2 ± 3.5	32.8 ± 1.5	31.4 ± 2.2	1.05 ± 0.05
	F		2.633	17.363	1.302	0.584	2.305	2.331	2.651	1.233	2.069	0.685	0.190
	P		0.065	0.000	0.289	0.629	0.093	0.091	0.063	0.312	0.122	0.567	0.903
(II) Termination clinical biochemistry results of thirteen weeks in SD rats ($\bar{x} \pm SD$)													
Female	Control	10	56.7 ± 11.2	194.6 ± 40.6	5.9 ± 0.6	65.4 ± 4.3	1.85 ± 0.33	0.60 ± 0.10	8.11 ± 0.63	67.6 ± 1.9	34.4 ± 0.8	33.2 ± 1.5	1.04 ± 0.05
	300	10	48.3 ± 8.3	157.7 ± 51.9	6.5 ± 0.5	68.5 ± 2.9	1.87 ± 0.16	0.54 ± 0.08	7.72 ± 0.53	70.1 ± 2.9	35.2 ± 1.5	34.9 ± 2.1	1.01 ± 0.06
	600	10	55.3 ± 8.7	213.4 ± 50.2	5.9 ± 0.7	66.4 ± 2.5	2.02 ± 0.32	0.54 ± 0.06	7.76 ± 0.82	67.8 ± 2.5	34.2 ± 1.4	33.6 ± 1.3	1.02 ± 0.04
	1200	10	54.7 ± 9.7	190.0 ± 52.7	6.5 ± 0.8	68.3 ± 3.8	2.01 ± 0.30	0.51 ± 0.15	7.41 ± 0.47	69.4 ± 2.3	35.0 ± 0.8	34.4 ± 1.9	1.02 ± 0.05
	F		1.525	2.224	2.581	1.751	0.975	1.023	2.063	2.526	1.496	1.982	0.521
	P		0.225	0.102	0.069	0.174	0.415	0.394	0.122	0.073	0.232	0.134	0.671
Male	Control	10	55.4 ± 10.5	178.6 ± 44.1	5.9 ± 0.6	64.0 ± 3.8	1.35 ± 0.17	0.58 ± 0.18	7.95 ± 0.74	67.8 ± 3.1	34.3 ± 0.9	33.4 ± 2.3	1.03 ± 0.06
	300	10	59.4 ± 7.4	181.6 ± 31.1	5.8 ± 0.8	64.7 ± 2.6	1.26 ± 0.30	0.59 ± 0.12	7.51 ± 0.43	66.0 ± 2.4	33.8 ± 0.6	32.2 ± 1.9	1.05 ± 0.05
	600	10	56.1 ± 11.5	159.3 ± 58.2	6.0 ± 0.5	64.3 ± 2.7	1.33 ± 0.26	0.55 ± 0.13	7.12 ± 0.55	67.9 ± 2.3	34.0 ± 0.4	33.9 ± 2.1	1.01 ± 0.06
	1200	10	57.0 ± 11.7	153.8 ± 58.9	5.9 ± 0.4	64.7 ± 2.8	1.36 ± 0.26	0.55 ± 0.11	7.60 ± 0.82	66.9 ± 2.0	33.7 ± 1.2	33.1 ± 1.2	1.02 ± 0.03
	F		0.280	0.785	0.192	0.127	0.333	0.259	2.720	1.257	1.030	1.395	1.457
	P		0.839	0.510	0.901	0.943	0.801	0.855	0.059	0.304	0.391	0.260	0.243

Table 8
Effects of *meso*-zeaxanthin on the organ-to-body weight ratios in SD rats ($\bar{x} \pm SD$).

Sex	Dose (mg/kg bw/day)	Number	Liver (g)	Live/Body (%)	Kidney (g)	Kidney/Body (%)	Spleen (g)	Spleen/Body(%)	Testes (Ovaries) (g)	Testes(Ovaries)/ Body(%)
Female	Control	10	7.99 ± 0.54	2.61 ± 0.15	2.32 ± 0.12	0.76 ± 0.04	0.54 ± 0.11	0.18 ± 0.04	0.16 ± 0.02	0.05 ± 0.01
	300	10	7.92 ± 0.90	2.62 ± 0.14	2.36 ± 0.19	0.78 ± 0.03	0.53 ± 0.08	0.18 ± 0.03	0.17 ± 0.03	0.06 ± 0.01
	600	10	7.73 ± 0.69	2.54 ± 0.26	2.28 ± 0.12	0.75 ± 0.06	0.56 ± 0.18	0.19 ± 0.06	0.15 ± 0.02	0.05 ± 0.01
	1200	10	7.37 ± 0.62	2.49 ± 0.16	2.23 ± 0.14	0.76 ± 0.05	0.58 ± 0.16	0.20 ± 0.06	0.16 ± 0.02	0.05 ± 0.01
	F		1.594	1.223	1.305	0.926	0.217	0.343	1.720	1.314
	P		0.208	0.315	0.288	0.438	0.884	0.794	0.180	0.285
Male	Control	10	13.78 ± 1.59	2.67 ± 0.13	3.54 ± 0.44	0.69 ± 0.07	0.68 ± 0.10	0.13 ± 0.02	3.15 ± 0.29	0.61 ± 0.06
	300	10	12.40 ± 1.27	2.53 ± 0.12	3.33 ± 0.19	0.68 ± 0.03	0.63 ± 0.07	0.13 ± 0.01	3.11 ± 0.19	0.64 ± 0.05
	600	10	13.31 ± 0.94	2.59 ± 0.12	3.52 ± 0.45	0.68 ± 0.06	0.72 ± 0.18	0.14 ± 0.03	3.11 ± 0.19	0.61 ± 0.05
	1200	10	13.82 ± 1.41	2.64 ± 0.23	3.61 ± 0.19	0.69 ± 0.05	0.71 ± 0.10	0.13 ± 0.02	3.10 ± 0.20	0.59 ± 0.04
	F		2.494	1.443	1.245	0.076	1.140	0.522	0.076	1.349
	P		0.076	0.246	0.308	0.973	0.346	0.670	0.972	0.274

area of small bile ducts, blood vessels, lymph vessels can be seen; and no special Kupffer cells was observed. Mild congestion was found (Landrum & Bone, 2001) in three rats within central vein of hepatic lobule in control the group (one male rat and two female rats) and the total number of animals is 20, expressed as ♂1♀2/20 (Wooten & Hammond, 2002) in high dose group: two male rats and two female rats, the total number of animals is 20, expressed as ♂2♀2/20 (Li et al., 2010) in intermediate dose group: one male rat and one female rat, the total number of animals is 20, expressed as ♂1♀1/20 (Bone et al., 1993) in low dose group: two female rats, the total number of animals is 20, expressed as♀2/20. A small amount of rats were found small round and pieces distributed vacuoles within cytoplasm liver cells (in control group: ♂1♀1/20; in intermediate dose group: ♂1/20; in low dose group: ♀2/20); granular degeneration of individual rat liver cells was found, showing the distribution of patchy (in intermediate dose group: ♂1/20); part of rats were found liver cells degeneration, large, round and pieces distributed vacuoles were found within cytoplasm (in high dose group: ♂3♀6/20; in intermediate dose group: ♂1♀5/20); part of rats were found inflammatory cell infiltration within liver lobule (in control group: ♂2♀2/20; in high dose group: ♂3♀5/20; in intermediate dose group: ♂2♀1/20; in low dose group: ♂1♀3/20); scattered inflammatory cell infiltration were found within hepatic portal area (in control group: ♂1♀1/20; in high dose group: ♂2♀1/20; in intermediate dose group: ♂1♀2/20; in low dose group:♀1/20); lives of small amount rats can be seen spotted liver cell necrosis with inflammatory cell infiltration (in control group: ♂1♀2/20; in high dose group: ♂3♀2/20; in intermediate dose group: ♂2♀2/20; in low dose group: ♂1♀2/20).

Kidney pathological examination of 90-d feeding study were as followed: kidney velamen intact, cortex and medulla obvious layered, no fibrosis, glomerulus not found filling glomeruli, atrophy, necrosis and other changes. the renal pelvis mucosal intact, no abnormal changes such as metaplasia. Part of renal tubular epithelial cells can be seen mild swelling in small amount of rats (in control group: ♀2/20; in high dose group: ♂2♀3/20; in intermediate dose group: ♂1♀2/20; in low dose group:♀2/20); part of renal cortical interstitial tissue vascular can be found mild dilatation and congestion in small amount of rats (in control group: ♂1/20; in high dose group:♀1/20; in intermediate dose group: ♂1♀1/20; in low dose group:♀2/20); Renal cortex can be found inflammatory cell infiltration in small amount of rats (in control group: ♂1/20; in high dose group: ♂1/20; in low dose group:♀1/20).

Spleen pathological examination of 90-d feeding study were as followed: spleen was normal, red pulp and white pulp can be observed, white pulp can seen arterial sheath, red pulp can be seen scattered lymphocytes and RBC, the proportion of red pulp and white pulp was normal. Spleen sinusoids can be found mild dilatation and congestion in small amount of rats (in control group:♀1/20; in high dose group: ♂1♀1/20; in low dose group: ♂1/20).

Stomach and intestine (small intestine, duodenum) pathological examination of 90-d feeding study were as followed: mucosal epithelium cells was normal; lamina propria, submucosa, muscularis and serosa were not found hemorrhage and edema; gastric and intestinal glands had no atrophy, proliferative changes.

Spermary pathological examination of 90-d feeding study were as followed: seminiferous tubules were not atrophy, arrangement of spermatogenic cells were normal, no abnormal changes of mesenchymal.

Ovary pathological examination of 90-d feeding study were as followed: levels of follicle can be seen, no abnormal changes of mesenchymal.

4. Discussion

To obtain first-hand information of the acute oral toxicity of *meso*-zeaxanthin, we examined the MTD because *meso*-zeaxanthin has not known to be toxic or to cause adverse effects in human being from reported literature. For male and female SD rats and ICR mice at 10.0 g/kg, the absence of symptoms and the lack of negative effect on growth have suggested that *meso*-zeaxanthin is non-toxic under these acute oral toxicity assay conditions. The MTD of *meso*-zeaxanthin in SD rats and ICR mice are both over 10.0 g/kg bw. According to the toxicity classes, the tested substance, *meso*-zeaxanthin, is considered harmless and nontoxic grade material.

We choose the Ames test and two *in vivo* assays for genotoxicity evaluation. Our results have demonstrated that *meso*-zeaxanthin is neither cytotoxic nor mutagenic at 5 mg/plate – the ICH recommended maximum test dosage level and other diluted dosage levels for *S.typhimurium* TA97, TA98, TA100, and TA102 in the absence and presence of a microsomal metabolizing system. The trials of the *in vivo* bone marrow erythrocyte micronucleus and sperm abnormality of mice were performed and found that at 5.0 g/kg, *meso*-zeaxanthin did not increase the numbers of micronucleated PCEs and abnormal sperms under the experiment conditions, nor affect the proportion of PCEs to total erythrocytes. The gavage feeding approach was selected because it is the intended route of administration to humans. Combining the negative results in the two *in vivo* studies with the negative Ames test results we conclude that *meso*-zeaxanthin has no genotoxicity under the testing conditions.

In the sub-chronic toxicity study, the results showed that the body weight, food consumption, food utilization index, midway (day-42), and termination (day-90) hematological tests in both

male and female rats of each testing dose level group had no statistically significant differences compared with the control group ($P > 0.05$). Blood chemistry parameters from the current study have found no statistically significant differences in comparison with that of the control group. However, the blood AST levels of the 1200 mg/kg male rat groups had significant differences (ANOVA, $P < 0.05$) compared with the negative control group at midway of the experiment. Necropsy and pathological examination showed case of illness with histological results of liver cells vacuolar degeneration increased significantly in 1200 mg/kg and 600 mg/kg dose group, as well as those with inflammatory cell infiltration within liver lobule and spotted liver cell necrosis with inflammatory cell infiltration increased significantly in 1200 mg/kg dose group compared with the negative control group, other indexes had no significant toxicity in all the tested level, suggesting that *meso*-zeaxanthin administered at the tested dosage within the range of 600–1200 mg/kg for 90 days has hepatotoxicity. The daily oral administration of *meso*-zeaxanthin at dose of 300 mg/kg bw/day was well tolerated in rats. Furthermore gross examination of internal organs like liver, kidney, spleen, testes, ovaries, and organ-to-body weight ratios were also found normal. The no-observed-adverse-effect-level (NOAEL) of *meso*-zeaxanthin in rats is 300 mg/kg bw/day when administered orally for 13 consecutive weeks. Our study are consistent with the results of Howard Foundation, in which the NOAEL *meso*-zeaxanthin was >200 mg/kg/day (Connolly et al., 2011).

5. Conclusion

In summary, the findings of no acute toxicity, no mutagenic effects and no harmful effects in hematology, clinical chemistry and histopathology in this safety assessment indicate that the use of *meso*-zeaxanthin is safe at dose of 300 mg/kg bw/day in rats. The no-observed-adverse-effect-level (NOAEL) of *meso*-zeaxanthin in rats is 300 mg/kg bw/day when administered orally for 13 consecutive weeks. Application of a 100-fold safety factor to the rat study, the suggested ADI value is 3 mg/kg bw/day. Hence to further confirm these findings of no toxicity in the application of human consumption, a longer term animal study and or year-long human clinical trial safety evaluation of meso-zeaxanthin would be suggested as dietary supplements and for prolonged use.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.foodcont.2013.02.007.

References

Bone, R. A., Landrum, J. T., Alvarez-Correa, C., Etienne, V., & Ruiz, C. A. (2003). Macular pigment and serum response to dietary supplementation with meso-zeaxanthin. *Investigative Ophthalmology & Visual Science, 44*, 405, E-Abstract.

Bone, R. A., Landrum, J. T., Cao, Y. S., Howard, A. N., & Alvarez-Calderon, F. (2007). Macular pigment response to a supplement containing meso-zeaxanthin, lutein and zeaxanthin. *Nutrition & Metabolism, 4*(12), 1–8.

Bone, R. A., Landrum, J. T., Friedes, L. M., Gomez, C. M., Kilburn, M. D., Menendez, E. M., et al. (1997). Distribution of lutein and zeaxanthin stereoisomers in the human retina. *Experimental Eye Research, 64*, 211–218.

Bone, R. A., Landrum, J. T., Hime, G. W., Cains, A., & Zamor, J. (1993). Stereochemistry of the human macular carotenoids. *Investigative Ophthalmology & Visual Science, 34*, 2033–2040.

Chang, C. J. G. (2006). *Thirteen-week oral (gavage) toxicity of meso-zeaxanthin in Han Wistar rats with a 4-week recovery*. Gene Logic Study Number: 1567-04370. Available (as of 24.01.13) at. http://www.howard-foundation.com/04370FinalReport101006.pdf 1–344.

Connolly, E. E., Beatty, S., Laugbman, J., Howard, A. N., Louw, M. S., & Nolan, J. M. (2011). Supplementation with all three macular carotenoids: response, stability, and safety. *Investigative Ophthalmology & Visual Science, 52*, 9207–9217.

Connolly, E., Beatty, S., Loughman, J., & Nolan, J. (2010). Meso-zeaxanthin ocular supplementation trial: MOST. *Investigative Ophthalmology & Visual Science, 51*, 514, E-Abstract.

Firdous, A. P., Preethi, K. C., & Kuttan, R. (2010). Antioxidant potential of meso-zeaxanthin a semi synthetic carotenoid. *Food Chemistry, 119*, 1096–1101.

Landrum, J. T., & Bone, R. A. (2001). Lutein, zeaxanthin, and the macular pigment. *Archives of Biochemistry and Biophysics, 385*, 28–40.

Li, B. X., Ahmed, F., & Bernstein, P. S. (2010). Studies on the singlet oxygen scavenging mechanism of human macular pigment. *Archives of Biochemistry and Biophysics, 504*, 56–60.

Loane, E., Kelliher, C., Beatty, S., & Nolan, J. M. (2008). The rationale and evidence base for a protective role of macular pigment in age-related maculopathy. *British Journal of Ophthalmology, 92*, 1163–1168.

Neuringer, M., Sandstrom, M. M., Johnson, E. J., & Snodderly, D. M. (2004). Nutritional manipulation of primate retinas, I: effects of lutein or zeaxanthin supplements on serum and macular pigment in xanthophyll-free rhesus monkeys. *Investigative Ophthalmology & Visual Science, 45*(9), 3234–3243.

Wooten, B. R., & Hammond, B. R. (2002). Macular pigment: influences on visual acuity and visibility. *Progress in Retinal and Eye Research, 21*, 225–240.