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Safety assessment of astaxanthin-rich microalgae biomass: Acute and subchronic toxicity studies in rats

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ABSTRACT

Astaxanthin, a natural nutritional component, is marketed as a dietary supplement around the world. The primary commercial source for astaxanthin is *Haematococcus pluvialis* (microalgae). The objective of the present study was to investigate the acute and subchronic toxicity of an astaxanthin-rich biomass of *H. pluvialis* (AstaCarox®). The oral LD_{50} of the biomass in rats was greater than 12 g/kg body weight. In the subchronic study, Wistar rats (10/sex/group) were fed diets containing 0%, 1%, 5% and 20% of the biomass (weight/weight) for 90 days. *trans*-Astaxanthin was quantifiable in the plasma of the high-dose treated group only. Compared to the control group, no treatment-related biologically significant effects of astaxanthin were noted on body weight or body weight gain. Biomass feeding did not affect hematological parameters. In the high-dose group, slightly elevated alkaline phosphatase and changes in some urine parameters and an increase in kidney weight in both sexes were noted. Histopathology examinations did not reveal adverse effects except for a marginal increase in pigment in the straight proximal tubule of the kidney in 5/10 female rats treated with the high-dose. These changes were not considered as toxicologically significant. Although the rats in high-dose group received about 9% more fat, it is unlikely that this confounding factor significantly altered the outcome. The no-observed adverse-effect-levels (NOAEL) of the astaxanthin-rich biomass for male and female rats were determined as 14,161 and 17,076 mg/kg body weight/day, or 465 and 557 mg astaxanthin/kg/day, respectively, the highest dose tested.

1. Introduction

Carotenoids are a large family of over 700 natural lipid-soluble pigments. These pigments are only produced by phytoplankton, algae, plants, and a limited number of fungi and bacteria. One of the dietary carotenoids, astaxanthin (Fig. 1) is found in the red pigment of crustacean shells (i.e. crabs, shrimp), salmon, and the asteroidean (Miki et al., 1982). The pigment was first isolated in 1937 from lobster (Kuhn and Soerensen, 1938). Astaxanthin is a member of a select group of carotenoids known as xanthophylls, or oxygenated carotenoids. In contrast to β-carotene, astaxanthin possesses no provitamin A activity and unlike some carotenoids, astaxanthin is not converted to Vitamin A in man (Jyonouchi et al., 1995). Because astaxanthin is naturally present in seafood such as salmon, red fish, shrimp, krill and lobster, it has a long history of human consumption (Torrissen et al., 1989; Turujman et al., 1997).

Astaxanthin and *Haematococcus* algae meal are approved as color additives in the feed of salmonid fish. Use of astaxanthin or the algae meal enhances the pink to orange-red color of the flesh that is perceived by the consumer as one of the important quality criteria. The regulation requires that the quantity of astaxanthin in finished feed, from *Haematococcus* algae meal when used alone or in combination with other astaxanthin color additive sources shall not exceed 80 mg/kg of the finished feed. Astaxanthin is commercially produced from both natural and synthetic sources. The primary natural source of astaxanthin for commercial uses is *H. pluvialis* (microalgae). The green microalgae *H. pluvialis*, a single-celled aquatic organism, synthesizes astaxanthin from the carotenoids, lycopene or phytoene. When this algae is exposed to harsh environmental conditions and ultraviolet light, it accumulates the highest level of astaxanthin and in this process, the algae become blood red in color.

In addition to its use as a color additive in feeds, astaxanthin is also marketed as a dietary supplement for its potential health benefits. Several *in vivo* and *in vitro* studies, including animal and human studies have shown that astaxanthin possesses a wide variety

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; FDA, food and drug administration; ICH, International Conference on Harmonization; NOEL, no-observed-effect level; OECD, Organization for Economic Co-operation and Development.

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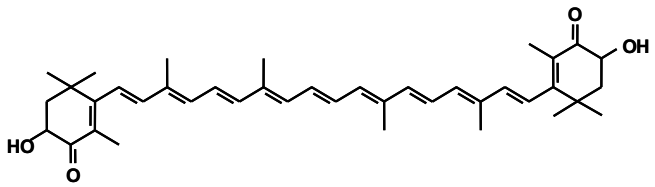

Fig. 1. Molecular structure of astaxanthin.

of biological activities, including as an antioxidant (Esterbauer et al., 1987; Naguib, 2000; Dore, 2005), as an antitumor agent (Tanaka et al., 1995), possesses anti-*Helicobacter pylori* effects (Wang et al., 2000), favorably affects blood pressure (Hussein et al., 2005a,b) as well as has a cardioprotective effect (Gross and Lockwood, 2004). Astaxanthin has been shown to be a more potent antioxidant than other carotenoids and vitamin E (Miki et al., 1982; Terao, 1989). The antioxidant-related health benefits of astaxanthin have been purported to be due to its unique chemical structure. Although the beneficial effects of astaxanthin have been investigated to some extent, its adverse effects have not been systematically studied or reported. In two separate subchronic studies in rats, effects of astaxanthin were investigated. In the study published in Japanese, Ono et al. (1999) reported that dietary exposure of *Haematococcus* extract at concentrations up to 5% (equivalent to 0.25% astaxanthin; ~125 mg/kg/day) to F344 rats for 90 days did not cause toxicological changes. In another subchronic toxicity study, Takahashi et al. (2005) reported that gavage administration of an astaxanthin-rich extract at doses up to 50 mg astaxanthin/kg/day to rats did not cause adverse toxicological effects. Both these studies did not follow good laboratory practices. Additionally, from safety perspective relatively low doses of astaxanthin were used in these studies and in the study by Takahashi et al. (2005) gavage route of administration was employed. The objective of the present study was to evaluate potential adverse effects, from feeding an astaxanthin-rich biomass of *H. pluvialis* (AstaCarox®) to rats at concentrations up to 20% in the diet and resulting in approximately 500 mg astaxanthin/kg/day intake in a subchronic toxicity study.

2. Materials and methods

2.1. Study design

The subchronic toxicity study was conducted according to a well-designed protocol. The study was conducted at Covance Laboratories Ltd. (North Yorkshire, England) in compliance with the United Kingdom Good Laboratory Practice Regulation 1997 and the organization for economic co-operation and development (OECD #408) principles on good laboratory practices. The subchronic study was designed to meet the known requirements of EC Directive 94/40/EC 1994.

2.2. Test substance

An astaxanthin-rich dehydrated and crushed aplanospore biomass of the green microalga *H. pluvialis* was provided by AstaCarotene (Gustavsberg, Sweden). The biomass is marked as AstaCarox® and contains ~3% astaxanthin (monoesters: 2.5%; diesters: 0.4%; and free: 0.1%). The composition of the biomass was as follows: moisture – 4%; fat – 45%; protein – 10%; fiber – 4%; ash – 10%; starch + sugar – 10%; carotenoids ~4%. Astaxanthin levels in the biomass were quantified by HPLC method (Odeberg et al., 2003). For the subchronic toxicity study, the biomass was administered orally by incorporation in the feed (SQC Rat and Mouse Maintenance Diet No. 1, Ground Fine from Special Diet Services Ltd., Witham, UK). The animals had access ad libitum to the diet for at least 13 weeks. The appropriate amounts of the biomass were thoroughly mixed with rodent feed by a standardized procedure to ensure homogeneity. The diets were mixed separately by group and placed in properly labeled containers. Compared to the animals in control group, the low, mid and high-dose group animals received about 0.45%, 2.25% and 9.0% more fat in their diet. The diet admixes were prepared weekly and stability and homogeneity testing confirmed that the dietary formulation for each achieved concentration of astaxanthin from the biomass remained constant during the course of the study.

2.3. Animals

For the acute toxicity study, Sprague–Dawley Crl:CD(SD)BR rats obtained from Charles River Italia (Italy) were used. In the subchronic study, male and female Wistar rats of the Crl:WI(GlX/BRL/Han)BR strain, about 28 days old at receipt, obtained from Charles River UK Limited (Margate, UK) were used. The animals were maintained according to standard guidelines. The animals were housed in groups of five in clean, stainless steel, wire-mesh cages under controlled conditions in a room ventilated with fresh air, with 15 air changes per hour. The room temperature was maintained at 22 ± 3 °C with relative humidity 55 ± 15% and a 12 h light/dark cycle. The animals were allowed to acclimatize for two weeks before the initiation of experiments with food and water available *ad libitum*.

2.4. Treatment

In an experiment designed to determine the oral LD_{50}, a *H. pluvialis* biomass suspension in Intralipid (emulsion containing soybean oil, egg yolk phospholipids, glycerol) diluting vehicle solution (Pharmacia & Upjohn, Milton Keynes, UK) was administered to Sprague–Dawley Crl:CD(SD)BR rats (5/sex/group) as a single oral dose of 12 g/kg body weight *via* gavage (two administrations of 20 ml/kg, spaced at about 2 h, containing 300 mg/ml *H. pluvialis* biomass). Animals were observed for 14 days for signs of morbidity or mortality. In another short-term repeat-dose toxicity study, rats (6/sex) were gavage administered *H. pluvialis* biomass suspension in Intralipid solution at a dose of 6 g/kg/day once per day for 14 consecutive days. The control group was treated with Intralipid vehicle. The animals were observed during the course of the study and at completion, hematology, blood chemistry, urine analysis, and pathology investigations were conducted.

In the subchronic study, Wistar strain rats (10/sex/group) were randomly divided into four groups. At study initiation, the animals were approximately six weeks old and their body weight was within ±20% of the overall mean of each sex. Males weighed between 141 and 173 g and females weighed between 122 and 145 g. Animals were fed a standard rat diet containing 0 (control), 10,000 (low dose), 50,000 (mid-dose) and 200,000 (high-dose) ppm astaxanthin-rich biomass of *H. pluvialis* (AstaCarox®). These dietary concentrations were based on a preliminary two-week study where no adverse effects of the biomass at concentrations up to 200,000 ppm were noted. In the subchronic study, the diets were provided *ad libitum* for a minimum of 90 consecutive days, until the day prior to the scheduled euthanasia. The formulations were prepared weekly. The test article for each group was weighed in a prelabeled container and then added directly to the pre-weighed diet in a mixing drum. The diet was mixed for 30 min and samples were analyzed for stability. The stability and homogeneity analysis was performed for each batch of test article. The dietary concentrations of the test article in each group remained unchanged each week (constant ppm).

2.5. Parameters investigated

2.5.1. Clinical observations, body weights and feed consumption

All animals were observed twice daily for morbidity and mortality. Clinical examinations were performed once daily. Detailed physical examinations were conducted on all animals weekly, beginning one week prior to initiation of the experiments and prior to the scheduled necropsy. Ocular examinations were conducted on all animals prior to the initiation of experiments and during week-12 of the study on control and high-dose animals. Individual animal body weights were recorded at least weekly, both before treatment and on the first day of the initiation of experiments. Mean body weights and mean body weight changes were calculated for the corresponding intervals. Final body weights (fasted) were recorded prior to the scheduled necropsy. The amount of feed consumed by animals in each cage was recorded weekly. Feed intake was calculated as g/animal/day for the corresponding body weight intervals. Percent feed conversion efficiency (FCE) was calculated weekly for each group using the equation: 100 × (group mean body weight − group mean feed consumption). The dose concentrations (mg/kg/day) were determined weekly for each group using the following equation: (dietary concentration in ppm × feed consumption) − (mid-period body weight × 7). The overall achieved dose concentrations (weeks 1–13) were calculated as the mean of the weekly values.

2.5.2. Plasma concentration of astaxanthin

Blood samples (~0.6 ml) for determination of plasma levels of *trans*-astaxanthin were collected from the lateral caudal vein on days 2, 8, 31 and 91 of the study. The blood was collected into vials containing lithium/heparin anticoagulant. Samples were collected at approximately the same time each day. Following centrifugation of the blood, the plasma was quickly deep frozen (−70 °C) and samples were stored for further analysis. These samples (days 2, 8, 31 and 91) were analyzed for astaxanthin concentration after the completion of study. The astaxanthin levels were determined after liquid–liquid extraction of plasma samples followed by high-performance liquid chromatography (HPLC) with UV–visible detection (Odeberg et al., 2003). The procedure was standardized using pure *trans*-astaxanthin (99.3%) and canthaxanthin as internal standards.

2.5.3. Clinical pathology

Urine and blood samples for clinical evaluations (urinalysis, hematology, and plasma chemistry) were collected from all animals prior to scheduled necropsy. Urine samples were collected overnight from all animals during week-12. Urine analysis parameters included: specific gravity, pH, total volume, microscopy of sediment, protein, glucose, ketones, bilirubin, reducing substances, blood, and urobilinogen. The animals were fasted overnight prior to the collection of blood samples. Blood was collected from the lateral caudal vein. Hematology parameters included: hemoglobin concentration, red blood cell count, packed cell volume, mean cell volume, mean cell hemoglobin, total and differential leukocyte count, platelet count, prothrombin time, activated partial thromboplastin time. The hematology parameters were analyzed by Technicon H1 analyzer, and/or using kit from Bayer Diagnostics Ltd., UK. Plasma chemistry parameters included: albumin, total protein, globulin, albumin/globulin ratio, total bilirubin, urea nitrogen, creatinine, alkaline phosphatase, alanine aminotransferase (ALT), aspartate aminotransferase (AST), glucose, total cholesterol, calcium, chloride, phosphorus, potassium, and sodium. The serum chemistry parameters (except globulin and AG ratio) were analyzed using reagent kits from Boeheringer Mannheim UK Ltd. and Hitachi 747 analyzer. Globulin and AG ratio were calculated.

2.5.4. Macroscopic and microscopic examinations

A complete necropsy was conducted on all rats. Animals were euthanized by intraperitoneal administration of sodium pentobarbitone overdose followed by exsanguination. The necropsies included, but were not limited to, examination of the external surface, all orifices, and the cranial, thoracic, abdominal and pelvic cavities, including viscera. Over 40 tissues and organs were collected and placed in 10% neutral-buffered formalin. The only exception was for the testes which were preserved in 10% Bouin's solution. At necropsy, the following organs were weighed from all animals: adrenal glands, brain, heart, kidneys, liver, ovaries (females), pituitary, prostate, spleen, testes + epididymides (males), and thyroid with parathyroids. For histopathological examinations, tissues were processed into paraffin blocks, sectioned at nominal 5 μm, mounted on glass microscope slides and stained with hematoxylin and eosin.

2.6. Statistical analysis

Data were analyzed using statistical methods and values were presented as mean with the standard deviation (SD) and the number of animals (N) used to calculate the mean. Body weight gain, necropsy body weight, hematology, clinical chemistry and urine analysis variable were analyzed using one-way analysis of variance (ANOVA), separately for each sex. A regression test was performed to determine whether there was a relationship between increased dose and response. Where a significant result ($P < 0.05$) was observed and any of the pair-wise comparisons were also significant, the regression result was not reported. Levene's test for equality of variances among the groups was also performed, and where this showed evidence of heterogeneity ($P < 0.01$), even after log-transformation, the data were reanalyzed using non-parametric methods. The non-parametric methods employed were the Kruskal–Wallis ANOVA, the Terpstra–Janckheere test for dose-related trend and the Wilcoxon rank sum test for pair-wise comparison.

Organ weights were analyzed using analysis of covariance (ANCOVA) and Dunnett's test was done for each sex separately, using the necropsy body weight as covariate. Additionally, Levene's test for equality of variances across the groups was also performed for all organ weights. In all cases, this showed no evidence of heterogeneity ($P \geqslant 0.01$).

3. Results

3.1. Acute and repeat-dose toxicity studies

In the acute studies, the oral LD_{50} of *H. pluvialis* biomass was greater than 12,000 mg/kg body weight suggesting that the biomass is practically non-toxic. In the 14-day toxicity study, daily gavage administration of the biomass at dose levels of 6 g/kg/day did not result in treatment-related deaths. Clinical observations and laboratory investigations did not reveal treatment-related adverse effects (data not shown). No treatment-related changes were noted at the post-mortem examination (organ weights and gross pathology data not shown).

3.2. Plasma concentration of astaxanthin

trans-Astaxanthin was not quantifiable in any of the control samples analyzed. Although plasma samples from all groups were collected, astaxanthin concentration was analyzed only in control and high-dose group. Plasma concentrations of astaxanthin from animals treated with 200,000 ppm of the biomass on days 2, 8, 31 and 91 ranged from 39.5 to 190.2 ng/ml. The presence of astaxanthin in plasma samples demonstrated exposure to astaxanthin. The concentrations of astaxanthin in plasma were generally slightly higher in female rats than in males. This appears to be related to lower body weights and higher feed consumption (relative to body weight), as females consumed 18–25% greater feed. No relationship was noted between the mean plasma concentrations and length of exposure. The results of these investigations suggest low bioavailability of astaxanthin.

3.3. Survival, clinical observations and body weights

All animals survived to the scheduled necropsy. Orange fur staining was recorded for animals that received astaxanthin. The intensity of the staining was dose-related, ranging from light to dark orange in color. In a similar fashion, the feces of animals that received astaxanthin were also stained. There were no other treatment-related clinical observations. Compared to the control group, no treatment-related biologically significant effects of astaxanthin at dose levels up to 200,000 ppm were noted on body weight (Table 1) or body weight gain (data not shown). These results indicate that feeding of diet containing the astaxanthin biomass at concentrations up to 20% to rats for 90

Table 1
Effect of astaxanthin-rich biomass of *H. pluvialis* on body weights in male and female rats

Week of study	Males				Females			
	0	10,000	50,000	200,000	0	10,000	50,000	200,000
0	154.0 ± 4.97	156.2 ± 8.66	157.1 ± 5.62	158.1 ± 9.04	131.9 ± 4.90	128.4 ± 5.98	134.6 ± 8.02	129.0 ± 8.10
1	197.4 ± 7.94	198.5 ± 10.61	199.5 ± 6.84	197.2 ± 12.62	151.4 ± 6.73	147.0 ± 7.52	153.9 ± 8.13	147.7 ± 9.34
2	235.9 ± 13.06	234.2 ± 14.75	234.8 ± 10.76	232.4 ± 13.83	164.0 ± 6.70	162.8 ± 7.75	170.1 ± 9.37	163.6 ± 9.82
3	267.9 ± 17.46	260.7 ± 17.07	265.1 ± 13.78	259.2 ± 17.72	176.7 ± 7.94	172.8 ± 8.91	182.3 ± 11.53	174.1 ± 10.51
4	292.1 ± 22.23	283.7 ± 20.66	288.5 ± 16.96	283.1 ± 21.03	186.3 ± 8.27	181.7 ± 12.10	190.9 ± 10.75	182.4 ± 11.65
5	316.2 ± 27.12	303.4 ± 23.46	309.6 ± 19.82	305.2 ± 24.94	194.8 ± 0.07	190.3 ± 11.39	199.8 ± 11.31	189.1 ± 11.22
6	330.7 ± 27.64	318.8 ± 24.95	325.6 ± 20.42	322.8 ± 27.29	199.8 ± 11.17	195.8 ± 12.47	206.5 ± 12.36	197.9 ± 12.49
7	345.3 ± 31.29	334.1 ± 28.09	339.8 ± 22.72	338.4 ± 29.63	205.2 ± 11.25	200.0 ± 11.94	212.7 ± 11.02	203.2 ± 12.71
8	352.2 ± 31.50	343.0 ± 28.09	351.8 ± 24.24	250.7 ± 29.99	212.4 ± 9.20	204.4 ± 14.56	217.4 ± 13.27	207.0 ± 12.44
9	361.5 ± 32.12	351.5 ± 29.48	361.2 ± 26.69	360.5 ± 30.15	216.4 ± 11.59	207.6 ± 14.33	220.0 ± 12.47	209.7 ± 13.51
10	368.4 ± 33.49	356.6 ± 29.26	366.0 ± 25.84	371.7 ± 30.46	215.8 ± 10.40	210.5 ± 13.68	221.3 ± 12.80	214.2 ± 13.70
11	374.0 ± 34.46	362.6 ± 30.27	374.3 ± 26.85	376.8 ± 31.31	219.2 ± 10.44	211.6 ± 12.71	222.2 ± 12.92	216.4 ± 13.13
12	375.6 ± 35.44	367.0 ± 29.35	378.0 ± 26.45	381.0 ± 29.62	219.7 ± 9.13	213.3 ± 14.72	224.2 ± 12.12	215.1 ± 12.94
13	381.0 ± 34.46	369.0 ± 30.31	379.8 ± 25.30	386.9 ± 29.661	219.3 ± 10.73	212.6 ± 11.18	223.0 ± 12.97	217.2 ± 14.05

The values are means ± SD, $n = 10$.

days had no adverse effects on clinical observations and body weights.

3.4. Feed and test article intake

The overall feed consumption of animals receiving the astaxanthin biomass was generally similar to that of the controls. Similarly, the feed conversion efficiency of treated animals was similar to controls (Table 2). In male rats, the mean feed efficiency (%) during the entire study period in all groups ranged from 10.9% to 12.1%, while in females it was 5.5% to 6.0%. The feed efficiency during first few weeks of study in all groups was higher in both male and female rats and then slowly declined. Average consumption (week 1–13) of the biomass and the resulting intake of astaxanthin (mg/kg/day) in different groups (achieved dose) based on nominal dietary concentrations are summarized in Table 3. The achieved dose levels of biomass and astaxanthin decreased gradually as the treatment period progressed, reflecting the continued growth of the animals in relation to their fairly constant level of food intake. The results indicate that compared to males, females consumed higher amounts (18–25% greater) of the biomass and consequently the astaxanthin. The higher intake may be due to lower body weights of female rats and relatively higher feed consumption on the basis of body weight compared to males. The dose concentration of the biomass and astaxanthin decreased gradually as the treatment period progressed, reflecting the continued growth of the animals in relation to their fairly constant level of feed intake. There were no treatment-related significant adverse effects of the astaxanthin biomass on feed consumption. These results demonstrated that feeding of the astaxanthin biomass at concentrations up to 20% in feed to rats for 90 days did not affect feed consumption.

Table 2
Effect of astaxanthin-rich biomass of *H. pluvialis* on group mean feed conversion efficiency in male and female rats

Week of study	Mean feed conversion efficiency (%)							
	Males				Females			
	0	10,000	50,000	200,000	0	10,000	50,000	200,000
1	29.5	27.5	26.1	26.4	16.8	15.7	15.3	16.3
2	25.7	23.6	22.9	23.5	11.0	14.2	13.9	14.9
3	19.0	16.2	17.8	17.4	10.2	8.2	9.9	9.6
4	14.5	13.9	13.5	15.8	7.5	7.5	6.6	6.9
5	15.0	12.0	13.1	14.9	6.4	6.6	6.8	5.8
6	9.5	10.3	10.1	11.9	4.1	4.4	5.4	7.6
7	9.8	10.0	9.3	10.7	4.3	3.7	5.0	4.7
8	5.0	6.3	8.4	8.9	6.0	3.7	4.1	3.5
9	6.6	5.9	6.3	6.8	3.0	2.6	2.2	2.2
10	4.7	3.5	3.3	7.7	*	2.5	1.2	4.0
11	3.8	4.1	5.8	3.5	2.9	1.0	0.6	2.0
12	1.1	3.4	2.8	3.2	0.5	1.6	1.8	*
13	3.7	1.4	1.3	4.1	*	*	*	2.0
1 to 13	11.7	10.9	11.2	12.1	5.5	5.5	5.6	6.0

* Body weight stasis or loss. $n = 10$.

Table 3
Average astaxanthin-rich biomass of *H. pluvialis* (AstaCarox®) and astaxanthin consumption in different groups of rats (achieved dose)

Group no.	*H. pluvialis* biomass (ppm) in feed	Average consumption (mg/kg/day)*			
		Males		Females	
		Biomass	Astaxanthin	Biomass	Astaxanthin
1	0 (Control)	0	0	0	0
2	10,000	741	24	910	30
3	50,000	3724	122	4402	144
4	200,000	14,161	465	17,076	557

* The data are an average value of two cages of rats (5 animals/cage).

3.5. Clinical pathology

3.5.1. Hematology

There were no treatment-related adverse effects of astaxanthin-rich biomass of *H. pluvialis* on hematology parameters in male and female rats (Table 4). However, some statistically significant differences were noted when the control and treatment groups were compared. In male rats, mean cell hemoglobin, mean cell hemoglobin and platelets showed a significant dose–response effect (regression test). However, in females a similar dose–response was not observed when a dose–response test was performed. The low platelet counts may result from decreased production in the bone marrow or increased breakdown of platelets in the bloodstream, spleen or liver. However, histological examination of bone marrow, spleen or liver did not reveal any changes. Additionally, no other symptoms of thrombocytopenia were noted. The slight but statistically significant decrease in platelet counts noted in the high-dose males was not considered to be of toxicological relevance in the absence of microscopic change in associated hemopoeitic tissues. The differences noted in these parameters were minimal and similar differences were not observed in females. Platelet numbers were marginally lower in high-dose animals when compared with the control group, attaining statistical significance in females. Prothrombin time and neutrophils were significantly decreased in high-dose treated males. A similar decrease was observed in females receiving mid-dose (50,000 ppm) of the astaxanthin biomass but not at the high-dose. Since these differences lacked correlating changes in other red cell parameters, were of small magnitude within the historical control values of the lab, and/or were not noted in a dose-related manner, they were not considered test article-related. There were no other statistically significant differences when the respective control and treatment groups were compared. These results indicate that dietary administration of an astaxanthin-rich biomass of *H. pluvialis* to rats at doses up to 200,000 ppm had no adverse hematological effects.

3.5.2. Plasma chemistry

There were no treatment-related biologically significant adverse effects of an astaxanthin-rich biomass of *H. pluvialis* on plasma chemistry parameters in male and female rats (Table 5). However, some statistically significant differences were noted when the control and treatment groups were compared. A slight, but statistically significant dose-related increase in alkaline phosphatase activity in treated males was evident (dose–response test). An increase in alanine aminotransferase in the mid-dose group receiving 50,000 ppm of the biomass and a decrease in potassium levels in the high-dose group were noted. Additionally, an increase in plasma protein at 10,000 ppm and an increase in albumin at 10,000 and 50,000 ppm in male rats were also observed. In female rats receiving 50,000 ppm of the astaxanthin biomass, a decrease in albumin to globulin ratio was noted. These differences were minimal and were not considered to be treatment-related. Compared to control group, total cholesterol concentration was significantly higher in animals receiving 50,000 and 200,000 ppm of the biomass. The slight increase in cholesterol levels noted in high and intermediate-groups may be associated with the high fat content of the test article matrix. The high-dose group animals received about 9% more fat in their diet compared to the control group. There were no other statistically significant differences when the respective control and/or treatment groups were compared. The changes noted in plasma chemistry parameters were within historical control values of the lab. The results of plasma chemistry analysis

Table 4
Effect of astaxanthin-rich biomass of *H. pluvialis* on hematological parameters in male and female rats

Parameter	Units	Males				Females			
		0	10,000	50,000	200,000	0	10,000	50,000	200,000
Hemoglobin	g/dL	16.0 ± 0.5	16.2 ± 0.3	16.0 ± 0.6	15.8 ± 0.5	14.8 ± 0.5	15.3 ± 0.5	14.8 ± 0.6	14.9 ± 0.2
RBC	10^6/cm	8.86 ± 0.4	8.98 ± 0.4	9.06 ± 0.4	8.97 ± 0.3	7.96 ± 0.3	8.21 ± 0.4	8.04 ± 0.3	8.11 ± 0.2
PCV	%	47.7 ± 2.0	47.8 ± 1.7	47.8 ± 2.0	47.1 ± 1.5	44.0 ± 2.0	45.4 ± 1.9	43.2 ± 1.3	43.9 ± 0.8
MCV	fL	53.9 ± 1.7	53.3 ± 1.3	52.8 ± 1.7	52.5 ± 1.1[a]	55.3 ± 1.0	55.3 ± 1.1	53.8 ± 1.4	54.1 ± 1.6
MCH	pg	18.1 ± 0.7	18.0 ± 0.8	17.6 ± 0.6	17.6 ± 0.5[a]	18.6 ± 0.5	18.7 ± 0.5	18.4 ± 0.5	18.4 ± 0.6
MCHC	g/dL	33.6 ± 0.7	33.8 ± 0.9	33.4 ± 0.6	33.5 ± 0.4	33.6 ± 0.9	33.7 ± 0.5	34.2 ± 0.7	33.9 ± 0.5
Platelet	10^3/cm	957 ± 129	937 ± 95	923 ± 94	867 ± 62[a]	956 ± 120	863 ± 64	879 ± 95	791 ± 109**
Prothrombin time	s	20.1 ± 1.0	19.9 ± 0.7	20.7 ± 0.4	21.1 ± 1.2*	20.5 ± 0.8	20.6 ± 0.6	19.4 ± 0.9*	19.8 ± 0.8
APTT	s	21.0 ± 2.3	22.6 ± 2.1	22.0 ± 2.4	23.0 ± 2.3	18.9 ± 1.9	18.6 ± 2.4	17.6 ± 1.6	17.9 ± 1.0
WBC	10^3/cm	6.6 ± 1.2	5.4 ± 1.4	6.5 ± 1.5	5.4 ± 1.1	3.9 ± 0.9	4.0 ± 1.2	3.6 ± 0.7	3.8 ± 0.8
Neutrophils	10^3/cm	1.1 ± 0.2	0.9 ± 0.2	1.0 ± 0.3	0.8 ± 0.2**	0.7 ± 0.2	0.6 ± 0.2	0.6 ± 0.2	0.5 ± 0.1
Lymphocytes	10^3/cm	5.3 ± 1.1	4.4 ± 1.4	5.3 ± 1.4	4.6 ± 1.0	3.1 ± 0.8	3.3 ± 1.0	2.8 ± 0.6	3.2 ± 0.8
Monocytes	10^3/cm	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Eosinophils	10^3/cm	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
Basophils	10^3/cm	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
LUC	10^3/cm	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Neutrophils	%	17 ± 5	17 ± 4	16 ± 3	14 ± 4	17 ± 4	14 ± 4	18 ± 4	15 ± 4
Lymphocytes	%	79 ± 6	80 ± 5	81 ± 3	83 ± 4	80 ± 5	82 ± 4	78 ± 4	82 ± 4
Monocytes	%	1 ± 1	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0
Eosinophils	%	2 ± 1	2 ± 1	2 ± 1	2 ± 0	2 ± 1	3 ± 1	2 ± 1	2 ± 1
Basophils	%	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
LUC	%	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

The values are means ± SD. *$P < 0.05$; **$P < 0.01$; RBC = red blood cells; PCV = packed cell volume; MCV = mean cell volume; MCH = mean cell hemoglobin; MCHC = mean cell hemoglobin concentration; APTT = activated partial prothrombin time; WBC = white blood cells. $n = 10$.
[a] Significant dose response test.

Table 5
Effect of astaxanthin-rich biomass of *H. pluvialis* on clinical chemistry parameters in male and female rats

Parameter	Units	Males				Females			
		0	10,000	50,000	200,000	0	10,000	50,000	200,000
AST	IU/L	64 ± 6	69 ± 3	69 ± 5*	64 ± 5	53 ± 7	52 ± 8	59 ± 6	56 ± 4
ALT	IU/L	32 ± 16	38 ± 10	38 ± 13	27 ± 3	25 ± 4	26 ± 5	24 ± 6	27 ± 4
AP	IU/L	173 ± 39	194 ± 36	196 ± 36	214 ± 40[a]	71 ± 29	89 ± 14	76 ± 14	89 ± 33
Sodium	mmol/L	141 ± 2	141 ± 1	142 ± 2	140 ± 2	139 ± 1	139 ± 1	139 ± 2	139 ± 1
Potassium	mmol/L	4.4 ± 0.4	4.1 ± 0.2	4.1 ± 0.2	4.0 ± 0.3*	3.6 ± 0.2	3.8 ± 0.3	3.7 ± 0.3	3.8 ± 0.3
Chloride	mmol/L	104 ± 1	105 ± 1	105 ± 2	105 ± 2	106 ± 2	105 ± 2	105 ± 2	106 ± 1
Calcium	mmol/L	2.70 ± 0.04	2.68 ± 0.05	2.69 ± 0.05	2.67 ± 0.06	2.75 ± 0.06	2.75 ± 0.06	2.72 ± 0.06	2.74 ± 0.08
Phosphorus	mmol/L	1.6 ± 0.2	1.4 ± 0.2	1.5 ± 0.3	1.5 ± 0.2	1.1 ± 0.2	1.2 ± 0.2	1.3 ± 0.3	1.1 ± 0.2
Urea	mmol/L	7.4 ± 1.4	7.1 ± 1.1	7.3 ± 1.4	6.3 ± 1.1	8.0 ± 0.8	8.2 ± 1.2	7.0 ± 0.9	7.8 ± 1.1
Total bilirubin	μmol/L	2.0 ± 0.6	2.0 ± 0.7	2.2 ± 0.4	1.7 ± 0.3	2.6 ± 0.4	2.8 ± 0.4	2.9 ± 0.7	2.6 ± 0.4
Creatinine	μmol/L	74 ± 11	73 ± 8	70 ± 8	68 ± 9	69 ± 6	70 ± 6	64 ± 5	65 ± 6
Protein	g/L	65 ± 2	67 ± 2	68 ± 3*	67 ± 3	68 ± 3	68 ± 2	67 ± 3	67 ± 3
Albumin	g/L	41 ± 2	43 ± 2*	44 ± 2*	42 ± 2	46 ± 3	48 ± 2	48 ± 2	48 ± 3
Globulin	g/L	25 ± 2	24 ± 2	24 ± 2	25 ± 2	22 ± 2	20 ± 2	19 ± 2*	19 ± 2
AG ratio		1.7 ± 0.2	1.8 ± 0.2	1.8 ± 0.2	1.7 ± 0.1	2.2 ± 0.3	2.5 ± 0.2	2.5 ± 0.3*	2.5 ± 0.3
Total Cholesterol	mmol/L	2.0 ± 0.3	2.2 ± 0.3	2.4 ± 0.3*	2.8 ± 0.4***	1.4 ± 0.3	1.7 ± 0.4	2.1 ± 0.5**	2.2 ± 0.5***
Glucose	mmol/L	7.7 ± 1.0	7.3 ± 0.4	7.3 ± 0.7	7.3 ± 0.9	5.8 ± 1.2	5.7 ± 0.6	5.7 ± 0.5	5.9 ± 0.6

The values are means ± SD. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; AST = aspartate aminotransferase; ALT = alanine aminotransferase; ALP = alkaline phosphatase; AG = albumin globulin. $n = 10$.
[a] Significant dose response test.

show that except for a dose-related increase in plasma cholesterol concentration, feeding a diet containing an astaxanthin-rich biomass of *H. pluvialis* at doses up to 200,000 ppm to rats for 90 days had no adverse effects.

3.5.3. Urinalysis

There were no treatment-related adverse effects on urinalysis parameters in male and female rats. However, some minor differences in the urinary composition of high-dose males and to lesser extent in high-dose females were noted. Males fed the high-dose (20%) of the biomass produced slightly but significantly lower volumes of urine ($P < 0.01$) with a slightly high specific gravity and low pH (not statistically significant). In addition to these changes, urinary protein, total reducing substances and calcium oxalate crystals were slightly high or more frequently observed and phosphate crystals were less frequently observed in the high-dose treated male group. Compared to control groups, reducing substances were more frequently observed in the urine of high-dose treated female rats. These changes appear to be related to the high dietary concentration of the biomass. The relationships between these effects and exposure to the biomass, as well as their toxicological significance, are uncertain but could be the result of normal biological variation. The changes observed in the urine analysis parameters were within the historical control values of the lab.

3.6. Organ weights

No treatment-related changes of biological significance in organ weights were noted in male and female rats (Table 6) following

Table 6
Effect of astaxanthin-rich biomass of *H. pluvialis* on group mean organ weights adjusted to overall necropsy body weight in male and female rats

Organ/tissue	Males				Females			
	0	10,000	50,000	200,000	0	10,000	50,000	200,000
Body weight	355 ± 32.6	355.1 ± 32.6	355.1 ± 32.6	355.1 ± 32.6	206.6 ± 11.6	201.0 ± 11.8	210.7 ± 14.5	205.2 ± 13.7
Adrenal	0.06 ± 0.01	0.069 ± 0.01	0.07 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	0.06 ± 0.01
Kidneys	1.62 ± 0.13	1.66 ± 0.16	1.65 ± 0.09	1.77 ± 0.17*	1.06 ± 0.11	1.10 ± 0.08	1.13 ± 0.09	1.18 ± 0.12**
Spleen	0.69 ± 0.16	0.65 ± 0.11	0.59 ± 0.07	0.70 ± 0.13	0.45 ± 0.07	0.45 ± 0.07	0.46 ± 0.10	0.48 ± 0.07
Liver	8.43 ± 1.14	7.94 ± 0.94	8.33 ± 0.89	8.65 ± 0.98	5.13 ± 0.50	5.04 ± 0.28	5.29 ± 0.52	5.51 ± 0.63
Heart	0.10 ± 0.06	0.93 ± 0.11	1.09 ± 0.16	1.06 ± 0.13	0.65 ± 0.07	0.64 ± 0.05	0.67 ± 0.09	0.68 ± 0.05
Brain	1.10 ± 0.08	1.10 ± 0.10	1.96 ± 0.05	1.99 ± 0.10	1.80 ± 0.05	1.78 ± 0.07	1.81 ± 0.05	1.81 ± 0.06
Pituitary	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
Thyroid/parathyroid	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
Prostate	0.76 ± 0.15	0.73 ± 0.13	0.63 ± 0.12	0.79 ± 0.13				
Testes/epididymides	5.16 ± 0.30	5.05 ± 0.81	5.08 ± 0.33	5.11 ± 0.44				
Ovaries					0.08 ± 0.01	0.07 ± 0.02	0.08 ± 0.02	0.08 ± 0.02

The values are means ± SD. *$P < 0.05$; **$P < 0.01$; $n = 10$.

administration of an astaxanthin-rich biomass of *H. pluvialis*. However, statistically significant higher kidney weights were noted in animals maintained on feed containing 200,000 ppm ($P < 0.01$) of the biomass. The increase in kidney weight appears to be a physiological adaptive change to eliminate the test article or its metabolite. The increases noted in kidney weights lacked correlating macroscopic and microscopic changes; therefore, these differences were not considered as treatment-related. The ratio of the organ weight-to-body-weight was normal for all tissues, including kidney (data not included). These results indicate that feeding of the biomass had no significant adverse effects on organ weights.

3.7. Macroscopic and microscopic examinations

There were no treatment-related macroscopic findings at the scheduled necropsy following administration of an astaxanthin-rich biomass of *H. pluvialis* to rats. All macroscopic changes noted were considered to be spontaneous and/or incidental in nature and unrelated to the treatment. Except orange coloration of the mucosal surface of the duodenum and caecum no other macroscopic findings suggestive of effects due to treatment were noted. There were no treatment-related histopathological findings. All findings observed were consistent with normal background lesions in clinically normal rats of the age and strain used in this study and were considered spontaneous and/or incidental in nature and unrelated to the treatment. Minor renal tubular pigmentation occurred in five of the 10 high-dose treated females compared to none in controls. This finding was characterized by fine brown granules, detectable only at high magnification, in the cytoplasm of tubular epithelium and in the outer medulla, corresponding with the P3 segment of the straight proximal tubule of the kidney. No increase in the tubular pigment was evident in males although hyaline droplet accumulation may have complicated its detection. There were no other microscopic findings suggestive of treatment-related effects. There was no evidence of significant mucosal changes associated with the gastrointestinal discoloration noted macroscopically. These results support the conclusion that feeding of an astaxanthin-rich biomass of *H. pluvialis* in feed to rats for 90 days had no adverse macroscopic or microscopic effects.

4. Discussion

Two previously published studies reported on the effects of subchronic administration of astaxanthin. Ono et al. (1999) reported that dietary exposure of F344 rats to *Haematococcus* extract at the highest dietary dosage of 5% (equivalent to 0.25% astaxanthin; ~125 mg/kg/day) for 90 days did not affect body weight, feed consumption, blood cell morphology, plasma chemistry, organ weight or histopathology. Takahashi et al. (2005) also reported that daily oral (gavage) administration of an astaxanthin-rich extract of *H. pluvialis* resulting in a daily intake of up to 50 mg astaxanthin/kg/day to Sprague–Dawley rats for 90 days, did not result in treatment-related adverse effects. The findings of the present study demonstrate that dietary exposure of male and female Wistar strain rats to an astaxanthin-rich *H. pluvialis* biomass for 90 days and resulting in intakes of up to 500 mg astaxanthin/kg/day is without biologically significant adverse effect on a series of health-related parameters. These observations further support the previous findings from gavage and feeding studies indicating that ingestion of astaxanthin is without adverse effect.

The results of present study demonstrated relatively high intakes of the astaxanthin biomass in female rats compared to male rats (Table 3). For example, average consumption of the biomass at the highest dose of exposure in females was 17,076 mg/kg/day compared to 14,161 mg/kg/day in males. Similar differences in the intake of the biomass were also noted between males and females in other groups. The resulting exposure to astaxanthin as evaluated by plasma levels of astaxanthin in the animals exposed to the highest dose of the biomass was also higher in females compared to males. These sex-related differences in the biomass intake and the plasma levels of astaxanthin appear to be related to differences in body weight and feed consumption in male and female rats. The final mean body weight of females in the high-dose group was 217 g and that of males was 387 g. The average daily feed consumption in females during week 13 in the high-dose group was 15 g/animal and in males it was 20 g/animal. It appears that the lower body weights of female rats and relatively high food intake based on body weight as compared to males may have resulted in a higher intake of the astaxanthin biomass in female rats compared to males.

The dietary administration of the astaxanthin-rich biomass of *H. pluvialis* to rats was associated with fur and feces staining in all treated groups. This treatment was also associated with discoloration of the gastrointestinal tract in several intermediate and high-dose group animals. The fur and feces coloration was likely due to the direct contact of the test article with the fur and gastrointestinal tract. In an earlier study, Takahashi et al. (2005) also reported orange color of stools, proventriculus mucous membrane, and caecal content following exposure to astaxanthin-rich *H. pluvialis* extract to rats. Histopathological observations of gastric mucosa did not reveal adverse findings. Hence these findings were thought to be the result of adhesion of the test substance pigment. In the high-dose group, an increase in kidney weight in both sexes was noted. The increase in kidney weight appears to be a physiological adaptive change to eliminate the test article constituents or its metabolite. The increases in kidney weights lacked correlating macroscopic and microscopic changes. Hence, these increases

in kidney weight were not considered as adverse treatment-related effects.

Minor changes were noted in the blood and urine parameters in some treated groups and minor histopathological changes were noted in the kidneys of the high-dose females. Histopathological observations revealed minor renal tubular pigmentation in five of the 10 high-dose treated females compared to none in controls. The pigmentation was characterized by fine brown granules, detectable only at high magnification in the cytoplasm of tubular epithelium and in the outer medulla. In male rats, no increase in the tubular pigment was evident, although hyaline droplet accumulation may have complicated its detection. The minor changes noted in the kidney of five of the 10 high-dose treated female rats were not considered as toxicologically significant. The slight reduction in platelet counts noted in the high-dose animals is not thought to be of toxicological significance in the absence of microscopic change in associated hemopoeitic tissues. The minor increase in plasma alkaline phosphatase activity noted in high-dose males may be an adaptive change in response to the administration of high-doses of the biomass. The slight increase in plasma cholesterol levels noted in the intermediate and high-dose groups may be associated with the high fat content of the test article as animals in these groups received approximately 2.25% and 9% more fat in their diet, respectively, compared to the control group. However, the higher plasma cholesterol levels were not associated with increases in liver weight or evidence of microscopic change in liver. These differences were considered as incidental as they were either limited to one sex, or lacked dose–response, or were not supported by other changes in related clinical parameters. The bulky nature of the highest dose level of the biomass (20% of the feed) used in the present study may have resulted in some of the changes noted in this group. Renwick et al. (2003) reported that the addition of bulky macro-ingredients to experimental diets, in amounts that are exaggerated relative to human intake, may cause nutritional imbalance. The high use levels of the biomass in the present study may have caused some unknown nutritional imbalances resulting in the noted biochemical and other changes in this group. This also suggests that the noted changes may not be the result of astaxanthin intake.

The results of a tissue distribution study of astaxanthin in rats following dietary exposure to 0.3%, 1%, and 3% of astaxanthin in feed for 7 and 14 days by Petri and Lundebye (2007) provide supportive data for the rapid elimination or catabolism of astaxanthin without long-term storage of astaxanthin in the body. Odeberg et al. (2003) investigated metabolism and elimination of astaxanthin in human subjects following a single oral dose of 40 mg astaxanthin. The plasma half-life of astaxanthin was determined as 16 h. In another human study (Osterlie et al., 2000), plasma elimination half-life of astaxanthin following a single oral dose of 100 mg astaxanthin was reported as 21 h. In the present study, plasma astaxanthin levels in the high-dose group during the early period of exposure (i.e. at the end of 2 or 8 days) was similar to that noted at the end of 31 or 91 days. These results support the conclusion that length of exposure to astaxanthin is not related to the plasma concentration. Observations from the present study also support the half-life of astaxanthin noted in earlier human and rodent studies. Evidence from human and rat studies also suggest that astaxanthin bioavailability is very low and it is unlikely to be accumulated in the body. The high variability of plasma astaxanthin noted in the present study is in agreement with the results found by Gradelet et al. (1996) in rats. In both studies, the animals had free access to the feed.

In summary, the results of this subchronic toxicity study indicate that this astaxanthin-rich biomass of *H. pluvialis* had no adverse effects in either male or female rats. Based on the results of this study, the no-observed-adverse-effect-level (NOAEL) of the biomass was found to be 20% in diet. The equivalent dose of the biomass for male and female rats was 14,161 and 17,076 mg/kg body weight/day, respectively. The resulting dose of astaxanthin was 465 and 557 mg/kg/day, respectively. The results of this study suggest that the currently recommended doses of astaxanthin as a dietary supplement of 2–6 mg/day (0.07–0.1 mg/kg/day for an average individual weighing 60 kg) are ~800 fold lower than the NOAEL.

Conflict of interest statement

These studies were supported by Fuji Chemical Industry Co., Ltd., the manufacturer and distributor of astaxanthin.

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