Fundamental Toxicological Sciences (Fund. Toxicol. Sci.)
Vol.1, No.4, 151-159, 2014

Original Article

Safety evaluation of mutagenecity, acute and subacute toxicity study of *Chlorella vulgaris* CK-22 in rats

Sayaka Himuro, Sugi Ueno, Naoto Noguchi, Takuya Uchikawa and Koji Watanabe

Department of Research & Development, Chlorella Industry Co., Ltd., 1343 Hisatomi, Chikugo, Fukuoka 833-0056, Japan

(Received November 10, 2014; Accepted November 18, 2014)

ABSTRACT — The aim of this study was to evaluate the safety of *Chlorella vulgaris* CK-22 as a food supplement. We examined mutagenicity, acute toxicity and subacute toxicity using Wistar rats administered *Chlorella* powder (CP). In the mutagenesis test, CP exhibited no mutagenecity in the *in vitro* assay. In the acute toxicity test, CP was administered orally at 0 mg/kg, 1,000 mg/kg, 2,000 mg/kg and 5,000 mg/kg body weight to Wistar rats (five animals/sex/group). No significance changes were observed test article-related during the 14-day observation period. The LD_{50} of CP was estimated to be more than 5,000 mg/kg body weight in rats. In the subacute toxicity test, CP was administered at 0%, 2.5%, 5% and 10% in pelleted rodent diet to Wistar rats (ten animals/sex/groups). No mortality or treatment-related clinical signs were observed in any of the groups during the 28-day observation period. In both sexes, renal histopathology was conducted in the control and 10% groups, because absolute and relative renal weights increased in the 10% groups compared to the control groups. Based on the histopathology of the kidneys, the no-observed-adverse-effect level (NOAEL) is greater 8.57 g/kg body weight/day for males and 8.62 g/kg body weight/day for females.

Key words: *Chlorella, Chlorella vulgaris,* Mutagenicity, Acute toxicity, Subacute toxicity

INTRODUCTION

Chlorella is a unicellular green algae approximately 3 to 8 μm in diameter. It grows in fresh water, lake and waterholes the world over. In 1890, the Dutch microbiologist M. W. Beijerinck first observed green algae cells under a microscope and named the organism "Chlorella". *Chlorella vulgaris* has high photosynthetic efficiency and cell-proliferating potency; it divides into 4 cells every 20 hours. It has abundant protein (as much as 50-60%), as well as nutritional components such as vitamins, minerals, and dietary fiber. In 1964, *Chlorella* was established by industrial mass culture in Japan; since then, it has been produced in Korea, Taiwan and Indonesia as well as Japan. Since *Chlorella* first appeared at wellness markets and health food stores, it has been widely distributed as supplement for human health (Yamaguchi, 1997).

Several studies have established the wide-ranging potential of *Chlorella* as a health supplement. After type II_a hypercholesterolemia patients took 3 g/day of *Chlorella* tablets for 3 months, serum total- and LDL-cholesterol levels were decreased significantly compared to control groups (Sansawa *et al*., 2002). When healthy pregnant women took 6 g/day of *Chlorella* tablets from gestational week 16-20 until the day of delivery, concentrations of antioxidants such as lutein, zeaxanthin and β-carotene in their breast milk were higher than those in the control group (Nagayama *et al*., 2014). Alzheimer's disease patients who took 8 g/day of *Chlorella* tablets for 2 months had lower erythrocyte phospholipid hydroperoxide concentrations than before supplementation (Miyazawa *et al*., 2013). In this way, *Chlorella* contributes to the health of humans through hypocholesterolemic action, carotenoid supplementation and antioxidant effects. In the aquatic field, *C. vulgaris* strain CK-22 is grown by rotifer culture for use as feed around the world due to the fast rate of cell growth (Maruyama *et al*., 1988).

Several types of *Chlorella*, e.g., *Chlorella vulgaris*, *Parachlorella beijerinckii* and *Chlorella pyrenoidosa*, have already been identified and are utilized throughout the world. Moreover, different strains have different morphologies, growth rates, nutrient components and growth conditions. It's important for us to evaluate the safety of

Correspondence: Sayaka Himuro (E-mail: sayaka_himuro@chlorella.co.jp)

Chlorella strains used as food and feed. We performed a mutagenecity test, single-dose test and 28-day repeated dose toxicity test of *C. vulgaris* CK-22 in Wistar rats.

MATERIALS AND METHODS

Preparation of *Chlorella* powder (CP)

In this study, we used *Chlorella vulgaris* strain CK-22, a unicellular green alga, as the *Chlorella* source. *C. vulgaris* CK-22 was originally identified by Fott *et al*., who described its morphological characteristics (Fott and Nováková, 1969). It also has been recently re-identified as *C. sorokiniana* based on the sequences of the 18S ribosomal RNA gene and the morphological characteristics. The algal cells were cultured in an outdoor pool, harvested, and washed with water using a centrifuge separator (5,000 × g). The obtained alga slurry was heated at 100°C for 3 min with a heat exchanger (Morinaga Engineering Co. Ltd., Tokyo, Japan) and was powdered using a spray-drier under the blower temperature of 170°C.

Mutagenic test

We used the *Umulac* AT kit (Protein Purify Co., Ltd., Isesaki, Japan) in an *umu*-test. We prepared ethanol and hot water extracts from CP as samples. The samples were prepared using the following methods. For the ethanol extract, 500 mg of CP was added to 10 mL of 80% ethanol (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and homogenized for 1 min. It was centrifuged at 25,000 rpm for 20 min. The supernatant was diluted to 50 mL with 10% dimethylsulfoxide (DMSO) (Wako Pure Chemical Industries, Ltd.). For the hot water extract, 500 mg of CP was added to 25 mL of distillated water, and heated at 100°C for 10 min. It was centrifuged at 25,000 rpm for 20 min. The supernatant was diluted to 50 mL with distilled water. We assayed using the *Umulac AT* kit according to the manufacturer's protocol. In this test, we used a single strain, *Salmonella typhimurium* NM2009. As the positive control chemicals, we used 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2) and 2-aminoanthracene (2-AA). Because most aromatic amines require metabolic activation to be genotoxic, an activation system was prepared containing a liver supernatant fraction (S9) from rats. 2-AA was metabolically activated to a mutagenic compound by reacting with S9-mix. 10% DMSO was used as the negative control. Ten µL of extract, 10 µL of S9-mix and 90 µL of the bacteria culture were added to a microplate with 96 wells, respectively. We tested without the S9-mix at the same time. The mutagenicity was estimated using *β*-galactosidase activity as an indicator of *umu* gene expression by measuring the absorbance at 620 nm by an absorption spectrometer (INTERMED JAPAN, Inc., Osaka, Japan). In this test, sample absorbance values more than twice the absorbance of the negative controls are considered to indicate sample mutagenicity.

Animals

Wistar rats were purchased from Japan SLC, Inc. (Shizuoka, Japan). Acute and subacute toxicity tests were carried out according to OECD Guideline 420 (OECD, 2001) and OECD Guideline 407 (OECD, 2008), respectively. Before the tests, all animals were acclimated for 7 days and had free access to water and pelleted rodent diet, CE-2 (CLEA Japan, Inc., Tokyo, Japan). Animals were housed in individual cages (one rat/cage) with a 12-hr light cycle (6:00 to 18:00) at 23 ± 0.5°C in 55 ± 5% relative humidity. The animals were cared for according to the NIH published guideline.

Acute toxicity test

In the acute toxicity test, forty Wistar rats (aged 9 weeks) were randomly divided (five animals/sex/groups). The vehicle group was administered 2 mL saline by gavage, while the exposed groups were administered a single dose of 1,000 mg/kg (Low dose), 2,000 mg/kg (Middle dose) and 5,000 mg/kg (High dose) body weight of CP in 2 mL distilled water by gavage. All animals had free access to water and CE-2 for 14 days.

Subacute toxicity test

With regard to the subacute toxicity test, eighty Wistar rats (aged 6-weeks) were randomly divided (ten animals/sex/groups). The control group was fed CE-2, and separate treatment groups were fed CE-2 containing 2.5% (Low dose), 5% (Middle dose) and 10% (High dose) CP for 28 days. They had free access to water and pelleted rodent diet during the test period.

Observation and examination

Acute toxicity test

Immediately after dosing, the animals were observed for toxicity signs, mortality and morbidity at hours 1, 2, 3 and 4. They were then kept under observation for toxicity signs throughout the test period. Individual body weights were recorded on days 1, 4, 7 and 14, and water intake was measured by weighing the drinking bottles on days 1, 7, and 14. At the end of administration, all rats were fasted for 16-hr, after which blood sampling from the abdominal aorta and autopsy was conducted under pentobarbital anesthesia. Following sacrifice, a thorough necropsy was performed on animals, and the following organs were

weighed after dissection: heart, lungs, brain, liver, spleen, kidneys, adrenals, thymus, thyroid, testes, epididymides, seminal vesicle, ovary and uterus.

Subacute toxicity test

The animals were observed for toxicity signs, mortality and morbidity twice a day during the test period. Individual body weights, food and water intakes were measured on days 7, 14, 21 and 28. After observation of external appearance on the day following the last dose, blood sampling from the abdominal aorta as well as autopsy were conducted under pentobarbital anesthesia after 16-hr fasting. Hematological parameters measured at Kurume Clinical Laboratories (Fukuoka, Japan) included white blood cell count (WBC), WBC differential counts (neutrophil, lymphocyte, monocyte, eosinophil and basophil), red blood cell count (RBC), hemoglobin (Hb), hematocrit (Ht), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and platelets (PLT). Serum biochemical parameters, also measured at Kurume Clinical Laboratories, included total protein (TP), albumin (ALB), lactase dehydrogenase (LDH), aspirate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), amylase (AMY), total bilirubin (T-BIL), creatinine (CRE), uric acid (UA), glucose (GLU), total cholesterol (TCH), non-esterified fatty acid (NEFA), triglyceride (TG), sodium (Na), potassium (K), chloride (Cl), calcium (Ca) and inorganic phosphorus (IP).

Following sacrifice a thorough necropsy was performed, and heart, lungs, brain, liver, spleen, kidneys, adrenals, thymus, thyroid, testes, epididymides, seminal vesicle, ovary and uterus were weighed after dissection. The ratios of each organ to the terminal body weight and brain weight (relative organ weight) were calculated.

After this macroscopic examination, a histopathological examination was performed only on the control and high-dose groups of both sexes, to determine whether abnormities appeared in hematology, clinical biochemistry, organ weights and gross necropsy of each group. All samples were fixed in 10% neutral buffer formalin and stained with hematoxylin-eosin.

Statistical analysis

Statistical analysis was carried out using Ekuseru-Toukei 2012 software (version 1.00, Social Survey Research Information Co., Ltd., Tokyo, Japan). Variance in data for body weight, food intake, water intake, hematology, serum biochemistry and organ weight was checked for homogeneity by Bartlett's procedure. When the data were homogeneous, one-way analysis of variance (ANOVA) was applied. In the heterogeneous cases, the Kruskal-Wallis test was used. When statistically significant differences were found, Dunnett's multiple test was employed for comparison between control and *Chlorella*-administered groups.

RESULTS

Mutagenic test

The absorbance values of the 10% DMSO (solvent control) in the absence and presence of S9-mix were 0.35 and 0.44, respectively (Table 1). In the absence and the presence of S9-mix, the absorbance values of ethanol extract were 0.30 and 0.39, respectively. In the absence and the presence of S9-mix, the absorbance values of hot water extract were 0.52 and 0.56, respectively. None of the absorbance values reached the level of twice the solvent control; hence, the test for CP mutagenicity was negative.

Table 1. *Umu*-test of test samples obtained from CP.

Sample	Test samples	Amount added/assay		*β*-Galactosidase (Absorbance)	
		(μL)	(μg)	S9 (-)	S9 (+)
Solvent Control (DMSO)		10		0.35	0.44
CP	80% Ethanol extract	10		0.30	0.39
	Hot water extract	10		0.52	0.56
AF-2*			0***	0.30	-
			0.3	1.21****	-
2AA**			0***	-	0.52
			0.3	-	1.54****

*: Positive control: 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide.

**: Positive control: 2-aminoanthracene.

***: 10% DMSO was used as the solvent control for AF-2 and 2-AA.

****: Two-old or greater compared with the solvent control was considered as positive.

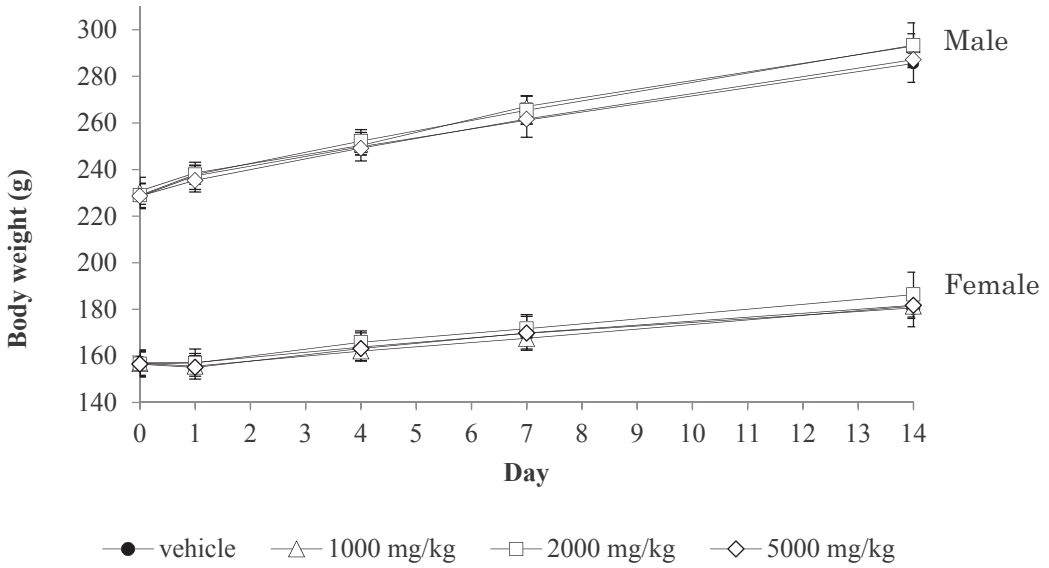

Fig. 1. Body weight changes of rats given a single dose of CP orally. Male and female rats were administered 0 (vehicle), 1,000, 2,000 and 5,000 mg/kg body weight of CP. Each point represents the mean ± S.D. of $N = 5$.

Single-dose oral toxicity

No deaths were observed in any groups. No mortality or adverse clinical signs were observed in any animal. CP-administered groups showed no significant differences in body weight compared to the control group (Fig. 1). Food and water consumption were statistically equivalent in all groups (data not shown). The absolute and relative (organ-to-body weight ratio) weight of all organs showed no statistically significant differences between control and treated groups (data not shown). In necropsy, no abnormalities were found in the CP-administered CP groups.

28-day oral toxicity

No deaths were observed in any groups. In all animals, no mortality or adverse clinical signs were observed. Body weight in 10% females showed a significant increase compared to the controls at the end of the test (Fig. 2). As compared to the controls, daily food intake was significantly increased in the 5 and 10% females (Table 2). The average daily CP intakes in the 2.5, 5 and 10% groups were 2.12, 4.06 and 8.57 g/kg/day for males and 2.12, 4.21 and 8.62 g/kg/day for females, respectively. Thus, daily CP intake was observed correlating with the compounding CP ratio as planned. Daily water intake significantly increased in 10% males compared to the controls.

One male rat in the 5% group died after receiving anesthesia. The fatality was connected to human error,

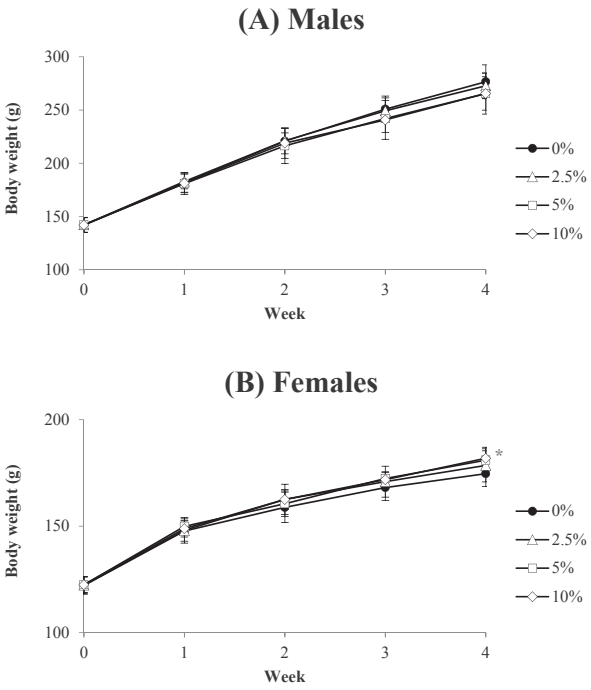

Fig. 2. Body weight changes of rats given CP orally for 28 days. Male (A) and female (B) rats were administered 0, 2.5, 5 and 10% CP.
* : Significantly different from the control group at $p < 0.05$. Each point represents the mean ± S.D. of $N = 10$.

Table 2. Parameters of male and female rats orally administered CP for 28 days.

Parameter	Males				Females			
	0% (n = 10)	2.5% (n = 10)	5% (n = 9)	10% (n = 10)	0% (n = 10)	2.5% (n = 10)	5% (n = 10)	10% (n = 10)
Final body weight (g)	290.29 ± 22.89	288.11 ± 13.14	284.64 ± 28.73	287.51 ± 22.09	180.61 ± 7.80	181.70 ± 8.02	186.44 ± 4.16	192.46 ± 6.03*
Food intake (g/day)	18.31 ± 1.21	19.19 ± 1.12	17.74 ± 1.33	18.78 ± 1.03	13.19 ± 0.07	13.76 ± 0.53	13.86 ± 0.32*	14.18 ± 0.27**
Water intake (g/day)	28.38 ± 1.86	30.12 ± 2.09	29.19 ± 2.15	31.80 ± 1.86*	22.73 ± 1.50	24.16 ± 0.63	24.19 ± 1.07	24.11 ± 0.96

Values are the mean ± S.D.

* , * * : Significantly different from the control group at $p < 0.05$, $p < 0.01$, respectively.

Table 3. Hematological parameters in rats orally administered CP for 28 days.

Parameter	Males				Females			
	0% (n = 10)	2.5% (n = 10)	5% (n = 8)	10% (n = 8)	0% (n = 8)	2.5% (n = 9)	5% (n = 10)	10% (n = 10)
WBC (× 10^3/μL)	3.31 ± 0.67	3.58 ± 0.82	3.44 ± 0.87	3.19 ± 0.66	2.96 ± 0.42	3.07 ± 0.56	2.71 ± 0.89	2.65 ± 0.78
Neutrophils (%)	20.38 ± 4.44	24.10 ± 5.43	26.36 ± 6.09	27.93 ± 6.66*	20.50 ± 3.49	20.39 ± 4.84	23.29 ± 3.94	23.17 ± 4.03
Lymphocytes (%)	76.99 ± 5.07	72.90 ± 5.69	70.55 ± 6.10	69.10 ± 6.73	76.81 ± 4.35	77.09 ± 5.02	73.42 ± 3.83	73.74 ± 4.45
Monocytes (%)	1.63 ± 0.89	1.85 ± 0.83	1.93 ± 0.78	1.94 ± 0.61	1.58 ± 0.90	1.32 ± 0.99	2.19 ± 0.80	1.78 ± 1.25
Eosinophils (%)	1.01 ± 0.33	1.12 ± 0.31	1.09 ± 0.30	0.96 ± 0.31	1.11 ± 0.22	1.20 ± 0.23	1.10 ± 0.32	1.31 ± 0.40
Basophils (%)	0.03 ± 0.09	0.03 ± 0.09	0.08 ± 0.15	0.08 ± 0.14	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
RBC (× 10^6/μL)	9.22 ± 0.28	9.48 ± 0.27	9.75 ± 0.47*	9.31 ± 0.34	8.25 ± 0.34	8.41 ± 0.33	8.42 ± 00.32	8.59 ± 033
Hemoglobin (g/dL)	16.18 ± 0.50	16.62 ± 0.43	17.11 ± 1.06	16.45 ± 0.50	15.13 ± 0.76	15.44 ± 0.67	15.41 ± 0.58	15.71 ± 0.50
Hematocrit (%)	48.98 ± 1.80	50.34 ± 1.14	51.00 ± 1.71	49.04 ± 1.99	46.31 ± 1.74	47.40 ± 1.81	47.51 ± 1.73	48.35 ± 1.89
MCV (μm^3)	53.11 ± 1.17	53.20 ± 1.75	52.50 ± 2.33	52.50 ± 2.00	56.25 ± 0.46	56.33 ± 1.50	56.50 ± 0.97	56.30 ± 1.25
MCH (pg)	17.33 ± 0.50	17.70 ± 0.48	17.63 ± 0.52	17.63 ± 0.52	18.25 ± 0.46	18.33 ± 0.50	18.20 ± 0.42	18.00 ± 0.00
MCHC (%)	33.11 ± 0.93	33.00 ± 0.82	33.50 ± 1.93	33.50 ± 1.07	32.63 ± 0.92	32.67 ± 0.50	32.40 ± 0.70	32.50 ± 0.71
Platelets (× 10^5/cmm)	5.76 ± 0.74	5.86 ± 0.61	5.51 ± 0.67	5.75 ± 0.62	5.00 ± 0.55	5.04 ± 0.43	4.97 ± 0.37	4.85 ± 0.38

Values are the mean ± S.D.

* : Significantly different from the control group at $p < 0.05$.

and so it was not considered to be test article-related.

In females, there were no significant changes of hematological and serum biochemical parameters among all groups (Tables 3 and 4). In males, significant increases of neutrophils and RBCs were observed in the 10% and 5% groups, respectively. A significant decrease of serum ALP and a significant increase of serum K was observed in all male CP-administered groups. A significant increase of serum TCH and a significant decrease of serum Cl was observed in the 2.5% group. Importantly, none of the changes of neutrophils, RBC, ALP, K, TCH and Cl levels in males were dose-dependent.

Relative cerebral weight (body weight ratio) was significantly increased in 5% females (Table 5). Absolute and relative renal weights (body weight ratio) were significantly increased in 10% males and females. Additionally, relative renal weight (brain weight ratio) was significant increased in 10% females.

In necropsy, one rat in the 5% females showed hydrops in the ovary. Because it was incidental (Motoyama *et al*., 1989), it was not considered test article-related. There were no macroscopic abnormalities in any other organs of all animals.

Because renal weights (absolute and relative) of 10% males and females increased compared with the controls, we performed histopathological examination of kidneys of the control and 10% groups in both sexes. These histopathological examinations produced no remarkable findings.

DISCUSSION

We examined mutagenicity, acute toxicity (single dose) and subacute toxicity (28-day repeated dose) using Wistar rats administered CP. We tested mutagenicity using both ethanol and hot water extracts from CP with the *umu*-test. CP indicated negative results under the absence and presence of metabolic activation. The test revealed that CP (*Chlorella* powder) was negative for mutagenicity.

In the acute test, single-dose oral administration of CP in male and female rats at 5,000 mg/kg body weight had no effect on mortality, clinical signs, body weight change, organ weights and gross observation, indicating that LD_{50} of CP in rats is more than 5,000 mg/kg body weight.

In the subacute test, there were no significant changes in clinical signs. Daily water intake was slightly increased in 10% males. Daily food intake was also slightly increased in 5 and 10% females. In 10% females, at 4 weeks, body weight was significantly increased as compared to controls. However, these increases were within the normal range throughout the test period in all animals.

On hematology, there was a significant increase in neutrophils in the 10% males. Because this difference was not observed in females and lacked significant correlative changes in other white cell parameters, it was not considered test article-related. Increase of RBC was observed in 5% males. Because of the lack of a dose relationship, it was not considered test article-related.

Regarding findings of serum biochemistry, a significant decrease in serum ALP was observed in all CP-administered males. Because this difference was not dose-dependent, it was not considered test article-related. A significant increase in serum K was observed in all administered males, but the values remained within the range observed in normal controls (Roy *et al*., 2010). The significant decrease of serum Cl in 2.5% males was within the normal range in rats (Delaney *et al*., 2003).

The relative renal weight (body weight ratio) of 5% females was slightly higher than that of controls. Because it was not dose-dependent, it was not considered test article-related. The absolute and relative renal weights in 10% males and females were statistically significantly increased as compared to controls. Values of CRE, UA, Na, K, Cl and IP as a renal function were within the normal range, such that no abnormality of kidney functional parameters was found. Moreover, renal histopathology was conducted in controls and 10% groups of both sexes. No pathological changes were discovered in the kidneys of control and 10% groups.

In conclusion, CP was negative for mutagenicity. As to the acute-dose test, the LD_{50} of CP in rats was found to be more than 5,000 mg/kg body weight. In the subacute test, the no-observed adverse effect level (NOAEL) was considered to be 8.57 g/kg body weight/day for males and 8.62 g/kg body weight/day. These results in this study suggest that *C. vulgaris* powder is safe as a food. However, the safety of CP over a longer test period at a higher level still needs to be examined. We plan to perform a long-term subchronic (90-day repeated dose) study of CP in which urinalyses is added to the battery of hematology, serum biochemistry, organ weight and histopathological assessments.

Conflict of interest---- The authors declare that there is no conflict of interest.

Table 4. Serum biochemical parameters in rats orally administered CP for 28 days.

Parameter	Males				Females			
	0% (n = 10)	2.5% (n = 10)	5% (n = 8)	10% (n = 8)	0% (n = 8)	2.5% (n = 9)	5% (n = 10)	10% (n = 10)
TP (g/dL)	6.17 ± 0.31	6.21 ± 0.20	5.96 ± 0.30	6.07 ± 0.29	5.53 ± 0.24	5.65 ± 0.39	5.52 ± 0.22	5.63 ± 0.45
ALB (g/dL)	4.66 ± 0.13	4.59 ± 0.23	4.39 ± 0.15	4.47 ± 0.24	4.22 ± 0.16	4.28 ± 0.32	4.21 ± 0.14	4.19 ± 0.31
LDH (IU/L)	3382.70 ± 819.95	3255.80 ± 407.12	3526.89 ± 449.69	3395.40 ± 696.00	3036.56 ± 478.63	3027.30 ± 313.85	2846.22 ± 361.75	2730.70 ± 459.81
AST (IU/L)	180.30 ± 31.76	1604.30 ± 14.11	171.00 ± 20.55	202.50 ± 90.41	160.33 ± 19.26	155.00 ± 14.52	157.00 ± 20.49	146.40 ± 19.40
ALT (IU/L)	48.10 ± 8.08	47.50 ± 5.78	42.67 ± 4.66	50.20 ± 10.86	41.11 ± 4.96	42.00 ± 6.67	40.44 ± 6.93	43.00 ± 10.10
ALP (IU/L)	933.70 ± 131.69	729.40 ± 61.37**	712.10 ± 74.00**	725.90 ± 75.22**	537.00 ± 71.70	547.00 ± 91.52	586.00 ± 94.00	535.50 ± 101.90
AMY (IU/L)	1472.50 ± 169.98	1704.00 ± 124.67	1671.56 ± 82.32	1736.10 ± 118.21	750.11 ± 139.50	794.20 ± 102.23	758.00 ± 114.84	797.10 ± 93.44
T-BIL (mg/dL)	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00	0.10 ± 0.00
CRE (mg/dL)	0.27 ± 0.03	0.27 ± 0.02	0.31 ± 0.10	0.27 ± 0.03	0.27 ± 0.03	0.27 ± 0.06	0.25 ± 0.03	0.25 ± 0.02
UA (mg/dL)	2.68 ± 1.56	1.77 ± 0.78	2.59 ± 0.94	1.79 ± 0.79	1.87 ± 0.87	1.67 ± 0.64	1.50 ± 0.46	1.25 ± 0.64
GLU (mg/dL)	73.00 ± 22.75	91.50 ± 16.51	83.44 ± 11.59	78.40 ± 10.04	76.89 ± 25.27	85.90 ± 22.14	84.00 ± 6.52	74.20 ± 10.09
TCH (mg/dL)	50.80 ± 6.97	64.60 ± 12.83**	58.11 ± 5.35	59.40 ± 8.80	77.00 ± 9.27	77.90 ± 11.06	76.56 ± 9.46	80.70 ± 9.09
NEFA (mmol/L)	604.00 ± 112.76	711.80 ± 141.29	720.22 ± 124.83	656.40 ± 94.76	763.44 ± 198.26	678.20 ± 115.79	625.44 ± 48.57	628.60 ± 116.61
TG (mg/dL)	51.70 ± 27.72	91.70 ± 40.45	87.33 ± 46.91	74.40 ± 23.13	40.67 ± 14.67	33.00 ± 11.03	34.44 ± 8.00	42.20 ± 12.73
Na (mmol/L)	145.50 ± 1.27	144.90 ± 1.73	144.33 ± 1.12	144.80 ± 2.04	144.67 ± 0.87	144.30 ± 0.82	144.44 ± 0.73	145.30 ± 1.42
K (mmol/L)	4.61 ± 0.34	5.06 ± 0.25*	4.99 ± 0.12*	5.12 ± 0.35*	4.34 ± 0.23	4.27 ± 0.11	4.36 ± 0.25	4.34 ± 0.32
Cl (mmol/L)	100.60 ± 1.58	99.00 ± 1.25*	99.56 ± 1.33	100.1 ± 1.52	100.33 ± 1.32	100.40 ± 1.26	101.33 ± 1.58	100.30 ± 2.00
IP (mmol/L)	10.44 ± 1.21	10.19 ± 0.76	9.59 ± 1.28	10.02 ± 1.37	8.73 ± 1.31	8.32 ± 1.32	8.47 ± 0.95	9.69 ± 1.07

Values are the mean ± S.D.

* , * * : Significantly different from the control group at $p < 0.05$, $p < 0.01$, respectively.

Table 5. Absolute and relative organ weights in rats orally administered CP for 28 days.

Parameter		Males				Females			
		0% (n = 10)	2.5% (n = 10)	5% (n = 9)	10% (n = 10)	0% (n = 10)	2.5% (n = 10)	5% (n = 10)	10% (n = 10)
Body weight (g)	Absolute	276.56 ± 15.91	272.76 ± 11.87	265.50 ± 19.37	265.57 ± 15.70	174.67 ± 6.02	178.54 ± 7.79	181.00 ± 4.40	181.89 ± 5.16
Heart (g)	Absolute	0.83 ± 0.06	0.83 ± 0.05	0.83 ± 0.07	0.82 ± 0.05	0.56 ± 0.02	0.57 ± 0.03	0.57 ± 0.03	0.58 ± 0.03
	/100g BW	0.29 ± 0.01	0.29 ± 0.01	0.29 ± 0.01	0.28 ± 0.01	0.31 ± 0.01	0.31 ± 0.02	0.31 ± 0.01	0.31 ± 0.01
	/100g brain	43.10 ± 3.29	42.80 ± 4.05	44.21 ± 3.81	41.93 ± 2.73	30.49 ± 1.53	31.47 ± 2.17	31.96 ± 1.78	31.18 ± 1.70
Lung (g)	Absolute	1.04 ± 0.24	0.95 ± 0.04	0.93 ± 0.08	0.97 ± 0.10	0.76 ± 0.05	0.77 ± 0.05	0.79 ± 0.07	0.76 ± 0.04
	/100g BW	0.36 ± 0.09	0.33 ± 0.02	0.33 ± 0.02	0.34 ± 0.05	0.42 ± 0.03	0.42 ± 0.02	0.43 ± 0.04	0.41 ± 0.01
	/100g brain	54.07 ± 11.52	48.55 ± 2.89	49.14 ± 3.99	49.88 ± 4.25	41.31 ± 3.73	42.53 ± 2.54	44.47 ± 3.61	40.51 ± 1.58
Brain (g)	Absolute	1.92 ± 0.13	1.95 ± 0.11	1.88 ± 0.07	1.95 ± 0.08	1.84 ± 0.08	1.81 ± 0.10	1.78 ± 0.08	1.87 ± 0.08
	/100g BW	0.66 ± 0.05	0.68 ± 0.05	0.67 ± 0.06	0.68 ± 0.05	1.02 ± 0.06	1.00 ± 0.06	0.96 ± 0.04*	1.01 ± 0.04
Liver (g)	Absolute	10.42 ± 0.98	10.40 ± 0.82	9.98 ± 1.59	10.26 ± 0.89	5.77 ± 0.23	6.06 ± 0.26	6.06 ± 0.37	6.17 ± 0.34
	/100g BW	3.59 ± 0.09	3.61 ± 0.19	3.49 ± 0.24	3.57 ± 0.17	3.20 ± 0.15	3.33 ± 0.10	3.25 ± 0.19	3.34 ± 0.20
	/100g brain	543.03 ± 44.24	535.10 ± 59.32	529.96 ± 86.80	526.06 ± 37.60	315.17 ± 18.78	334.87 ± 23.81	339.97 ± 16.86	330.70 ± 19.42
Spleen (g)	Absolute	0.71 ± 0.08	0.70 ± 0.05	0.67 ± 0.09	0.70 ± 0.07	0.52 ± 0.04	0.51 ± 0.03	0.54 ± 0.03	0.53 ± 0.04
	/100g BW	0.24 ± 0.02	0.24 ± 0.02	0.24 ± 0.02	0.24 ± 0.02	0.29 ± 0.02	0.28 ± 0.02	0.29 ± 0.01	0.29 ± 0.02
	/100g brain	36.96 ± 4.48	36.05 ± 3.57	35.76 ± 4.96	35.77 ± 2.89	28.59 ± 2.72	28.41 ± 1.88	30.31 ± 1.96	28.50 ± 1.64
Kidneys (g)	Absolute	2.45 ± 0.16	2.55 ± 0.19	2.47 ± 0.20	2.72 ± 0.29**	1.54 ± 0.09	1.59 ± 0.11	1.59 ± 0.13	1.71 ± 0.12**
	/100g BW	0.85 ± 0.06	0.89 ± 0.07	0.87 ± 0.06	0.95 ± 0. 10*	0.86 ± 0.05	0.88 ± 0.05	0.85 ± 0.07	0.93 ± 0.05*
	/100g brain	127.64 ± 6.39	131.04 ± 11.67	130.97 ± 11.77	139.50 ± 12. 67	84.24 ± 5.63	87.74 ± 4.50	89.18 ± 5.12	91.61 ± 4.21**
Adernals (mg)	Absolute	53.59 ± 9.51	55.70 ± 13.60	45.91 ± 6.80	53.76 ± 10.21	69.14 ± 3.61	66.25 ± 6.24	67.44 ± 6.19	68.67 ± 7.36
	/100g BW	18.42 ± 2.65	19.34 ± 4.67	16.23 ± 2.54	18.85 ± 3.99	38.37 ± 2.95	36.48 ± 3.21	36.20 ± 3.52	37.14 ± 4.08
	/100g brain	2787.61 ± 446.64	2868.67 ± 758.30	2430.18 ± 301.63	2762.06 ± 518.26	3775.27 ± 281.85	3657.94 ± 342.51	3786.12 ± 352.21	3671.37 ± 332.36
Thymus (g)	Absolute	0.36 ± 0.07	0.36 ± 0.07	0.32 ± 0.05	0.32 ± 0.09	0.25 ± 0.05	0.30 ± 0.06	0.26 ± 0.05	0.29 ± 0.04
	/100g BW	0.13 ± 0.03	0.12 ± 0.02	0.11 ± 0.02	0.11 ± 0.03	0.14 ± 0.02	0.16 ± 0.03	0.14 ± 0.03	0.15 ± 0.02
	/100g brain	19.02 ± 3.92	18.45 ± 3.32	17.02 ± 2.88	16.22 ± 4.16	13.73 ± 2.53	16.37 ± 3.27	14.65 ± 2.96	15.29 ± 2.10
Thyroid (mg)	Absolute	14.86 ± 3.56	14.72 ± 2.37	13.93 ± 2.02	15.64 ± 3.58	11.93 ± 2.53	13.03 ± 1.55	10.62 ± 2.70	11.66 ± 1.66
	/100g BW	5.12 ± 1.21	5.10 ± 0.70	4.94 ± 0.83	5.44 ± 1.23	6.58 ± 1.21	7.18 ± 0.86	5.70 ± 1.48	6.32 ± 0.99
	/100g brain	767.12 ± 165.58	756.89 ± 128.16	742.08 ± 119.99	804.27 ± 195.06	652.98 ± 149.03	722.30 ± 110.57	596.52 ± 154.22	623.46 ± 80.69
Prostate (g)	Absolute	0.37 ± 0.07	0.38 ± 0.12	0.32 ± 0.09	0.36 ± 0.09				
	/100g BW	0.13 ± 0.03	0.13 ± 0.04	0.11 ± 0.03	0.13 ± 0.03	-	-	-	-
	/100g brain	19.65 ± 4.92	19.68 ± 5.48	16.86 ± 4.35	18.82 ± 4.94				
Testes , Epididymis (g)	Absolute	3.82 ± 0.27	3.76 ± 0.23	3.80 ± 0.35	3.74 ± 0.20				
	/100g BW	1.32 ± 0.06	1.31 ± 0.08	1.34 ± 0.09	1.31 ± 0.08	-	-	-	-
	/100g brain	199.56 ± 16.29	193.58 ± 18.52	201.43 ± 16.15	192.18 ± 11.05				
Seminal vesicle (g)	Absolute	0.87 ± 0.09	0.86 ± 0.10	0.84 ± 0.11	0.89 ± 0.15				
	/100g BW	0.30 ± 0.03	0.30 ± 0.04	0.30 ± 0.04	0.31 ± 0.05	-	-	-	-
	/100g brain	45.41 ± 5.81	44.24 ± 5.88	44.89 ± 6.69	45.72 ± 7.36				
Ovary, Uterus (g)	Absolute					0.50 ± 0.09	0.47 ± 0.06	0.54 ± 0.05	0.54 ± 0.09
	/100g BW	-	-	-	-	0.28 ± 0.05	0.26 ± 0.03	0.29 ± 0.03	0.29 ± 0.04
	/100g brain					27.23 ± 4.40	26.11 ± 3.71	30.13 ± 3.56	28.61 ± 3.96

Values are the mean ± S.D.

* , * * : Significantly different from the control group at $p < 0.05$, $p < 0.01$, respectively.

REFERENCES

Delaney, B., Carlson, T., Frazer, S., Zheng, T., Hess, R., Ostergren, K., Kierzek, K., Haworth, J., Knutson, N., Junker, K. and Jonker, D.E. (2003): Evaluation of the toxicity of concentrated barley beta-glucan in a 28-day feeding study in Wistar rats. Food Chem. Toxicol., **41**, 477-487.

Fott, B. and Nováková, M. (1969): A monograph of the genus *Chlorella*. The fresh water species. In Studies in Psychology. Plaque: Academia (Fott, B. ed.), pp.10-74.

Maruyama, I., Nakamura, T., Matsubayashi, T., Ando, Y. and Maeda, T. (1988): Fatty acid composition of rotifers fed with *Chlorella* and yeast. Suisanzoshoku, **36**, 259-263.

Miyazawa, T., Nakagawa, K.,Takekoshi, H., Higuchi, O., Kato, S., Kondo, M., Kimura, F. and Miyazawa, T. (2013): Ingestion of Chlorella reduced the oxidation of erythrocyte membrane lipids in senior Japanese subjects. J. Oleo. Sci., **62**, 873-881.

Motoyama, K., Sumi, A., Miura, M., Yano, J., Morino, T., Matsumoto, K. and Yamamoto, H. (1989): Six-month chronic toxicity study of miporamicin in rats. Jpn. J. Antibiot., **42**, 2447-2471.

Nagayama, J., Noda, K., Uchikawa, T., Maruyama, I., Shimomura, H. and Miyahara, M. (2014): Effect of maternal Chlorella supplementation on carotenoid concentration in breast milk at early lactation. Int. J. Food Sci. Nutr., **65**, 573-576.

OECD (2001): Test No. 420 : Acute Oral Toxicity – Fixed Dose Procedure. OECD Publishing.

OECD (2008): Test No. 407 : Repeated Dose 28-day Oral Toxicity Study in Rodents. OECD Publishing.

Roy, B., Sarkar, A.K., Sengupta, P., Dey, G., Das, A. and Pal, T.K. (2010) Twenty-eight days repeated oral dose toxicity study of gemifloxacin in Wistar albino rats. Regul. Toxicol. Pharmacol., **58**, 196-207.

Sansawa, H., Inoue, K. and Shirai, T. (2002): Effect of Chlorella tablet ingestion on mild hypercholesterolemic patients. Nippon Shokuhin Kagaku Kogaku Kaishi, **49**, 395-400.

Yamaguchi, K. (1997): Recent advances in microalgal bioscience in Japan, with special reference to utilization of biomass and metabolites: a review. J. Appl. Physiol., **8**, 487-502.